Mechanistic basis of MAGUK-organized complexes in synaptic development and signalling

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Abstract | Membrane-associated guanylate kinases (MAGUKs) are a family of scaffold proteins that are highly enriched in synapses and are responsible for organizing the numerous protein complexes required for synaptic development and plasticity. Mutations in genes encoding MAGUKs and their interacting proteins can cause a broad spectrum of human psychiatric disorders. Here, we review MAGUK-mediated synaptic protein complex formation and regulation by focusing on findings from recent biochemical and structural investigations. These mechanistic-based studies show that the formation of MAGUK-organized complexes is often directly regulated by protein phosphorylation, suggesting a close connection between neuronal activity and the assembly of dynamic protein complexes in synapses.

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The postsynaptic sides of excitatory synapses (referred to in the rest of this paper as synapses) contain membrane-spanning neurotransmitter receptors and a submembrane thickening enriched with scaffold proteins, signalling enzymes and cytoskeletal proteins, which is known as the postsynaptic density (PSD). PSDs have pivotal roles in orchestrating synaptic structure and function; however, our understanding of the mechanistic basis of the formation of PSDs and their dynamics is rudimentary.

The slow progress in understanding the formation and dynamic assembly of PSDs is, in part, a result of their physical structure. PSDs are complex protein aggregates and are extremely large, insoluble in water, chemically heterogeneous and densely packed, but also rather mobile; these features make the detailed biochemical characterization of PSDs extremely difficult. We do not yet understand how PSD aggregates specifically form and disassemble beneath postsynaptic membranes, how the formation of PSD aggregates affects the activities or specificities of PSD-related enzymes, or how PSD aggregates communicate with various protein machineries, such as actin-remodelling complexes and the apparatus of protein synthesis and degradation, both within and outside the PSD.

Membrane-associated guanylate kinases (MAGUKs) are a family of scaffold proteins that are mainly concentrated at the sites of cell–cell junctions. They have vital roles in diverse cellular processes, including cell polarity regulation, cell–cell adhesion and synaptic signal transduction[1–3]. Excitatory synapses are particularly enriched in proteins from the MAGUK family, including members of the Discs large homologue (DLG), calcium/calmodulin-dependent serine protein kinase (CASK) and membrane-associated guanylate kinase inverted (MAGI) subfamilies[4–6]. MAGUK scaffolds are crucial in the development and plasticity of synapses[7]. Mutations in the genes encoding MAGUKs are known to cause various nervous system disorders[8]. In this Review, we summarize some of the progress that has been made over the past few years in mechanistic-based studies of PSD protein–protein interactions. We focus on the MAGUKs and the protein complexes assembled in synapses by this family of proteins in view of the important roles MAGUKs have in determining synaptic structure and function.

The postsynaptic density

The PSD was initially discovered in electron microscopy (EM) studies of neurons, in which it was observed to be an electron-dense thickening located beneath the postsynaptic membrane[9,10]. Subsequent biochemical and EM analyses of purified PSDs from various types of neurons revealed that PSDs are composed of densely packed proteins that form disc-shaped mega-assemblies a few hundred nanometres in width and ~30–50 nm in thickness[11–15]. Recent proteomic studies have generated a quantitative molecular inventory of the proteins making up PSDs[16]. It is now known that PSDs are composed of thousands of different proteins with a wide range of abundances; these proteins include neurotransmitter receptors, cell adhesion molecules, signalling enzymes,

scaffold proteins, cytoskeleton proteins, membrane trafficking proteins and molecular motors, as well as protein synthesis and degradation machineries[4,12,13,17] (FIG. 1).

EM studies using gold-particle-conjugated antibodies to detect specific PSD proteins[14,15] and more recent super-resolution light microscopic imaging studies[18–23] have shown that the proteins in PSDs form distinct layers along the axo-dendritic axis of synapses with a sequential order of membrane-spanning glutamate receptors and cell adhesion molecules, MAGUKs, synapse-associated protein 90 (SAP90)/postsynaptic density protein 95 (PSD95)-associated proteins (SAPAPs) and SRC homology 3 (SH3) and multiple ankyrin repeat domains protein (SHANK) scaffolds, and the actin cytoskeleton contacting the interior face of PSDs (FIG. 1). High-resolution EM tomographic investigations have shown that the main PSD scaffold PSD95 (also known as DLG4) is arranged in a pillar-shaped pattern that lies perpendicular to the PSD membrane, with the N terminus palmitoyl group of PSD95 contacting the membrane and its C-terminal guanylate kinase (GK) domain facing the inside of the dendritic spine[15] (FIG. 1). Given this arrangement and its extremely high abundance in PSDs (studies have calculated that the concentration of PSD95 at PSDs is ~100 μM, based on the average size of PSDs and the average number of PSD95 molecules in each PSD[16,24]), PSD95 can be viewed as the most crucial organizer of PSD structure and is able to interface with upstream membrane-spanning glutamate receptors and the downstream SAPAP–SHANK complexes, and to shape the architecture of PSDs.

The architecture of PSDs is highly dynamic, both during development and in mature neurons[25–27]. In response to diverse neuronal stimuli, PSD proteins undergo assembly and disassembly, clustering and diffusion, and membrane insertion and removal processes, which are tightly associated with various forms of synaptic plasticity (for example, long-term potentiation (LTP) and long-term depression)[25–29]. Various super-resolution optical imaging studies of both fixed and living neurons have offered extraordinary rich information on the structure and dynamics of PSD at a resolution of ~25 nm and have revealed several new observations and concepts[17,20–23,30,31]. First, major scaffold proteins, such as PSD95 and SAPAPs, and glutamate receptors form co-clustered nanodomains that are ~50–80 nm in diameter; each PSD typically contains a few such nanodomains. As such, the receptors and scaffold proteins are further enriched in these nanodomains within the already enriched PSD assembly. Second, the juxtaposition of clustered receptors (for example, AMPA-type glutamate receptors (AMPARs)) and presynaptic neurotransmitter-releasing sites positively correlates with the postsynaptic response activity (FIG. 1). Third, even though they are generally less mobile than their counterparts located outside nanodomains, both receptors and scaffolds in nanodomains are still highly dynamic under basal conditions and changes in neuronal activity can further modify their clustering and dynamic properties. Last, increasing or decreasing the levels of scaffold proteins, such as PSD95, in synapses can cause the enlargement or shrinkage of AMPAR nanodomains, respectively. Thus, although the overall morphology of a PSD in a given synapse can be relatively stable within minutes after stimulation, the molecular components and the internal subcompartmental organization within this structure can change rapidly.

MAGUKs and their binding partners

MAGUK subfamilies. MAGUKs can be classified into several different subfamilies: the DLGs, CASK, the palmitoylated membrane proteins (MPPs), the Zonula occludens (ZO) proteins, the caspase activation and recruitment domain (CARD)-containing MAGUK proteins (CARMAs) and the MAGI subfamily proteins[32] (FIG. 2a). The DLG subfamily consists of SAP97 (also known as DLG1), PSD93 (also known as DLG2), SAP102 (also known as DLG3) and PSD95. These proteins are crucial in the dynamic regulation of glutamatergic synaptic signalling and apical–basal cell polarity[1,33–35]. CASK is found at both the pre- and postsynaptic sides of excitatory synapses; it modulates synaptic vesicle trafficking and neurotransmitter release as well as postsynaptic signalling[36,37]. The MAGI subfamily proteins are broadly expressed in various tissues and cells, such as the brain (for example, MAGI2 is expressed at inhibitory synapses) and various epithelia, and they are known to have crucial roles in the proper development and function of tissues[6,38]. The MPP subfamily has an important role in establishing and maintaining cell polarity in many cell populations, including neurons[39,40]. The functions of the ZO and CARMA subfamilies of MAGUKs are better known in tissues other than brain and will not be covered in this Review. Not surprisingly, mutations in genes encoding the MAGUK family proteins are linked to human diseases, including many forms of cancer and neurological disorders[3,8,41].

Domain organization of MAGUKs. By definition, every member of the MAGUK family contains a GK-like domain (FIG. 2a). Except for the MAGI subfamily members, the rest of MAGUKs invariably contain a PDZ–SH3–GK tandem (PSG) in their C-terminal end (FIG. 2a). In addition to this common domain architecture, some MAGUKs contain LIN2–LIN7 (L27), CARD, CaM kinase-like, WW and further PDZ domains (FIG. 2a). These domains are all known to function as protein–protein interaction modules[42–45] and thus MAGUKs are ideally suited as scaffold proteins for assembling large signalling complexes. Except for the CaM kinase-like domain in CASK, which can function both as a specific protein–protein interaction module[45] and as a protein kinase specifically phosphorylating neurexins[46], none of the other domains found in MAGUKs has catalytic activities.

