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View from the PEAKs: Insights from structural studies on the PEAK family of pseudokinases

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The PEAK family of pseudokinase scaffolds, comprising PEAK1 (originally termed SgK269), PEAK2 (SgK223, the human orthologue of rat Pragmin) and PEAK3 (C19orf35), have emerged as important regulators and integrators of cellular signaling and also play oncogenic roles in a variety of human cancers. These proteins undergo both homo- and heterotypic association that act to diversify signal output. Recently, structural and functional characterization of PEAK3 and its protein–protein interactions have shed light on PEAK signaling dynamics and the interdependency of PEAK family members, how PEAK dimerization regulates the binding of downstream effectors, and how 14-3-3 binding acts to regulate PEAK3 signal output. These important advances form the basis of this review.

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Introduction

Pseudokinases - an overview

Protein kinases are critical components of cellular signal transduction cascades, classically catalysing the transfer of the γ-phosphate of ATP to the side chains of specific Ser, Thr or Tyr residues on target substrates in order to regulate substrate activity, localization and/or protein–protein interactions [1,2]. The human complement of protein kinases comprises 535 members [2] and the vast majority of these share conserved sequence elements critical for phosphotransferase activity, specifically: the Lys residue within the VAIK (Val-Ala-Ile-Lys) motif that together with the Gly-rich loop mediates ATP binding; the Asp residue of the DFG (Asp-Phe-Gly) motif that co-ordinates Mg^{2+} in complex with ATP; and the Asp from the HRD (His-Arg-Asp) loop that acts as the catalytic base during phosphoryl transfer [1]. Approximately 10 % of protein kinases are classified as pseudokinases due to amino acid substitutions in one or more of these sequence elements that disable phosphotransferase activity [1,2]. The pseudokinases themselves can be subclassified into four classes depending on nucleotide and cation binding activity: those that bind neither (Class I); that bind nucleotides only (Class II); cations only (Class III); and those that bind both but are still catalytically inactive (Class IV) [3].

Importantly, despite loss of catalytic function, pseudokinases play key roles in cellular signaling via three broad mechanisms. First, by acting as molecular switches. This is best exemplified by the pseudokinase mixed lineage kinase domain-like (MLKL), which functions in the necroptosis cell death pathway. Here, in the inactive conformation of MLKL, the pseudokinase domain interacts with and restrains the four helical bundle 'killer' domain. Phosphorylation of MLKL on the pseudokinase domain activation loop (A-loop) by the upstream kinase receptor-interacting protein kinase 3 (RIPK3) triggers a conformational change that releases the 'killer' domain and promotes MLKL oligomerization and plasma membrane translocation and permeabilization [4]. Second, by functioning as allosteric regulators. For example, the pseudokinase HER3 directly transactivates the related family member and active tyrosine kinase EGFR via heterodimerization [5], and the pseudokinase domain of JAK2 associates with the adjacent tyrosine kinase domain to negatively regulate the activity of the latter [6,7]. Third, pseudokinases can function as scaffolds that mediate the assembly of signal transducing and/or regulatory protein complexes. For example, the Tribbles (TRIB) pseudokinases recruit specific E3 ubiquitin ligases, including COP1, via a conserved C-terminal motif, and specific protein targets for ubiquitin-directed degradation, such as C/EBPα, via the pseudokinase domain [8]. The TRIBs also interact

with MEK kinases via a different C-terminal motif and modulate AKT/FOXO signaling via poorly understood mechanisms [8].

The PEAK family of pseudokinases

The PEAK family of pseudokinases in humans, comprising PEAK1 (originally termed SgK269), PEAK2 (SgK223, the human orthologue of rat Pragmin) and PEAK3 (C19orf35), are classified as scaffolds and have emerged as important regulators and integrators of signaling downstream of a variety of cell surface receptors, including specific growth factor receptors and integrins, and cytoplasmic tyrosine kinases [9]. They share a conserved overall architecture, with three main common features: a C-terminal pseudokinase (PsK) domain; regulatory α-helices that flank the PsK domain and together form the split helical dimerization (SHED) domain; and N-terminal intrinsically disordered regions (IDRs), of ∼1200, 900 and 130 amino acid residues in PEAK1-3 respectively [9] (Figure 1a). The IDRs harbour phosphorylation-dependent binding motifs that mediate selective interaction with the src homology (SH)2 domains of specific signaling proteins including Grb2 ($PEAK1^{Y635}$ and $PEAK3^{Y24}$), Tensin-3 ($PEAK1^{Y635}$) and C-terminal Src kinase (Csk) ($PEAK2^{Y411}$) and the phosphotyrosine binding (PTB) domain of Shc1 ($PEAK1^{Y1188}$) (Figure 1a and b), as well as 14-3-3 ($PEAK3^{R66\text{-}P71}$) [10–15]. The IDRs also harbour phosphorylation-independent binding sites for particular SH3 domain-containing proteins eg CrkII ($PEAK1^{P1152\_K1158}$, $PEAK2^{P812\_K818}$, $PEAK3^{P56\_K62}$) [9].