Targets on PSD membranes. Many synaptic transmembrane proteins — including glutamate receptors (both NMDA-type glutamate receptors (NMDARs) and AMPARs), ion channels and synaptic adhesion molecules — contain conserved PDZ-binding motifs (PBMs) at their C-terminal cytoplasmic tails (FIG. 1). Interactions between these PBMs and the PDZ domains of MAGUKs are known to be crucial in the trafficking, clustering, targeting or

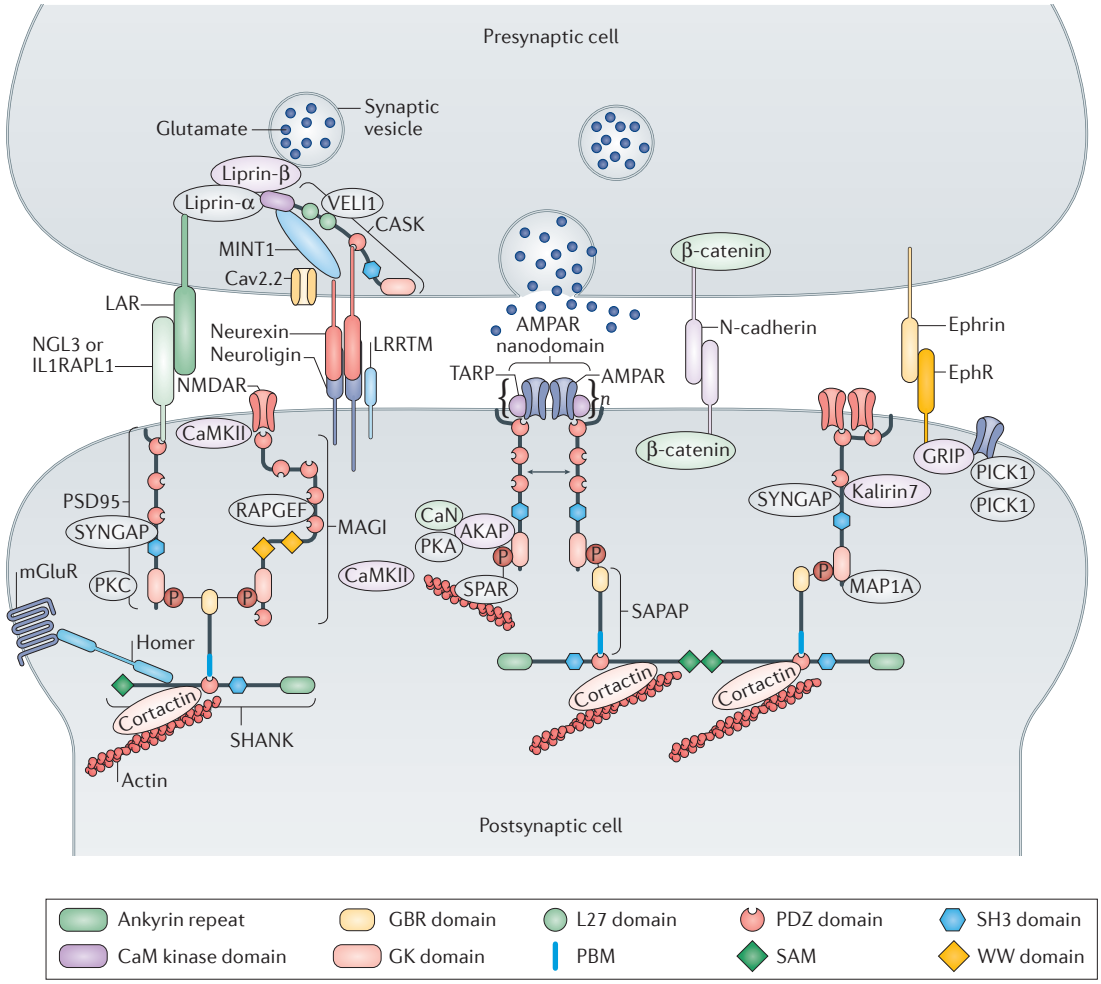
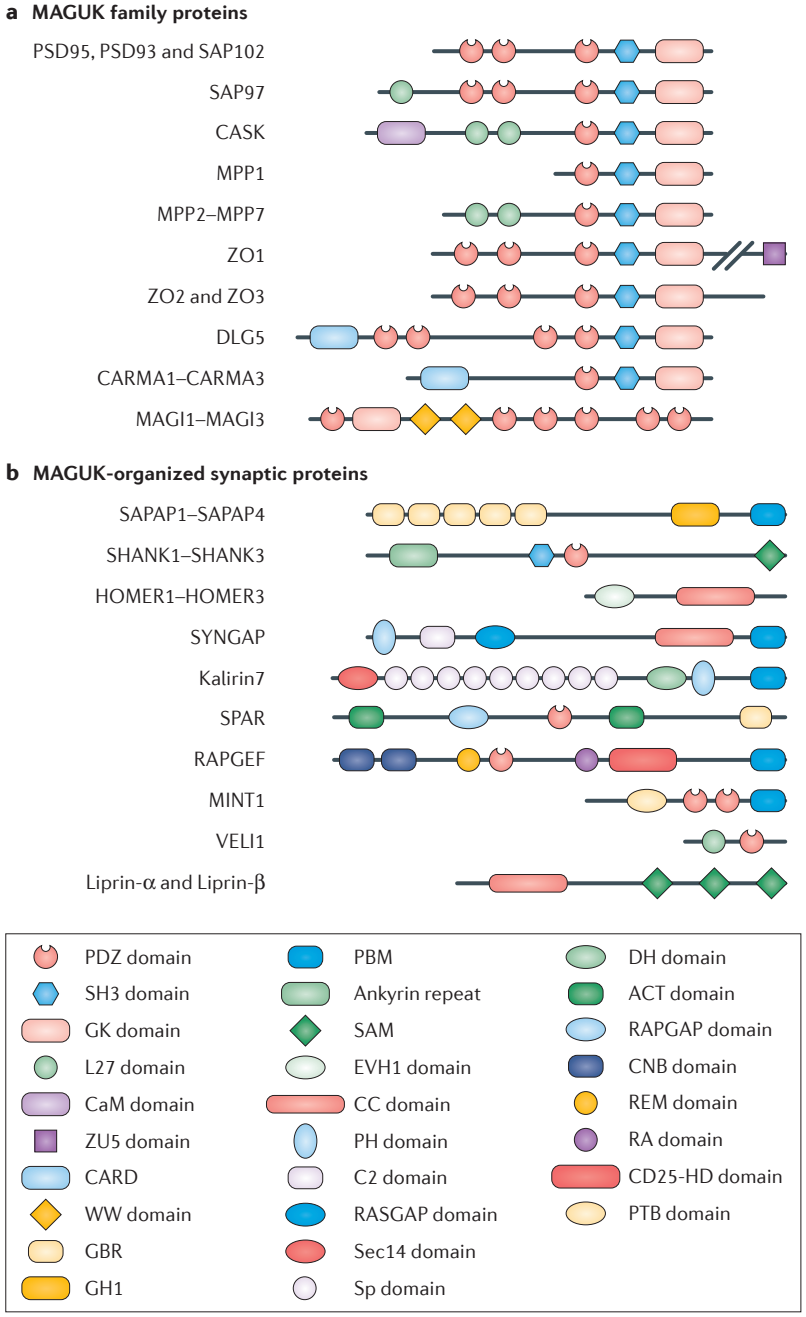


Figure 1 | **Scaffold protein-mediated protein complex organizations in excitatory synapses.** Scaffold proteins, in particular membrane-associated guanylate kinases (MAGUKs), have central roles in interfacing synaptic membrane receptors or channels with signalling enzymes and cytoskeletal proteins deep in the postsynaptic density (PSD). The domain structures of Discs large homologue (DLG) MAGUKs and members of two of their interacting postsynaptic scaffold proteins, synapse-associated protein 90 (SAP90)/PSD95-associated protein (SAPAP) and SRC homology 3 (SH3) and multiple ankyrin repeat domains protein (SHANK), are shown in the figure because the DLG–SAPAP–SHANK complex is thought to be one of the most critical proteins that determine the overall architecture of PSDs (the rest of the proteins are drawn using simple schematics). The DLG–SAPAP–SHANK complex and glutamate receptors often form co-clustered dynamic nanodomains (the AMPA-type glutamate receptor (AMPAR) cluster shown at the centre of the postsynaptic side represents one of these nanodomains), which are aligned with presynaptic neurotransmitter release sites, allowing strong postsynaptic responses following neurotransmitter release. The clustering of AMPARs may be facilitated by the multimerization of DLG MAGUKs, as indicated by the two-way arrow. A growing list of synaptic adhesion proteins serves to connect pre- and postsynaptic membranes and to regulate synaptic development and plasticity. Many such synaptic adhesion proteins (for example, interleukin-1 receptor accessory protein-like 1 (IL1RAPL1)) can bind to MAGUKs by their cytoplasmic tails. The synaptic protein complexes that are organized by scaffold proteins are often dynamic and can be regulated by synaptic activity-induced protein modifications such as phosphorylation (indicated by red circles with a 'P' at the centre). In addition, several small GTPase regulatory proteins such as SYNGAP, RAP guanine nucleotide exchange factor (RAPGEF), spine-associated RAP GTPase activating protein (SPAR) and kalirin 7 are also known to be intimately involved in the dynamic assemblies of synaptic protein complexes. Selected presynaptic protein complexes involved in neurotransmitter release and synaptic adhesion are also shown here. The domain structure for the presynaptic MAGUK calcium/calmodulin-dependent serine protein kinase (CASK) is also shown. AKAP, A-kinase anchor protein; CaMKII, calcium/calmodulin-dependent protein kinase II; CaN, calcineurin; Cav2.2, voltage-gated calcium channel subunit-α Cav2.2; EphR, ephrin receptor; GBR, GK-binding region; GK, guanylate kinase-like; GRIP, glutamate receptor-interacting protein; L27, LIN2–LIN7; LAR, leukocyte common antigen related; LRRTM, leucine-rich repeat transmembrane protein; MAGI, membrane-associated guanylate kinase inverted; MAP1A, microtubule-associated protein 1A; mGluR, metabotropic glutamate receptor; NGL-3, netrin-G3 ligand; NMDAR, NMDA-type glutamate receptor; PBM, PDZ-binding motif; PDZ, PSD95–DLG1–Zonula occludens 1; PKA, protein kinase A; PKC, protein kinase C; PICK1, protein interacting with C kinase 1; SAM, sterile α-motif; TARP, transmembrane AMPAR regulatory protein; VELI1, vertebrate lin-7 homologue 1.



◀ Figure 2 | **Domain organization of MAGUKs and their related synaptic proteins. a** | Schematic diagrams of the domain organization of the membrane-associated guanylate kinase (MAGUK) family proteins. As their names indicate, each MAGUK contains a guanylate kinase-like (GK) domain, which has lost its nucleotide kinase activity and become a phosphoprotein-binding module. Every MAGUK member contains at least one PDZ (PSD95–DLG1–ZO1) domain, which is known to bind to short carboxy-terminal tails of various receptors or ion channels (FIG. 1). Except for membrane-associated guanylate kinase inverted (MAGI) proteins, all MAGUKs contain a PDZ–SH3–GK (PSG) tandem in their domain organizations and such a domain organization pattern has been conserved throughout MAGUK evolution. The PSG tandems of MAGUKs are increasingly recognized to function as structural and functional supramodules in binding to their specific target proteins. **b** | Domain organization of selected proteins forming MAGUK-assembled synaptic complexes. In addition to the major scaffold proteins that form complexes with various MAGUKs, we have also included several MAGUK-binding small GTPase regulatory proteins to emphasize the potential dynamic regulation of postsynaptic density protein complex assembly. CaMK, calcium/calmodulin-dependent protein kinase; CARD, caspase activation and recruitment domain; CARMA, CARD-containing MAGUK protein; CASK, calcium/calmodulin-dependent serine protein kinase; CC, coiled-coil; CDC25-HD, RASGEF domain; CNB, cyclic nucleotide binding; DH, DBL homology; DLG, Discs large homologue; EVH1, Enabled/VASP homology 1; GBR, GK-binding region; GH1, GKAP homology domain 1; L27, LIN2–LIN7; PBM, PDZ-binding motif; PH, pleckstrin homology; PSD, postsynaptic density protein; PTB, phosphotyrosine-binding; RA, RAS-associating; RAPGAP, RAP GTPase-activating protein;RAPGEF, RAP guanine nucleotide exchange factor; RASGAP, RAS GTPase-activating protein; REM, RAS-GEF-like; SAM, sterile α-motif; SAP, synapses-associated protein; SAPAP, SAP90/PSD95-associated protein; Sec14, Sec14p-like; SH3, SRC homology 3; SHANK, SH3 and multiple ankyrin repeat domains protein; Sp, spectrin-like; SPAR, spine-associated RAP GTPase activating protein; VELI1, vertebrate lin-7 homologue 1; ZO, Zonula occludens; ZU5, ZO1–UNC5.

removal of these synaptic transmembrane proteins[4,29,47,48]. PSD95 (and probably also PSD93 and SAP102) directly associates with the PBMs of the NR2 subunits of NMDARs via its PDZ1 and PDZ2 domains[49]. By contrast, PSD95 indirectly interacts and recruits AMPARs through binding, via its PDZ domains, to the PBMs of stargazin and other transmembrane AMPAR regulating proteins (TARPs)[50–52].

These direct and indirect interactions are essential for the trafficking, anchoring and clustering of both NMDARs and AMPARs at synapses during synaptic development and signalling[48,52–54]. Knockdown of PSD95 disrupts the nanodomain organization of AMPARs[23] and downregulation of AMPARs disturbs the sub-synaptic clustering of PSD95 (REF. 31). The distribution of the receptors and scaffolds in nanodomains is not uniform within the spine and varies greatly between different synapses and even within the same synapse at different times. Both NMDARs and AMPARs have been found in either the central or peripheral regions of synaptic membranes[31]. Importantly, synapses with AMPARs clustered in nanodomains tend to have larger mean excitatory postsynaptic currents than synapses with uniformly distributed receptors[23]. Thus modification of receptor–scaffold interactions within the nanodomains could amplify or decrease synaptic transmission, even in the absence of altered receptor numbers[31]. However, the molecular mechanisms that determine both the density and size of the receptors and scaffolds in nanodomains are largely unknown.

An increasing body of evidence indicates that the interactions between MAGUKs and cell adhesion molecules may provide a direct *trans*-synaptic link to align the postsynaptic compartment with release sites for presynaptic neurotransmitters[55,56]. Cell adhesion molecules — including neurexins and neuroligins, leucine-rich repeat transmembrane proteins (LRRTMs), synaptic cell adhesion molecule (SYNCAM), N-cadherin, ephrin and Eph receptors, and the type IIa receptor protein tyrosine phosphatases and their postsynaptic binding partners such as tropomyosin receptor kinase C (TRKC), SLIT and NTRK-like proteins (SLITRKs), interleukin-1 receptor accessory protein-like 1 (IL1RAPL1), netrin-G3 ligand (NGL3) and SALM3 — function as bridges across the synaptic cleft, playing essential parts in promoting synapse formation and plasticity[57–59] (FIG. 1). Neurexins and neuroligins, which form one of the best-studied adhesion molecule pairs in synapses, interact with each other via their extracellular domains with exquisite specificities[60–62]. The C-terminal tail PBMs of neurexins and neuroligins interact with the PDZ domains of CASK and PSD95 (or MAGI2 at inhibitory synapses) at presynaptic and postsynaptic sites, respectively[40,63,64]. The CASK CaM-kinase-like domain binds to the three sterile alpha motif domains of liprin-α, a core scaffold protein in the presynaptic active zone, thus coupling the synaptic vesicle exocytosis and release machinery to the adhesion molecules[45,65] (FIG. 1). Such MAGUK-aligned *trans*-synaptic protein interaction networks may also facilitate the retrograde modulation of presynaptic maturation and neurotransmitter release by PSD95 and neuroligins[66–68]. Fitting with the crucial functions of MAGUK-organized *trans*-synaptic complexes, mutations in *CASK* have been found in patients with X-linked mental retardation (XLMR), FG syndrome (also known as Opitz–Kaveggia syndrome) and cerebellar hypoplasia[41,69–72]. Mutations in the genes of several adhesion molecules (such as neurexins, neuroligins and contactin-associated protein-like 2 (CNTNAP2; also known as CASPR2)) are associated with autism, schizophrenia and XLMR[73–76].

Non-membrane targets. Numerous non-transmembrane synaptic MAGUK-associated proteins have been identified in the past two decades (some of these are shown in FIG. 2b). Although most of these MAGUK-associated proteins are scaffold proteins without enzymatic activities, several enzymes have been found to directly interact with MAGUKs in synapses. Some of these MAGUK-interacting enzymes are small GTPase regulatory factors, such as RAS–RAP-GTP activating protein (GAP) SYNGAP, spine-associated RAP-GAP (SPAR) and RAP guanine nucleotide exchange factor (GEF) RAPGEF, and RHO GEF kalirin 7 (FIG. 2b), indicating that intimate connections exist between synaptic activities and small G-protein-regulated signalling events. The PSD95-binding protein SYNGAP is particularly interesting, as, being a catalytically active enzyme, it exists at a near-equal molar amount of PSD95 and thus counts as one of the most abundant proteins in PSD[24,77]. Downregulation of SYNGAP leads to premature hippocampal spine formation and over-activation of excitatory synapses in young mice[78]; this effect might explain the molecular basis of cases of epilepsy and autism that have been linked to *SYNGAP* mutations[79–81]. The association of SYNGAP with PSD95 can be rapidly regulated by neuronal activity. A few minutes after chemical LTP induction, SYNGAP was found to disperse from the PSD and thus promote spine formation, presumably via RAS-mediated actin cytoskeleton formation[82]. The rapid, LTP-induced SYNGAP dispersion from PSDs is a good example of the highly dynamic nature of the PSD structure. Kalirin 7 directly interacts with PDZ domains of DLG MAGUKs via its C-terminal PBM and regulates spine morphogenesis at excitatory synapses[83]. Kalirin 7 knockout mice showed a decrease in spine density in the hippocampus and abnormal anxiety-like behaviour[84].