Reflecting the presence of these binding sites, the PEAKs function as scaffolds, assembling complexes of signaling proteins in specific subcellular compartments, including focal adhesions (Figure 1c). To support their scaffolding function, the PEAKs dimerize via the SHED domains to form homo- and heterotypic complexes [11,16–20] (Figure 1a). Since each family member exhibits a characteristic signaling potential defined by its suite of binding partners, heterotypic association with another family member provides a mechanism to diversify and regulate signal output. For example, PEAK1 requires PEAK2 to promote cell migration and Stat3 activation, and PEAK3-induced cell invasion in 3D cultures is regulated quantitatively and temporally by the presence of PEAK1 and PEAK2 [11,16]. The mechanism and role of PEAK dimerization is expanded upon later in this review.

In terms of function, the PEAKs regulate a variety of biological endpoints, including cell morphology, migration, invasion and proliferation, and in general, promote transition from an epithelial to more fibroblastic phenotype [10,11,15,21,22]. These properties are exemplified in the case of PEAK1, which plays a critical role in regulating the qualitative nature of signal output from the epidermal growth factor receptor (EGFR) over time following EGF simulation. Here, PEAK1 binding to the Shc1 scaffold leads to a change in output from cell proliferation/survival to cell invasion and morphogenesis [15]. Other less-well characterized roles for the PEAKs include PEAK1-mediated regulation of angiogenesis via regulation of vascular endothelial growth factor receptor 2 (VEGFR2) expression [23] and the involvement of the mouse orthologue of PEAK2 as a component of the Notch transcriptional activation complex [24]. Importantly, the ability of the PEAKs to regulate endpoints relevant to oncogenesis underpins their roles in a variety of malignancies, which include breast and pancreatic cancer (for PEAK1) [10,25,26], pancreatic and colorectal cancer (PEAK2) [27,28] and acute myeloid leukemia (PEAK3) [20].

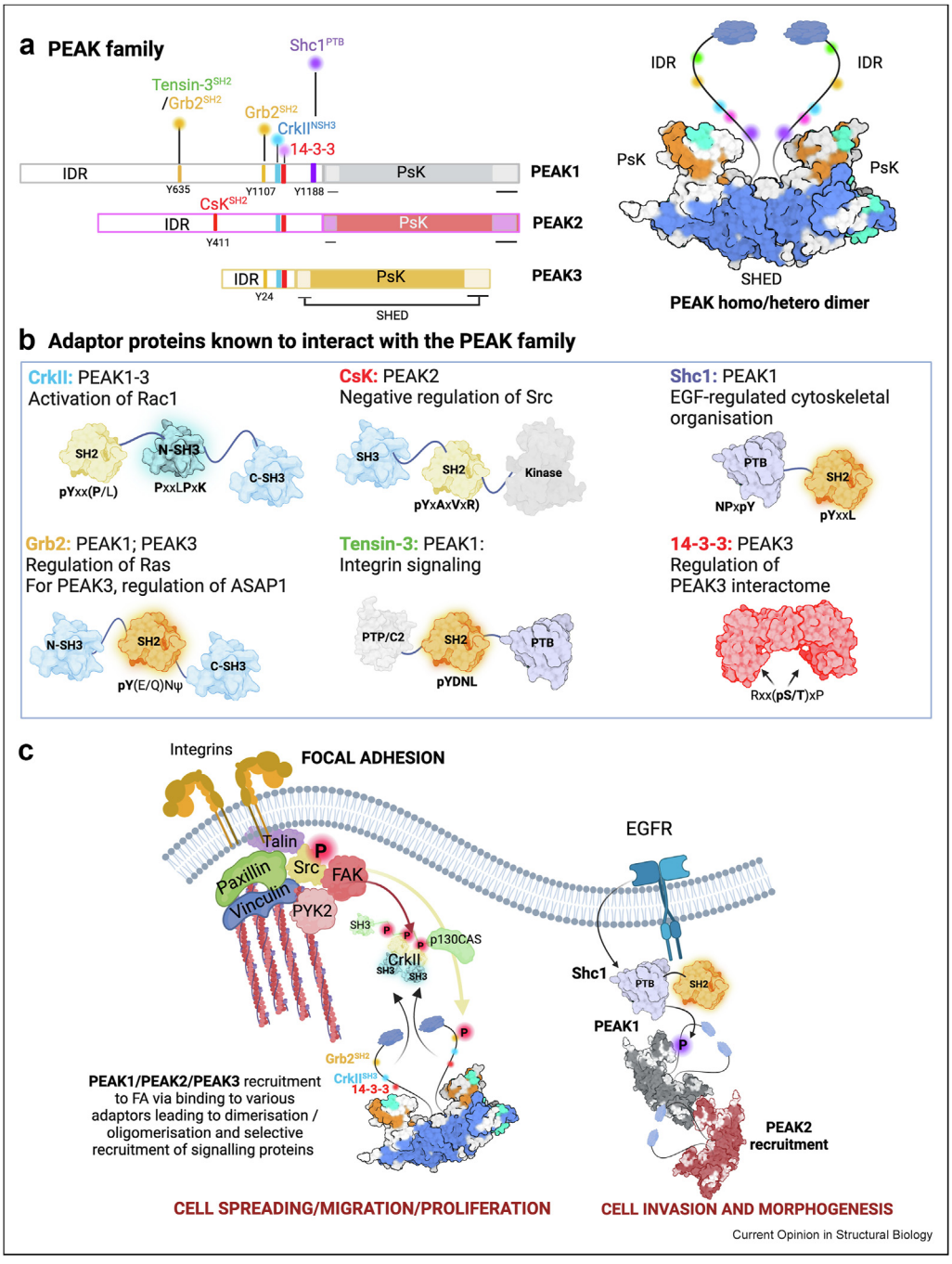
In this review we describe structural insights into PEAK family signaling, with a particular focus on the characterization of PEAK3 over the last two years and the insights it provides.