Dendritic spines contain a network of highly branched actin molecules that are concentrated at sites immediately beneath the PSD. The actin cytoskeleton stabilizes and modulates the dynamic PSD structure in response to neuronal activity inputs[25,27,85–87]. Super-resolution imaging studies have shown that the actin cytoskeleton is unevenly distributed and can be rapidly reorganized within synapses[20,28,88]. Activity-promoted remodelling of the actin cytoskeleton is understood to be the foundation of synaptogenesis and synaptic plasticity[27,86,89]. The PSD95–SAPAP–SHANK complex, the key postsynaptic scaffold protein network, provides a direct link between membrane receptors–cell adhesion molecules and the actin cytoskeleton, and thus facilitates effective activity-dependent dendritic remodelling. SAPAP is essential for the recruitment of SHANK proteins to excitatory synapses and is known to interact with the GK domain of PSD95 (REF. 90). The PSD95 GK domain–SAPAP interaction requires the phosphorylation of the N terminal 14 amino acid repeats of SAPAP (termed the GK-binding region (GBR))[3,91] (FIG. 2b). The C-terminal PBM of SAPAP specifically binds to the PDZ domain of SHANK[92]. SHANK directly binds to several actin nucleation-initiating proteins such as cortactin and the actin-related protein 2 (ARP2)–ARP3 complex, promoting the docking of the actin cytoskeleton to the PSD[20,92–94]. In addition, PSD95 can directly bind to cytoskeleton-associated proteins such as microtubule-associated protein 1A (MAP1A) and cysteine-rich PDZ-binding protein (CRIPT) via its GK and PDZ3 domains, respectively[95,96]. The SAPAP and SHANK family proteins have been strongly implicated in the pathophysiology of various psychiatric disorders such as obsessive–compulsive disorder and autism[97–101].

Dendritic remodelling
A biochemical process of dendritic spines in which the actin cytoskeleton undergoes a rapid change in shape and structure in response to various stimuli.

Structural basis of MAGUK interactions

We focus here on the structural basis of MAGUK–target interactions. We begin by briefly updating recent findings relating to PDZ domain–target interactions; a more comprehensive overview of such interactions was covered in our recent review[102].

PDZ domain-mediated target interactions. PDZ domain-containing proteins are highly abundant in excitatory synapses[4] and every member of the MAGUK family contains at least one PDZ domain. The best-known function of PDZ domains is to bind to a short stretch of amino

acids at the C-terminal tail of target proteins, although PDZ domains can also associate with internal peptide segments[103]. In addition, many PDZ domains can interact with phospholipids[104]. An important theme emerging from recent studies is that additional amino acid residues beyond the canonical PDZ domains and PBMs, including PDZ domains from MAGUKs and PBMs from MAGUK targets (referred to as 'extended PDZ domains' and 'extended PBMs', respectively), often have crucial roles in forming highly specific and stable PDZ domain-containing protein–target complexes[102,105].

PSD95 interacts with ion channels and glutamate receptors via its first two PDZ domains, which are connected by a short and highly conserved link[4,102]. Several studies have shown that the inter-domain orientation between PDZ1 and PDZ2 of PSD95 is partially fixed, facilitating synergistic binding of PDZ12 tandem to multimeric targets[106–108] (FIG. 3a). This multivalency effect has been implicated in the PSD95-mediated clustering of membrane receptors and ion channels[109] (FIG. 3a). The discovery of the bivalent target interaction mode of PSD95 also prompted the development of multimeric inhibitors to block associations of PSD95 with its physiological targets such as NMDARs and TARPs with high specificity and potency[108,110,111].

Inactivation no after potential D (INAD) assembles the core components of the photo-transduction signalling pathway of fly photoreceptors via its five PDZ domains[112]. When flies are kept in the dark, two cysteine residues (Cys606 and Cys645) near the PBM-binding pocket of PDZ5 are in the reduced form, which allows the binding of its target with high affinity. On exposure to light, the two Cys residues are oxidized to form an intramolecular disulfide bond and this leads to deformation of the PBM-binding pocket and dissociation of the target from PDZ5 (REFS 113,114) (FIG. 3b). The light-dependent INAD PDZ45 domain–target interaction indicates that, in addition to passively organizing signalling complexes, scaffold proteins can actively participate in signalling processes by directly regulating their binding to target proteins. Given that MAGUKs, analogous to INAD, function to organize protein complexes in key signalling pathways in synapses, it may not be surprising that similar active signalling regulations are also adopted by some MAGUK scaffolds.

Long-distance, conformation-coupled allosteric regulation appears to be a common strategy in regulating PDZ domain–target recognition. Such a conformational coupled PDZ–target interaction was initially observed in the interaction between the PSD95 PDZ3 domain and its target, CRIPT peptide[115]. An α-helix extension at the C-terminal end of the canonical PDZ boundary stabilizes the conformation of the PDZ domain and therefore increases binding to this peptide. A similar C-terminal helix extension-induced enhancement of target-binding affinity was also observed for the PDZ2 domain of sodium–hydrogen exchange regulatory cofactor 1 (NHERF1)[116]. Another example of allosteric regulated interaction is partitioning defective protein 6 (PAR6), a master cell polarity regulator that has crucial roles in neurogenesis and axon development and is known to bind to MPP5 (also known as PALS1 MAGUK)[117]. PAR6 contains a semi-CDC42–RAC-interactive binding (CRIB) motif immediately N terminal to its PDZ domain. The semi-CRIB motif, together with the PDZ domain of PAR6, associates with CDC42 in a GTP-dependent manner[118]. In the absence of CDC42, the semi-CRIB sequence is unstructured and the PDZ domain interacts with its target (for example, MPP5) with a low affinity. On binding to CDC42, the semi-CRIB motif adopts a β-strand conformation, which physically couples CDC42 and the PAR6 PDZ domain together by forming a continuous, intermolecular β-sheet[118] (FIG. 3c). The binding of CDC42 to the semi-CRIB sequence induces obvious conformational changes to the βA–βB-loop of the PAR6 PDZ domain and positions a highly conserved Lys (Lys165) in a conformation favourable for PBM binding[117] (FIG. 3c). Such conformation-coupled allosteric regulation of PDZ–target interactions may offer mechanistic explanations about activity-dependent modifications of protein interaction networks in synapses.

GK domains as phosphoprotein-binding modules. The MAGUK GK domains evolved from the yeast GK enzyme, which catalyses the transfer of phosphate groups from ATP to GMP. During evolution, the GK domain lost its enzyme activity, although the crucial residues that bind to the phosphate group of GMP are conserved[119,120]. Rather than functioning as a nucleotide kinase, the GK domains of MAGUKs bind to a large number of proteins from synapses and other cellular compartments[90,96,121–123].

A major advancement in understanding MAGUK GK domains came from the discovery that the GK domains of DLG MAGUKs specifically recognize their targets in a phosphorylation-dependent manner[91]. This discovery was led by an earlier finding showing that phosphorylation of a Ser residue in the central link region of the scaffold protein LGN (also known as GPSM2) is required for SAP97-mediated spindle orientation during asymmetric cell divisions in early brain development[124–126]. In searching for the interaction mechanism between SAP97 and LGN, it was discovered that the SAP97 GK domain binds to a phosphorylated LGN peptide with a submicromolar dissociation constant. Removal of the phosphate group from Ser401 of LGN eliminated SAP97 GK domain–LGN binding[91]. The molecular basis of SAP97 GK domain-mediated phospho-LGN recognition was elucidated by the crystal structure of the GK domain in complexes with a phosphorylated LGN peptide (pLGN)[91]. The pLGN peptide in the complex adopts a one-turn α-helix followed by a β-strand conformation, occupying the concave groove formed by the GMP-binding subdomain and the 'CORE' subdomain of the SAP97 GK domain (FIG. 4a). The phosphate group of pLGN forms extensive interactions with several residues in the GMP-binding subdomain (FIG. 4b). Additional hydrophobic interactions between pLGN and the SAP97 GK domain (at the ψ1, ψ2 and ψ3 sites) further enhance formation of the complex[3,91] (FIG. 4b).

Similar phosphorylation-dependent binding exists between DLG and LGL, the key components of the master cell polarity regulatory Scribble complex (DLG–LGL–SCRIB)[127–130]. LGL2 directly interacts with the PSD95 GK domain and the interaction strictly requires

Multivalency effect
Binding avidity enhancement brought by synergistic actions of individual interaction sites between multi-domain scaffold protein and multivalent target interactions.

Allosteric regulation
Binding of an effector molecule to a specific site of a protein causes conformational changes far away from the binding site and thus alters the function of the protein.

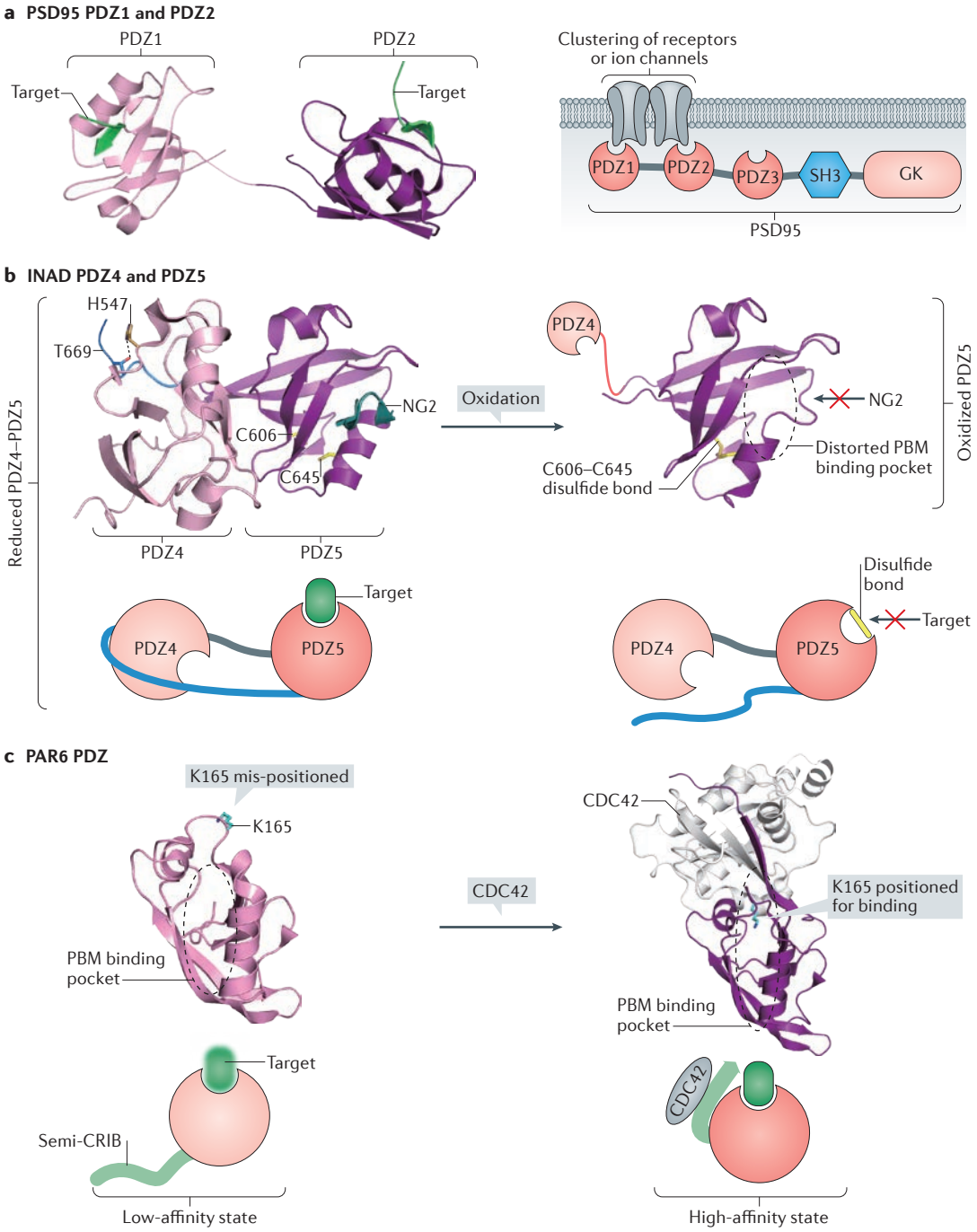


Figure 3 | **Regulated PDZ–target interactions. a** | The ribbon diagram on the left shows a representation of the structure of the postsynaptic density protein 95 (PSD95) PDZ1 (PSD95–DLG1–Zonula occludens 1) and PDZ2 tandem with each domain in complex with a target peptide (PDB code: 2KA9; REF. 106). The right-hand image highlights that the near-parallel alignment of the two target-binding channels of the PDZ1–PDZ2 tandem allows DLG membrane-associated guanylate kinases (MAGUKs) to bind to multimeric receptors and ion channels such as NMDA-type glutamate receptors, or to promote dimerization of its binding targets on synaptic membranes. **b** | Inactivation no after potential D (INAD) PDZ4–PDZ5 tandem in its reduced form (left) forms a complex with the NG2 peptide (PDB code: 3R0H; REF. 114); whereas when PDZ5 is uncoupled with PDZ4 (right), it becomes oxidized and has a distorted PDZ-binding motif (PBM) binding pocket and thus is incapable of binding to NG2 (PDB code: 2QKT; REF. 113). As MAGUK PDZ domains also form supramodules, it is possible that MAGUK scaffolds may adopt analogous active signal regulation roles. **c** | Ribbon diagrams showing partitioning defective protein 6 (PAR6) PDZ alone (PDB code: 2LC7; left) and in complex with cell division control protein 42 homologue (CDC42) (PDB code: 1NF3; right; REF. 118). The binding of CDC42 to the semi-CDC42–RAC-interactive binding (CRIB) region of PAR6 PDZ converts PAR6 into a conformational state with a higher affinity for its target, as highlighted by the repositioning of K165 in the target-binding groove of the PDZ domain. DLG, Discs large homologue; GK, guanylate kinase-like; PBM, PDZ-binding motif; SH3, SRC homology 3 domain.

Phosphoprotein-binding modules
Protein domains such as the SH2 domain, the GK domain and the FHA domain that can specifically recognize phosphorylated proteins.

the phosphorylation of at least one of the three conserved phosphorylation sites located in the central linker of LGL2 (REF. 127). The structures of the PSD95 GK domain in complex with phospho-LGL2 (pLGL2) peptides reveal that these peptides bind to the GK domain in a similar fashion to pLGN, consolidating the concept that the DLG GK domains function as specific phosphoprotein-binding modules[127]. These structural studies revealed that DLG GK domains recognize their phospho-targets mainly via two discrete sites: a phospho-Ser-binding site (phospho-site) and hydrophobic sites (ψ1–ψ3). In addition to these two common binding sites, other polar residues (charged sites