Structural characterization of PEAK1-3

The PEAKs dimerize via conserved SHED domains

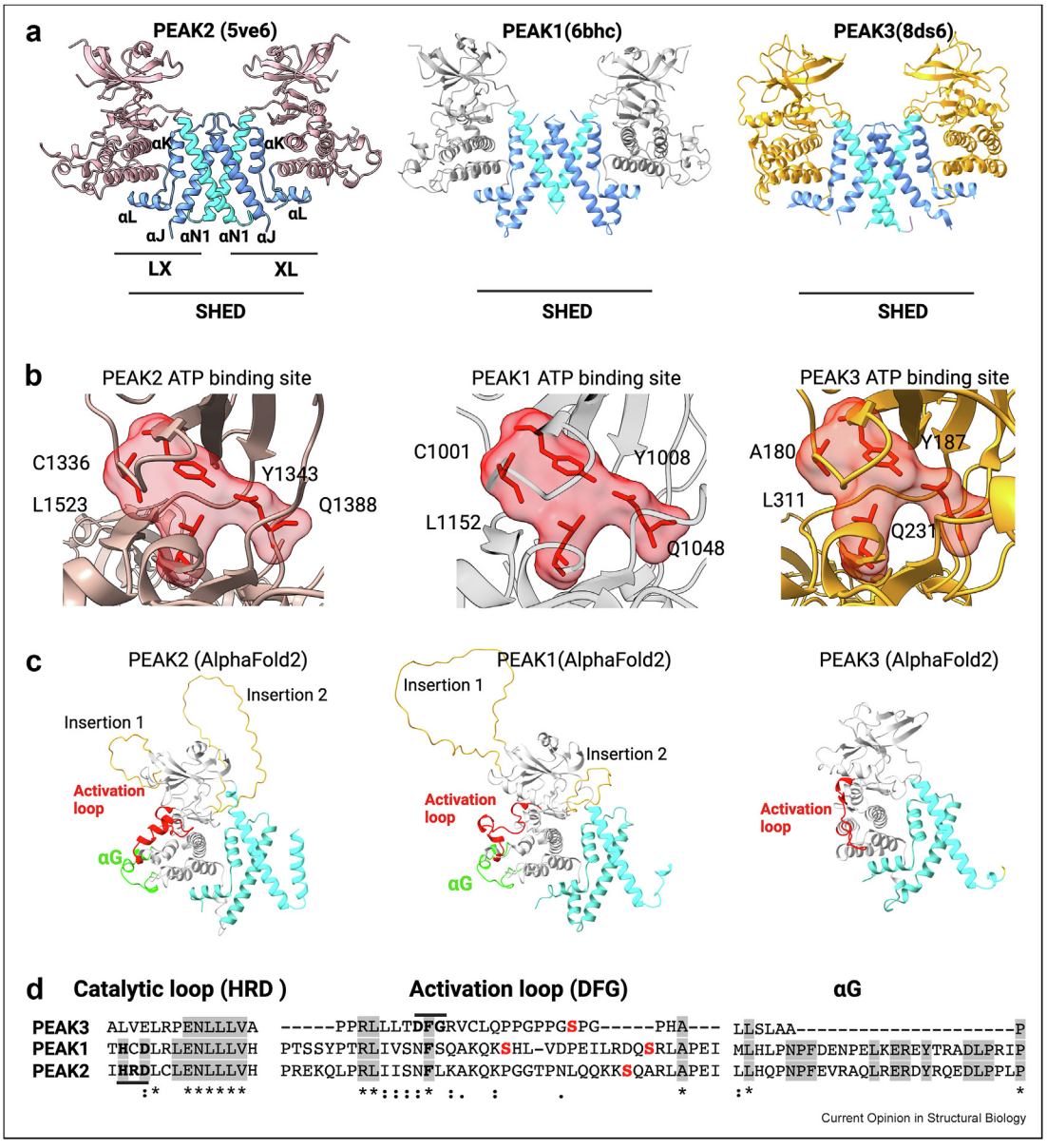
The first PEAK protein structure to be resolved by X-ray crystallography was that of the PsK domain and flanking α-helices from PEAK2 [17]. In the monomeric form of this protein, the N-terminal helix αN1 directly interacts with the C-terminal helices αJ and αK, while αL helix slides under the αI helix of the PsK domain forming a 'XL'-shaped helix bundle. Moreover, consistent with previous biochemical studies demonstrating that PEAK1 and PEAK2 undergo homo- and heterotypic association [16], the PEAK2 structure revealed a dimeric form, with the αN1 and αJ helices from one monomer exhibiting hydrophobic interactions with the corresponding αJ and αN1 helices from the second monomer to form a unique 'XX'-shaped four-helix bundle dimerization domain [17] (Figure 2a). Further evidence for the ability of PEAK2 to dimerize with sub-nanomolar affinity was obtained from analytical ultracentrifugation and small angle X-ray scattering [17], and the same dimeric structure was observed for the Rat orthologue Pragmin [29]. Importantly, the 'hot-spot' residues critical to dimerization exhibit a high degree of conservation across the PEAK family, and subsequent X-ray crystallography and Cryo-EM structures of PEAK1 and PEAK3, respectively, determined that these proteins also dimerize via the same mechanism [18,30] (Figure 2a). In addition, the significant conservation of the dimerization interface is consistent with heterotypic interaction of the PEAKs demonstrated in cells [11,16]. Definition of the PEAK1 structure led to this novel four helix bundle being termed the split helical dimerization (SHED) domain. A search for similar domains revealed that the SHED domain exhibits structural similarity to the N-helix - C-terminal region (CTR) of the protein