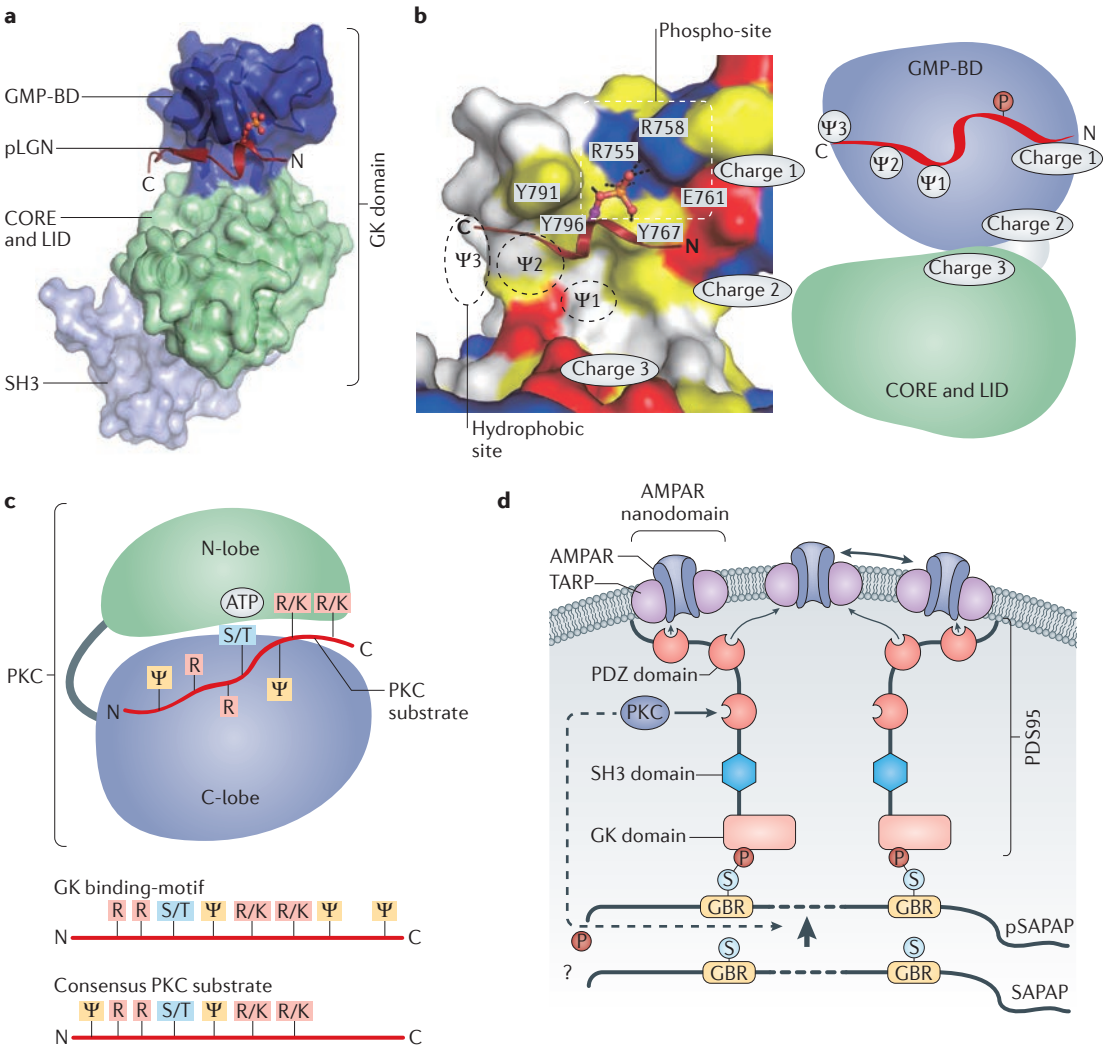


Figure 4 | **Mechanistic basis governing the phosphorylation-dependent target binding of MAGUK GK domains.** **a** | The images shows the structure of the synapse-associated protein 97 (SAP97) SRC homology 3 (SH3)– guanylate kinase-like (GK)–phosphorylated LGN (pLGN) complex (PDB code: 3UAT; REF. 91). The GK domain consists of the GMP-binding domain (GMP-BD), originally defined in yeast guanylate kinase, and a helical subdomain known as the 'CORE and LID'. The phosphate group of the pLGN is shown as a stick-and-ball structure. **b** | The surface representation (left) and schematic diagram (right) show the SAP97 GK domain–pLGN interface. In this representation, the hydrophobic residues are in yellow, the positively charged residues are in blue, the negatively charged residues are in red, and the rest of the amino acids are in grey. The pLGN peptide binds to the 'phospho-site' and the 'hydrophobic sites' (ψ1–ψ3) of the SAP97 GK domain. Additional polar residues (charged sites 1–3) can further enhance the binding affinity and specificity of this interaction. **c** | Schematic diagram showing the substrate recognition pattern of protein kinase C (PKC) derived from the structure of atypical PKC in complex with a substrate peptide[133]. Note that the consensus GK-binding motif fits well with the PKC substrate recognition motif. **d** | Schematic diagram showing that PKC PSD95–Discs large homologue 1–Zonula occludens 1 (PDZ)-binding motif-based docking to PSD95 PDZ domains can promote PKC-mediated phosphorylation of SAP90/postsynaptic density protein 95 (PSD95)-associated protein (SAPAP) and subsequently enhance PSD95–SAPAP complex formation. The formation of the PSD95–SAPAP complex can further promote clustering and formation of AMPA-type glutamate receptor (AMPAR) nanodomains. The connection between AMPARs and PSD95 is through the AMPAR accessory protein transmembrane AMPAR regulatory protein (TARP) and PDZ domains of PSD95. GBR, GK-binding region.

1–3) can further enhance the binding affinity and specificity of DLG GK domains[91,127] (FIG. 4b). Amino acid sequence analysis reveals that the residues forming the phospho-site and the hydrophobic sites are highly conserved among the majority of the MAGUK family proteins, including DLGs, CASK, MPPs and MAGIs[3]. Consistent with this analysis, the structures of the GMP-binding subdomains of the GK domains of DLGs, CASK, MPP1 and MPP5 are highly similar to each other, suggesting that binding to phosphoproteins is probably a common feature of the GK domains of DLGs, CASK, MPPs and MAGIs. It is noted that ZO1, DLG5 and CARMA GK domains lack most of the key residues found in the phospho-site and hydrophobic sites in DLG GK domains[3], suggesting that these three MAGUK GK domains do not function as phosphoprotein-binding modules.

The discovery of phosphorylation-dependent MAGUK GK domain–target interactions is particularly interesting for understanding MAGUK-mediated organization of synaptic protein complexes. SAPAP1 was shown to interact with the GK domains of PSD95 and MAGI2 (also known as S-SCAM) nearly 20 years ago[90,131]. The SAPAP1–GK domain interactions have been narrowed to several GBR motifs in the N terminus of SAPAP1. Amino acid sequences of the SAPAP1 GBR motifs are highly similar to those of the LGN and LGL2 peptides[91], suggesting that PSD95 and MAGI2 GK domains also bind to SAPAP1 in a phosphorylation-dependent manner. Indeed, phosphorylated SAPAP1 peptides bind to PSD95 GK domains with an affinity comparable with that of pLGN binding to such domains; removal of the phosphate group from the SAPAP1 phospho-peptides eliminates their binding to the PSD95 GK domain[91]. Interestingly, the Ser residues in GBR1 and GBR5 (Ser54 and Ser201 in human SAPAP1, which are conserved in all SAPAP isoforms) have been reported to be phosphorylated *in vivo*[132]. The phosphorylation-dependent interaction between PSD95 GK domains and SAPAP indicates that the GK domain-mediated synaptic complex assembly is a regulated event, which is probably coupled to changes in synaptic activity. Further studies are required to validate whether such a switch is indeed used in physiological settings, and which kinase is involved in the phosphorylation of SAPAP.

Analysis of the known MAGUK GK domain binding phospho-targets comes up with a highly conserved GK domain binding motif of -R/K-R/S-X-pS/T(0)-ψ-R/K-R/K-ψ-, where ψ represents hydrophobic residues, X denotes any residue and pS/T represents phospho-Ser or phospho-Thr. We note with extreme interest that this GK domain binding motif bears a high similarity to the protein kinase C (PKC) substrate recognition and phosphorylation motif that we characterized recently[133] (FIG. 4c; see Supplementary information S1 (figure)). Furthermore, LGN and LGL2 have been shown to be phosphorylated by atypical PKC (aPKC), a member of the PKC family, on the Ser residues responsible for binding to MAGUK GK domains[126,128,129]. It is well known that the PKC family of enzymes is actively involved in regulating synaptic activity[134–137]. In addition, PKCs are highly abundant in PSDs (~10 μM)[24,138]. Several PKC kinases contain PBMs in their C termini (Supplementary information S1 (figure)). It is possible that PKC PBMs could bind to MAGUK PDZ domains in synapses and thus dock the kinases to allow them to phosphorylate their specific targets (for example, SAPAP) (FIG. 4d). It is tempting to speculate that MAGUK GK domain-mediated target interactions may be regulated by the neuronal activity-modulated changes in PKC activity. As both PSD95 and SAPAP are important for synaptic development and plasticity, phosphorylation-dependent PSD95–SAPAP complex formation may represent a mode of synaptic activity regulation.

The MAGUK PDZ–SH3–GK supramodule. All of the MAGUK family proteins, except MAGIs, share the common PSG core architecture (FIG. 2a) and this PSG organization has been a conserved feature of MAGUKs throughout evolution[139]. The SH3 domain and the GK domain of MAGUKs have been shown to form an integrated structural unit, as the folding of the SH3 domain requires a stretch of ~10 residues immediately following the C-terminus of the GK domain[91,119,120]. Several groups have provided evidence showing direct interactions between the PDZ and SH3 domains[140–143], although in some cases the interactions are only transient. A recently solved structure of the MPP5 PSG in a complex with the cytoplasmic tail of crumbs (CRB-CT, which is conserved in all isoforms of crumbs) revealed that all three domains of the MPP5 PSG, which form a stable and integral structural unit, are involved in binding to CRB-CT. The interaction between MPP5 PSG and CRB-CT is of much higher affinity than the interaction between the isolated MPP5 PDZ domain and CRB-CT (K_d ~70 nM versus ~6 μM)[40] (FIG. 5). Similarly, the affinity between the CASK PSG and the cytoplasmic tails of neurexins is much higher than that between the CASK PDZ domain alone and neurexin cytoplasmic tails[40]. This study offers an important mechanistic explanation of the long-standing disparity between the promiscuous MAGUK PDZ domain–target interactions that have been observed in numerous biochemistry-based studies *in vitro* and the functionally highly specific MAGUK–target interactions that have been observed in studies based on genetics and/or cell biology *in vivo*[1,4,34]. Mechanistically, in the MPP5 PSG–CRB-CT complex, the C-terminal PBM of CRB-CT fits into the canonical target-binding groove of the PALS1 PDZ domain. Importantly, the SH3 domain is also intimately involved in binding to the residues of PBM via stable structural coupling between PDZ and SH3 domains, resulting in a clasp that physically embraces the CRB-CT PBM (FIG. 5b). The residues forming the PDZ–SH3 clasp are unique among MAGUK PSGs and therefore partially determine the binding specificities for various MAGUK PSGs and their targets[40]. Unexpectedly, an eight-residue fragment upstream of the CRB-CT PBM binds to the GK domain of the MPP5 PSG following a manner analogous to phospho-peptide binding to the DLG GK domain, except that the residue corresponding to phospho-Ser in MAGUK GK domain target peptides is a Glu residue (FIG. 5a). This observation raises the possibility that other MAGUK PSG tandems may also use similar strategies to recognize their targets and bind to them with high affinity and specificity. In addition, bindings between

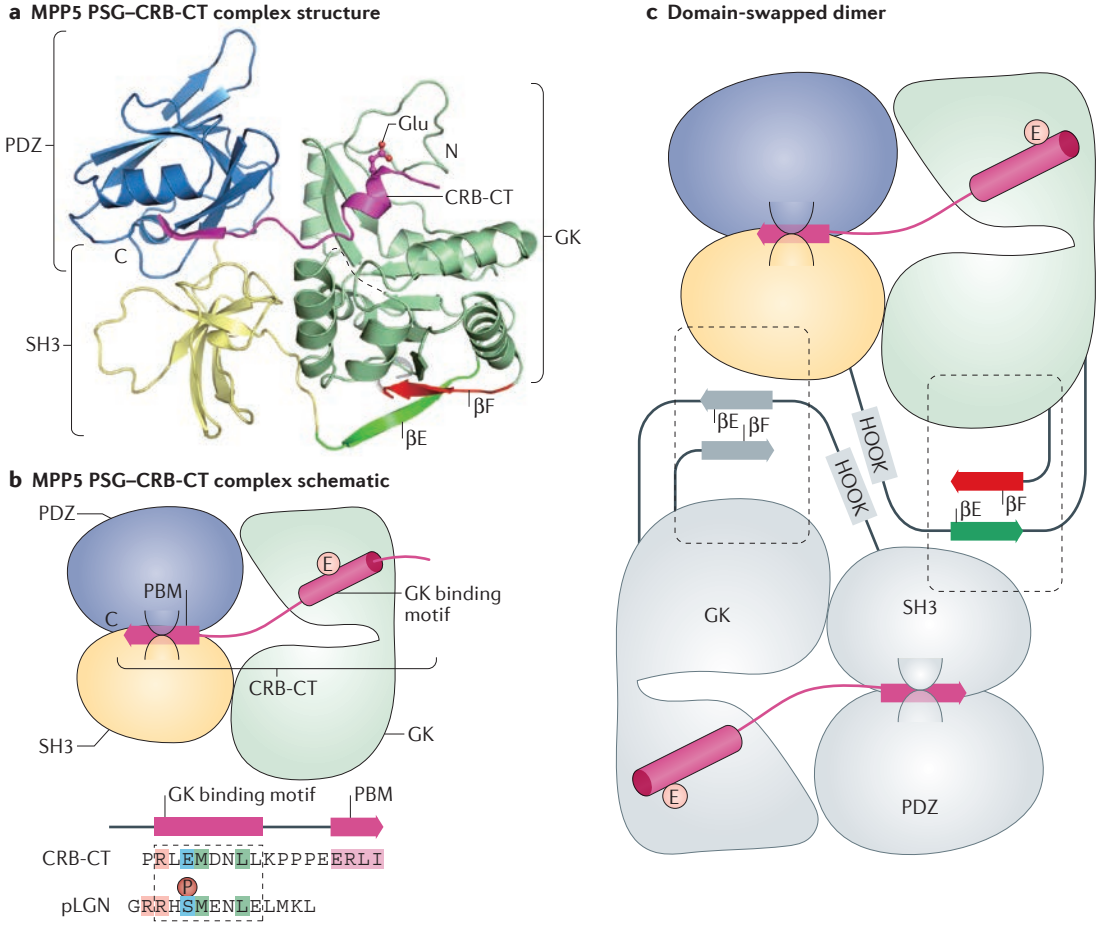


Figure 5 | **Formation of the MPP5 PSG supramodule and CRB-CT complex. a** | This panel shows a ribbon diagram of the MPP5 PSG–CRB-CT complex structure (PDB code: 4WSI; REF. 40). The Glu residue corresponding to the phospho-Ser in the phospho-target of the guanylate kinase-like (GK) domain is shown as a stick-and-ball model. **b** | This panel provides a schematic diagram of the MPP5 PSG–CRB-CT complex structure. The structure-based sequence alignment of CRB-CT and phosphorylated LGN (pLGN) shows that the amino-terminal helical region of CRB-CT resembles the helical region of pLGN that binds to the Discs large homologue 1 (DLG1) GK domain described in FIG. 4a. The highlighted residues in the alignments are directly involved in binding to membrane-associated guanylate kinase (MAGUK) GK domains. **c** | The MPP5 PSG forms a symmetrical domain-swapped homodimer. The flanking sequences of the GK domain of one monomer form a pair of β-strands (βE and βF, highlighted in the dashed box) that interact with the partial SRC homology 3 (SH3) domain from another monomer unit. The 'HOOK' region connecting the SH3 and GK domains provides enough space for the domain-swapping to occur. PBM, CRB-CT, cytoplasmic tail of crumbs; PDZ-binding motif; PDZ, PSD95–DLG1–Zonula occludens 1; PSG, PDZ–SH3–GK.

Supramodules
Two or more protein modules arranged in tandem in a protein that interact with each other to form a high-order structure with functions distinct from those of the individual or simple sum of the modules.

Domain-swapped dimer
Two identical protein molecules associate to form dimer by exchanging identical structural elements, such that native intramolecular interactions are replaced by their intermolecular counterparts.

MAGUK PSG targets and MAGUK GK domains could be constitutively on if the GK recognition motif contains a negatively charged Glu or Asp, or be a kinase activity-regulated event if the GK recognition motif contains a Ser or Thr (FIG. 5b). Examples of such MAGUK PSG and target interactions include, but are not limited to, the PSD95–neuroligin[64], CASK–neurexin[144] and CASK–SYNCAM[145] complexes. A salient message is that, via binding to PSG supramodules, interactions between MAGUKs and their membrane targets are not only highly specific, but can also be regulated.

Another interesting finding from the MPP5 PSG–CRB-CT complex structure is that the MPP5 PSG forms a symmetrical domain-swapped homodimer (FIG. 5c). In this domain-swapped dimer, the flanking sequences of the GK domain of one monomer form a pair of β-strands (βE and βF) that interact with the βA–βD strands of the partial SH3 domain from another monomer unit, forming the complete, intermolecular SH3 fold (FIG. 5c). As the MPP5 PSG is a monomer in solution when unbound from CRB-CT, the formation of the domain-swapped MPP5 dimer in the complex is probably induced by binding to CRB-CT[40]. The coupling of the SH3 domain and the GK domain via SH3 fold completion is a common property of MAGUKs[91,119,120] (FIG. 6a,b). Mechanistic insights into this process have come from the rat SAP97 SH3–GK tandem. Tyr710 from βE inserts into the folding core of the SH3 domain and Trp904 from βF packs extensively with the residues forming the hydrophobic core of the SH3 domain (FIG. 6a; see Supplementary information S2 (figure)). Residues involved in βE and βF coupling are highly conserved among the majority of MAGUK