Figure 1



Structure and signaling roles of the PEAK family of pseudokinase scaffolds. a. Left panel, schematic representation of PEAK1-3 structure. The short black lines indicate the regulatory helices that comprise the split helical dimerization (SHED) domain. Binding sites for specific adaptor and 14-3-3 proteins are indicated. IDR, intrinsically disordered region; PsK, pseudokinase domain; SH, Src homology; PTB, phosphotyrosine binding. Right panel, structure of a PEAK dimer highlighting the role of the SHED domain interface. **b.** Schematic representation of particular adaptor-type signaling proteins known to associate with the PEAKs. The conserved binding motifs for particular protein–protein interaction domains are indicated. The recruitment profile of each adaptor to the individual PEAKs, and the main adaptor-associated signaling pathways, are also highlighted. **c.** Schematic representation of PEAK1-3 signaling downstream of integrins and the epidermal growth factor receptor (EGFR). The PEAK signaling complex downstream of the EGFR is assembled at a relatively late (20 min) timepoint following EGF stimulation and also contains PPP1CA, PPP1CC, DAB2IP and ASAP2 [15].

Figure 2



Structural characteristics of the PEAK SHED and PsK domains. a. Structure of the different PEAK dimers indicating how dimerization is mediated via the conserved SHED domains. The constituent α-helices of the SHED domain (αN1, αJ, αK, αL and αM) in PEAK2 are labelled. LX and XL refer to shapes of particular helical bundles [17]. **b.** Structure of the ATP binding site of the different PEAKs. Residues occupying the site where ATP would normally bind are shown in red in semi-transparent surface representation. **c.** The ColabFold Google Colab notebook called AlphaFold2 [33] was used to predict the structure of PEAK1 (Entry Q9H792), PEAK2 (Entry Q86YV5) and PEAK3 (Q6ZS72). The predicted models with the highest lDDT score were used to conduct our structural analysis. Predicted AlphaFold 2 structure of the PEAK PsK domains are depicted in cartoon representation using ChimeraX [42]. Key structural elements are highlighted, including the insertions in PEAK1 and PEAK2, the A-loop (red) and the αG (green). **d.** Alignment of conserved kinase domain regions in the different PEAKs. The HRD and DFG motifs, and potential phosphorylation sites in the A-loop, are highlighted in bold black and red, respectively.

kinase PINK1 [18,31]. The role of the PINK1 N-helix-CTR region requires further elucidation, but protein–protein interaction roles, specifically in PINK1 oligomerization and association with the translocase of the outer mitchondrial membrane (TOM) complex, have been recently reported [31,32].

The PEAK pseudokinase domains exhibit unique features

The structural analysis of the PEAK2 PsK domain revealed a canonical bi-lobal kinase fold but with atypical features. These included the 'hijacking' of the catalytic lysine of the consensus VAIK motif of active

protein kinases by Gln1048 as well as an ATP binding pocket occluded by the side chains of residues Tyr1008, Cys1001, Gln1048 and Leu1152 [17] (Figure 2b). Subsequent analysis of PEAK1 X-ray crystallographic and PEAK3 Cryo-EM structures also revealed an occluded ATP binding pocket, with the 'occupying' tyrosine, leucine and glutamine residues being conserved across the PEAK family [18,30] (Figure 2b). Overall these data indicate that the PEAKs should lack ATP binding ability and can be considered as 'true' pseudokinases, results corroborated by the absence of nucleotide binding in thermal shift assays undertaken on PEAK1 and PEAK2 [3], and in nucleotide-analogue binding assays performed on PEAK2 [29].

However, several notable differences are observed among the published PEAK structures [17,18,30]. First, PEAK1 and PEAK2 exhibit two insertions of different length between the β4 and β5 strands and between the catalytic and A-loops in the N-lobe, respectively, features that are largely absent in PEAK3 as depicted in the respective AlphaFold2 models [33] (Figure 2c). As these insertions were either deleted to facilitate the structural characterisation of PEAK1 and not resolved in PEAK2, the structural role of these insertions remains unclear. Second, only PEAK2 has a conserved HRD motif in its catalytic loop (Figure 2d). Third, unlike PEAK1 and PEAK2, PEAK3 lacks the regulatory αG helix [30], an important interface for engagement and regulation of partner kinases [34] (Figure 2c–d). Fourth, the A-loop is resolved and is significantly shorter in the PEAK3 structure and adopts an extended conformation, whereas it is disordered in PEAK1 and PEAK2 structures (Figure 2c and d). However, all PEAK proteins contain exposed serine residues in their A-loop, suggesting potential regulatory mechanisms through phosphorylation (Figure 2d) [35]. Fifth, only PEAK3 possesses a fully-conserved DFG motif characteristic of *bona fide* kinases (Figure 2d) with the Phe in a 'DFG-in' orientation typical of active kinases [36]. The Phe also exhibits this orientation in the PEAK1 structure and PEAK2 predicted AlphaFold2 structure. Sixth, the αC helix in PEAK3 is fully resolved, unlike in PEAK1 and PEAK2, and adopts a conformation observed in active protein kinase A. However, despite PEAK3 exhibiting certain structural features characteristic of an active kinase, the occluded ATP site (Figure 2b) suggests that catalytic function is unlikely, thus classifying PEAK3 as a pseudokinase similar to PEAK1 and PEAK2.

Functional insights from the PEAK structures

PEAK insights into the functional role of dimerization

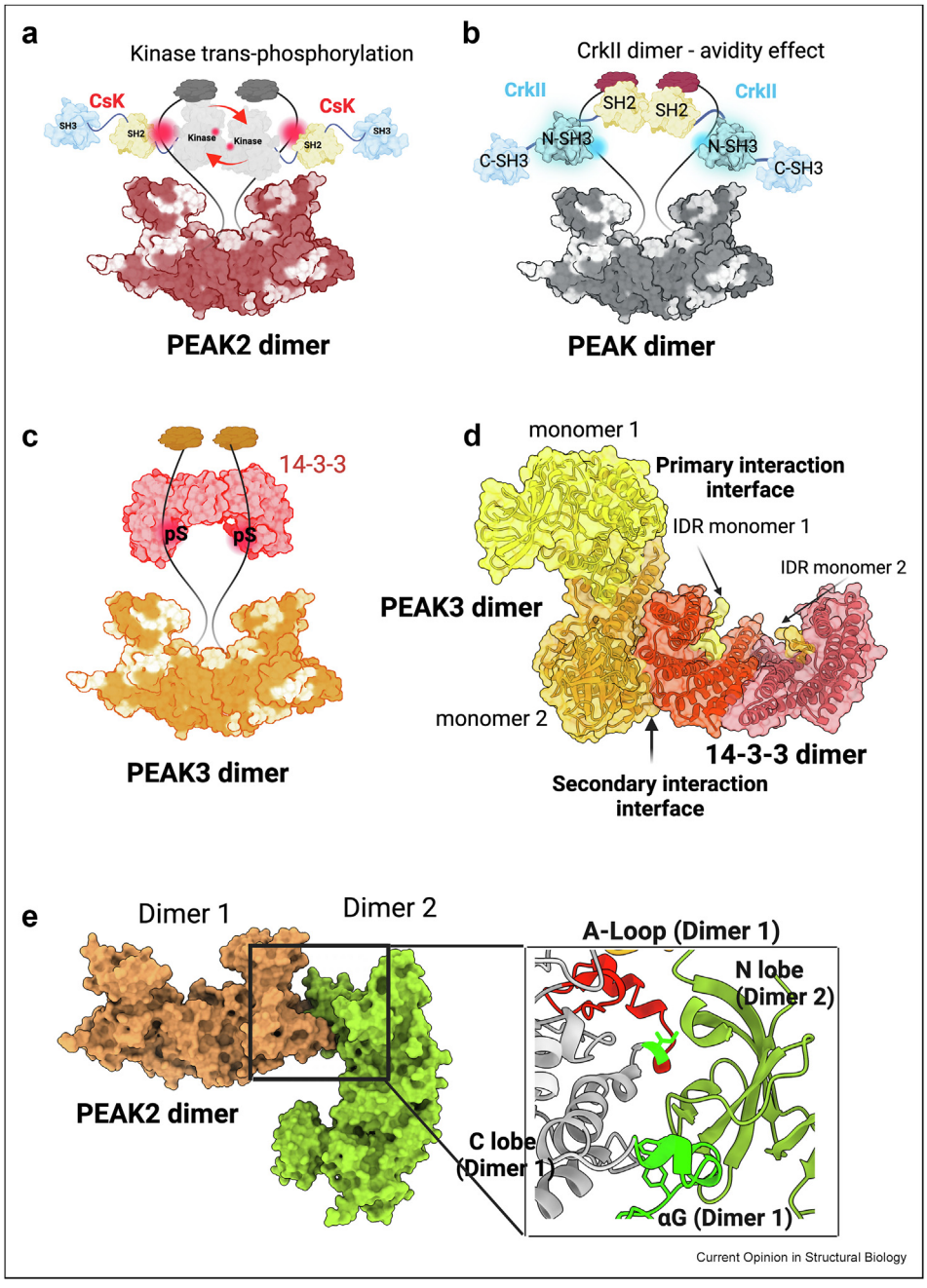
Dimerization is well-recognized as a key regulatory mechanism for *bona fide* protein kinases. For example, in the case of the erbB family of receptor tyrosine kinases, this mediates both kinase activation and signal diversification [37,38]. Although the PEAKs are pseudokinases, their ability to regulate biological endpoints such as cell migration is also dependent on dimerization potential [11,16,17,19,20], and recent structural, biochemical and biophysical analyses have provided new insights into the underpinning mechanisms.