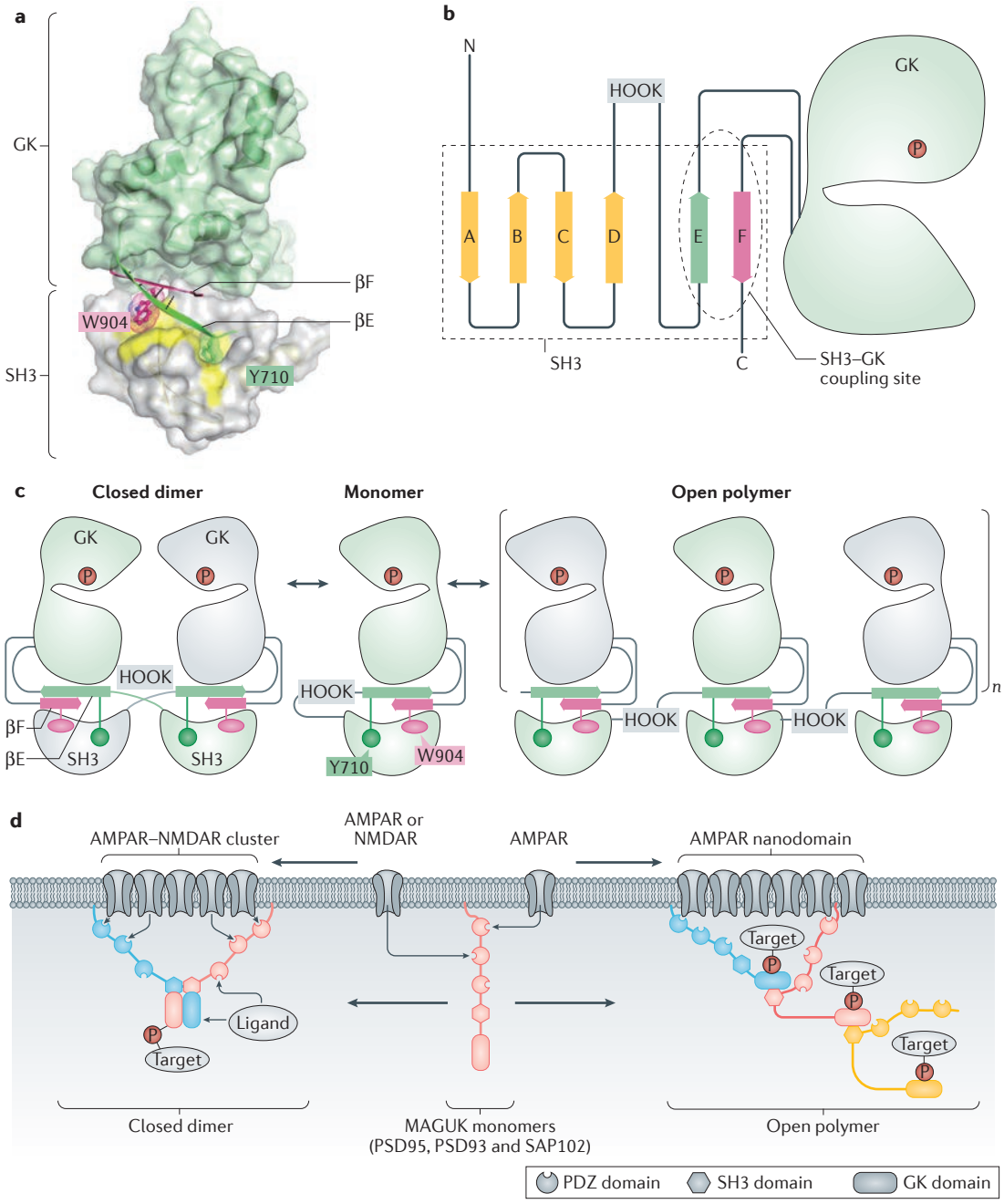


Figure 6 | **Mechanistic basis of possible formations of MAGUK polymers. a** | The surface diagram of the synapse-associated protein 97 (SAP97) SRC homology 3 (SH3)– guanylate kinase-like (GK) tandem (PDB code: 3UAT; REF. 91) shows that βE from the SH3 domain and βF at the carboxy-terminal end and outside the GK domain 'stitch' the SH3–GK domain together into an integral structural unit. The βE–βF-mediated coupling of SH3 and GK domains is a highly conserved feature in many membrane-associated guanylate kinases (MAGUKs) (Supplementary information S2 (figure)). **b** | βE–βF couple with the βA–βD to complete the SH3 fold in MAGUKs. Note that the phospho-target-binding site in GK domain is far away from the coupling site formed by βE and βF. **c** | A schematic model depicting the formation of 'closed dimers' or 'open polymers' of MAGUK SH3–GK tandems. If the βE and βF strands of an SH3–GK tandem form an intramolecular β-sheet, this SH3–GK tandem forms a monomer as shown in the middle of the panel. When two neighbouring SH3–GK tandems exchange their βE strands with each other, this results in the formation of a closed dimer, as depicted in the left of the panel. Alternatively, swapping of βE can occur between one SH3–GK with the next one in a relayed manner, so the domain-swapping results in the formation of an SH3–GK polymer as depicted in the right-hand panel. **d** | The models depict possible dimerization or multimerization of AMPA-type glutamate receptors (AMPARs) and/or NMDA-type glutamate receptors (NMDARs) beneath PSD membranes via binding to DLG MAGUKs. The formation of the closed SH3–GK domain-swapping-mediated DLG dimer can cause the formation of small AMPAR–NMDAR clusters (left). The formation of the open DLG polymer can induce formation of very large AMPAR receptor clusters or nanodomains (right). The binding of phosphorylated target proteins (shown as ovals) to the GK domain of DLG MAGUKs may have regulatory roles in the formation of DLG dimer or polymers. PDZ, PSD95–DLG1–Zonula occludens 1.

family proteins (Supplementary information S2 (figure)), indicating that formation of the SH3–GK supramodule is a common feature of MAGUKs. As the folding of MAGUK GK domains and interactions between GK domains and their phosphorylated target proteins do not require SH3 domains[91] (FIGS 4,6b) and the SH3 domains of MAGUKs are not likely to bind to canonical proline-rich sequences[119], the formation of SH3–GK supramodules via βE and βF coupling is probably important for PSG tandem-mediated target recognitions, as exemplified by the MPP5 PSG–CRB-CT complex.

Various studies have provided some evidence of the importance of βE and βF coupling-mediated MAGUK SH3–GK assembly in brain development and function. For example, truncation of the C-terminal 14-residue of DLG in *Drosophila melanogaster* (the *dlg*^{sw} allele)[33] led to imaginal disc overgrowth and failure of pupariation in larvae. As the deletion of these amino acids (corresponding to βF, as shown in FIG. 6a) in fly DLG does not affect binding of its GK domain to phosphorylated targets such as Pins[91], the *dlg*^{sw} mutant phenotype is most likely to be caused by impaired SH3–GK coupling. A recent study showed that the SH3 and GK domains of CARMA1 interact with each other in a way similar to that of DLG MAGUKs and that the interaction is indispensable for CARMA1-mediated activation of nuclear factor κB (NF-κB)[146]. A recent human genetic study identified mutations causing Tyr728 in CASK (corresponding to Tyr710 in βE of DLG1; FIG. 6a) to be replaced by Cys, which led to two brothers having microcephaly and X-linked intellectual disability with nystagmus, and a mutation leading to Trp919 in CASK (equivalent to Trp904 in βF of SAP97, as shown in FIG. 6a) being changed to Arg, which was associated with X-linked intellectual disability in a male individual[147], highlighting the importance of coupling between the SH3 and GK domains in CASK.

The structures of the SH3–GK supramodule that have been solved for PSD95 (REFS 119,120), SAP97 (REF. 91) and ZO1 (REFS 140,141) (either in their apo- or GK domain–target bound forms) show that βE–βF-mediated SH3–GK couplings are intramolecular in nature. The structure of the MPP5 PSG–CRB-CT complex indicates that target binding can induce intermolecular SH3–GK coupling[40] (FIG. 5c). Note that all MAGUKs contain a long insertion following βE that is known as the HOOK region (FIG. 6b), which could serve as a flexible linker for MAGUKs to form domain-swapped dimers or even polymers through βE–βF-mediated SH3–GK coupling[119] (FIG. 6c,d). Such intra- and intermolecular couplings between SH3 and GK domains can have profound implications in assembling MAGUK-mediated high-order protein complexes in synapses and other cellular settings. For example, DLG MAGUKs in the PSD may form an SH3–GK tandem-mediated closed dimer (FIG. 6c) similar to the MPP5 PSG dimer (FIG. 5c). It is also possible that DLG MAGUKs may form an open polymer via another form of intermolecular SH3–GK coupling (FIG. 6c). As the βE–βF pair coupling is highly conserved among most of the MAGUK family proteins, it is tempting to speculate that different members of DLG MAGUKs may form homo- and/or hetero-oligomers. The PSG target-binding-induced oligomerization observed in PALS1 is also interesting because it suggests that MAGUKs may undergo target-dependent oligomerization (FIG. 6d). Target-binding-regulated DLG oligomerization might provide a mechanistic explanation for how DLG MAGUK-assembled glutamate receptor nanodomains form and their dynamic nature (FIG. 6d).

Conclusions and perspectives

In addition to being key scaffold proteins that organize synaptic signal transduction complexes, MAGUKs are indispensable for embryonic development and tissue homeostasis in both invertebrates and vertebrates. Whole genome duplications during evolution have resulted in multiple paralogues in each subfamily of MAGUKs in mammals[139], presumably fulfilling their diverse functional and cognitive requirements[35]. The existence of such paralogues in mammals has imposed significant technical challenges in dissecting the functions of individual MAGUKs, at least partly because of functional redundancy and compensatory effects among subfamily members. Creative manipulations based on genetic and/or molecular biology from different laboratories in the past few years have used some interesting approaches to meet these challenges[35,54,148,149]. Recent developments of CRISPR/Cas9-based efficient gene editing techniques are likely to accelerate functional studies of individual MAGUKs in different tissues and at specific developmental stages.

In parallel, mechanistic-based studies of MAGUKs in the past few years have considerably expanded our understanding of the molecular bases governing interactions between MAGUKs and their targets and the dynamic regulation of such interactions. However, many questions remain to be answered. For example, although our understanding of the general target-binding properties of DLG MAGUKs is emerging and reaching some level of consensus, it is not clear whether each member has distinct target binding and regulation properties. This is an important question, as different DLG MAGUKs have been suggested to have distinct cognitive functions in higher mammals[35], but the mechanistic basis governing the functional diversity of such highly similar MAGUKs is not clear. It is not known whether allosteric regulation between different target-binding sites might exist within MAGUKs (for example, whether a ligand binding to the GK domain can regulate the target-binding properties of the PDZ–SH3 tandem of MAGUK PSG and vice versa). Whether different paralogues of MAGUKs can interact with each other to form oligomers also remains unclear. If they do, how do they interact and what are the functional implications? The MAGI subfamily members only contain a partial GK domain and lack a SH3 domain; little is known about the target-binding properties of this subfamily of MAGUKs.

Mutations in genes encoding many of the MAGUKs are associated with various human diseases, including psychiatric disorders and cancers. Thus it will be of great interest to uncover the mechanisms by which these mutations alter MAGUK properties in the hope of informing therapeutic development in these disorders.

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Competing interests statement
The authors declare no competing interests.

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