First, PEAK dimerization can promote activation of an associated binding partner. This is exemplified by regulation of C-terminal Src kinase (CSK) by the rat orthologue of PEAK2, Pragmin. This pseudokinase associates with the SH2 domain of CSK upon Src family kinase (SFK)-mediated phosphorylation of Pragmin Y391 (PEAK2 Y411) in the IDR region, and overexpression of Pragmin leads to CSK activation and increased tyrosine phosphorylation of Pragmin as well as many other cellular proteins [21,29]. Importantly, while a loss-of-function mutation in the Pragmin dimerization interface did not affect CSK association, it markedly reduced Pragmin-mediated CSK activation [29]. This suggests a model whereby recruitment to the Pragmin dimer promotes dimerization and activation of an associated tyrosine kinase, in this case CSK (Figure 3a). Importantly, the recent characterization of PEAK3 by the Roche group indicates that in the case of this scaffold, SHED-dependent dimerization promotes activation of the associated tyrosine kinase PYK2 and its downstream signalling to Akt [20].

Second, PEAK dimerization can enhance recruitment of specific SH2- and SH3-containing adaptor proteins and 14-3-3. The PEAKs undergo SHED-dependent homo- and hetero-dimerisation with nanomolar affinity and likely oligomerization through the αG/A-loop with micromolar affinity. In addition, they also contain IDRs replete with SH2 and SH3 motifs that drive the selective assembly of multivalent adaptor signalling complexes. While the affinity of individual SH2 and SH3 sites is in the micromolar range, the multiplicity of these sites combined with the multivalency of each adaptor (Figure 1b), their interactions with other multivalent scaffolds (eg p130Cas or NEDD9) and PEAK dimerisation/oligomerization, drive molecular crowding and result in significant avidity effects [14].

The affect of PEAK dimerization on adaptor protein binding was originally demonstrated for PEAK3, where loss of dimerization potential led to a marked reduction in binding to the adaptor CrkII [11,19]. A recent study using recombinant proteins has clarified the underpinning mechanism, demonstrating that CrkII can exist in both monomeric and dimeric forms, with dimerization being mediated via the SH2 domain. Furthermore, stable interaction between CrkII and dimeric PEAK1 and PEAK2 was only detectable for dimeric CrkII [14]. This underscores the crucial role of avidity in assembly of PEAK/CrkII complexes (Figure 3b). Interestingly, both dimerization-induced kinase activation and avidity

Figure 3



Regulatory roles of PEAK dimerization and oligomerization. a. Activation of an associated kinase. This is exemplified by PEAK2-mediated activation of CSK kinase activity, through binding of CSK to PEAK2 via its SH2 domain. **b.** Enhancement of adaptor protein binding via avidity effects. The example shown is PEAK3-mediated CrkII binding. **c.** Association of PEAK3 with 14-3-3 is also dimerization-dependent. The schematic highlights the role of the primary interaction interface involving the conserved 14-3-3 binding motif present in each PEAK3 monomer. **d.** PEAK3/14-3-3 interaction also involves a secondary interaction interface between one PEAK3 monomer and 14-3-3. **e.** Structure of the PEAK2 oligomerization interface. Left panel: Potential dimerization interface based on crystal packing of PEAK2 (pdb 5ve6) between two PEAK dimers. Right panel: Close up of this interface showing oligomerisation interaction made through the A-loop (red) and the αG (green) of dimer 1 with the N-lobe of dimer 2.

effects on adaptor binding might contribute to the observed requirement for dimerization potential in tyrosine phosphorylation of PEAK3 [11]. Here, dimerization may promote activation of a Src family kinase or the PEAK3-associated kinase PYK2 [11,29]. In addition, an alternative, but not mutually exclusive explanation, is that dimerization is required to recruit PEAK3 to sites of CrkII clustering in cells, such as focal adhesions (Figure 1c) and PEAK3 undergoes tyrosine phosphorylation at such sites [14]. Consistent with this model, mutation of the CrkII SH3 domain binding site in PEAK3 leads to loss of PEAK3 tyrosine phosphorylation [11]. Of note, the related adaptor CrkL also undergoes dimerization, in this case mediated via the C-terminal SH3 domain [39], suggesting that avidity may play a broader role in regulation of adaptor protein recruitment to receptors and scaffolds that undergo dimer- or oligomerization.

Recent studies on the association of PEAK3 with 14-3-3 have demonstrated that this binding interaction is also dependent on PEAK3 dimerization [14,30], and this is also likely explained by avidity effects. This is because 14-3-3 proteins exist as dimers and can accommodate the binding of two consensus 14-3-3 binding motif phosphopeptides derived from each partner of the PEAK3 dimer (Figure 3c). However, it is important to note that despite the high conservation of the consensus 14-3-3 sites across the PEAK family (Figure 1a), PEAK3 exhibits a 10-fold higher binding affinity for 14-3-3 compared to PEAK1 and PEAK2. Intriguingly, besides the canonical interaction between the consensus 14-3-3 binding motif in PEAK3 and the conserved amphipathic groove of 14-3-3, defined as the primary interface, secondary interactions were observed between specific residues from the SHED and PsK domain of PEAK3 and 14-3-3. This secondary interface is thought to stabilize an unusual binding mode for 14-3-3, with PEAK3 sitting outside of the 14-3-3 cradle on one monomer [30] (Figure 3d). The contribution of this secondary interface to PEAK3/14-3-3 binding may explain the observed higher affinity of 14-3-3 for PEAK3 compared to PEAK1 and PEAK2 [14], as residues from the dimerisation domain helices of one monomer of the PEAK3 dimer involved in this interaction are mostly non-conserved in PEAK1 and PEAK2.

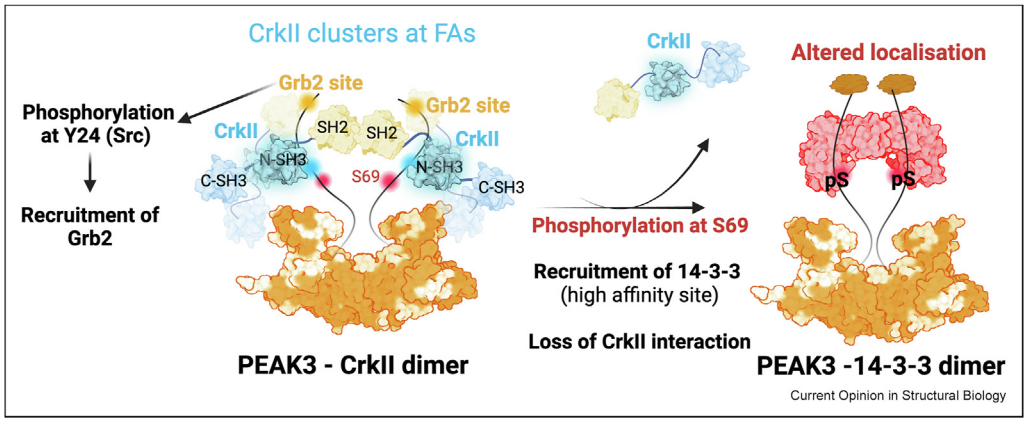
The PEAK pseudokinase domains and higher order oligomers

While multiple lines of evidence support the critical signaling and biological roles of PEAK dimerization, questions remain as to whether the PEAKs can form higher-order oligomers. To date, oligomers of PEAK proteins have been observed when purifying PEAK proteins *in vitro*. In the case of PEAK1 and PEAK2, analytical ultracentrifugation indicated the clear presence of oligomeric forms, and analysis of the crystallographic data of PEAK2 revealed a novel oligomerization interface involving specific α-G helix and A-loop residues in one dimer and a groove in the N-lobe of the dimer partner [17] (Figure 3e). Importantly, site-directed mutagenesis demonstrated the importance of these interactions in the assembly of PEAK2 higher-order oligomers *in vitro*, as well as in the formation of PEAK2 homotypic complexes in cells. Moreover, the oligomerization interface appeared important for PEAK2-enhanced cell migration [17]. The recent PEAK3 cryo-EM data also suggest the existence of higher-order PEAK3 oligomers (both in the presence and absence of 14-3-3) [30], although the mechanism by which this oligomerisation occurs is unclear. Consequently, PEAK oligomerization represents an important area for future mechanistic and functional studies.

Regulation of PEAK signaling

Previous work demonstrated that following growth factor stimulation, scaffold proteins exhibit dynamic changes in their interactomes, and this can regulate receptor output over time [15]. Recent work demonstrated that this concept also applies to the PEAKs, with PEAK3 exhibiting marked temporal changes in its binding partners following EGF treatment of cells. These changes are characterized by loss of tyrosine phosphorylation and decreased binding to Grb2, CrkII and ASAP1 early after EGF stimulation [11]. In this context, the interplay between the protein tyrosine phosphatase PTPN12 and particular SFKs regulates PEAK3 tyrosine phosphorylation and consequently the recruitment of binding partners dependent on this mechanism [11]. However, recent studies have identified additional regulatory processes. The first involves the phosphorylation of PEAK3 on Ser69, which is embedded within the 14-3-3 consensus motif discussed earlier. Critically, this consensus motif lies adjacent to the binding site for CrkII (Figure 1). In an series of elegant biophysical experiments using isothermal calorimetry to measure the binding affinity of CrkII to a tandem peptide harbouring both the CrkII and 14-3-3 binding sites, Roy and colleagues demonstrated that prebinding of 14-3-3 to the phosphorylated peptide led to a 6-fold reduction in affinity of CrkII binding compared to the uncomplexed and unphosphorylated peptide [14]. Furthermore, the authors speculated that due to avidity effects, this negative co-operativity is likely to be more pronounced when binding to dimeric PEAK3 is considered, a concept supported by the total absence of binding of dimeric CrkII to the dimeric PEAK3/14-3-3 complex. Therefore, the tandem site functions as a molecular switch to control CrkII binding and signal output of PEAK3 (Figure 4). Importantly, the physiological relevance of this switch was confirmed by cellular studies demonstrating that the PEAK3 S69A mutant not only loses 14-3-3 binding but exhibits increased tyrosine phosphorylation and CrkII and Grb2

Figure 4



The PEAK3 tandem CrkII/14-3-3 binding site acts as a molecular switch that regulates PEAK3 signal output. PEAK3 binds to clustered CrkII at focal adhesions via the $CrkII^{NSH3}$, with binding promoted by avidity effects reflecting SH2-mediated CrkII dimerization. This localizes PEAK3 to a subcellular region with active Src that phosphorylates PEAK3 on Y24, leading to Grb2 binding. Phosphorylation of PEAK3 at S69 creates a high-affinity binding site for 14-3-3, resulting in the formation of a highly stable PEAK3:14-3-3 dimer:dimer and destabilizes CrkII binding to the adjacent site, disfavoring PEAK3/CrkII complexes. Possible outcomes are altered PEAK3 subcellular localization and/or termination of PEAK3/CrkII signaling.

association and also significantly increased ability to promote cell motility compared to PEAK3 wildtype (WT) [14].

In parallel to the Roy et al. study, the Jura laboratory demonstrated that 14-3-3 binding regulated the subcellular localization of PEAK3, with the PEAK3 S69A mutant predominantly localizing to the nucleus of COS-7 cells, whereas the WT protein exhibited a uniform cytoplasmic distribution. Moreover, characterization of the PEAK3 S69A interactome by mass spectrometry revealed a remarkable expansion compared to PEAK3 WT, with binding to a type 2A serine/threonine phosphatase complex and protein kinase D paralogs only detected for the mutant [30]. Consequently 14-3-3 binding harnesses the scaffolding potential of PEAK3, which is likely to reflect direct effects on accessibility of binding sites in PEAK3 and indirect effects via regulation of co-localization with particular partners.

Conclusions

While recent studies have provided valuable insights into the mechanism, regulation and function of signaling by the PEAK scaffolds, several critical questions remain. First, although dimerization is clearly critical to their signal output, it appears likely that formation of higher order oligomers also occurs in cells. The strongest evidence for this is provided by PEAK2, where an oligomerization interface has been defined and functionally validated both *in vitro* and *in vivo*. Additionally, preliminary evidence for higher order structures has also been obtained for both PEAK1 and PEAK3 [17,30]. Furthermore, given the multivalency of many adaptor proteins, adaptor protein binding may facilitate and stabilize the assembly of PEAK oligomers. Here, it will be important to address whether PEAK complexes undergo liquid–liquid phase separation to demix from the bulk environment to create a consensed phase that compartmentalizes and regulates PEAK signaling [40]. This possibility is supported by the multivalency of the PEAK scaffolds and their adaptor binding partners, and the presence of IDRs in the PEAKs. Indeed, a precedent for scaffold proteins exhibiting this behaviour is provided by linker for activation of T cells (LAT), which recruits Grb2 and Sos1 to form phase-separated clusters at the plasma membrane [40].

Second, whereas dimerization of other members of the kinase superfamily is often regulated (for example, by specific growth factor ligands in the case of the erbB family), the mechanisms governing homo- or heterotypic association of the PEAKs are yet to be fully unravelled. Studies on purified PEAK2 PsK-domain, including its SHED domain, revealed a nanomolar affinity for dimerization but micromolar for oligomerization [17]. Using αG/A-loop oligomerisation mutants, the same study also demonstrated isolation of the PEAK1-PEAK2 heterodimer. While the dimerization affinity is high, it is possible that this process (and oligomerization) is regulated by post-translational modification, particularly phosphorylation. Such modifications may regulate the orientation and/or binding activity of key interaction interfaces, influencing the dimerization and oligomerization states of the PEAK proteins. Evidence indicating that phosphorylation may play a key role is provided in a recent preprint, which reports that CAMK2-mediated phosphorylation of PEAK1 positively regulates its heterotypic association with PEAK2 [41]. A likely region for

phosphorylation-dependent regulation of the PEAKs is the A-loop. In this context, each PEAK protein possesses a unique A-loop sequence harboring known or potential phosphorylation sites (Figure 2d). Conformational changes within the A-loop following phosphorylation are likely to affect the positioning of the αC helix and consequently the N-lobe, which would have implications for the relative positioning of the SHED domain. Moreover, since the αG/A-loop regulate oligomerisation of PEAK2 *in vitro* (Figure 3e), A-loop phosphorylation may also govern oligomerisation processes.

Third, while significant progress has been made in defining the different PEAK interactomes, the mechanism of association with specific binding partners, such as PYK2 in the case of PEAK3, requires structural insights. In addition, it is highly likely that additional partners remain to be identified, as highlighted by the recent report that PEAK1 directly binds and activates CAMK2 [41]. Fourth, it will be important to develop therapeutic stategies directed against the PEAKs, given the important roles played by these pseudokinases in multiple human cancers. Here, while PEAK dimerization will be challenging to target via a small molecule-based approach, given the affinity of the interaction and the size of the corresponding interface, the N-lobe groove and A-loop involved in PEAK1/2 oligomerization may prove more amenable [17]. This could also represent a binding site to direct a proteolysis-targeting chimera (PROTAC) protein degrader [9]. Furthermore, the identification of therapeutically 'actionable' targets coupled to the PEAKs, such as PYK2 and CAMK2, may lead to treatment approaches involving pharmacological inhibition of key downstream pathways.

Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

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* of special interest
** of outstanding interest

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