When two’s a crowd - Structural mapping of PEAK pseudokinase interactions identifies 14 3 3 as a molecular switch for PEAK3/Crk signaling.

Isabelle Lucet (✉ lucet.i@wehi.edu.au)  
The Walter and Eliza Hall Institute of Medical Research  
https://orcid.org/0000-0002-8563-8753

Michael Roy  
The Walter and Eliza Hall Institute of Medical Research  
https://orcid.org/0000-0003-0198-9108

Minglyanna Surudoi  
The Walter and Eliza Hall Institute of Medical Research  
https://orcid.org/0000-0003-3464-8633

Ashleigh Kropp  
The Walter and Eliza Hall Institute of Medical Research

Jianmei Hou  
Cancer Program, Biomedicine Discovery Institute, Monash University

Weiwen Dai  
The Walter and Eliza Hall Institute of Medical Research

Joshua Hardy  
The Walter and Eliza Hall Institute of Medical Research  
https://orcid.org/0000-0002-8014-8552

Lung-Yu Liang  
Walter and Eliza Hall Institute of Medical Research

Thomas Cotton  
The Walter and Eliza Hall Institute of Medical Research

Bernhard Lechtenberg  
Walter and Eliza Hall Institute of Medical Research  
https://orcid.org/0000-0002-5674-6894

Toby Dite  
The Walter and Eliza Hall Institute of Medical Research

Xiuquan Ma  
Biomedicine Discovery Institute, Department of Biochemistry and Molecular Biology, Monash University  
https://orcid.org/0000-0001-7227-1289

Roger Daly  
Monash University  
https://orcid.org/0000-0002-5739-8027

Onisha Patel  
The Walter and Eliza Hall Institute of Medical Research  
https://orcid.org/0000-0001-6701-7139

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When two’s a crowd - Structural mapping of PEAK pseudokinase interactions identifies 14-3-3 as a molecular switch for PEAK3/Crk signaling.

Michael J. Roy1,2,#, Minglyanna G. Surudoi1,2, Ashleigh Kropp1,2, Jianmei Hou3,4, Weiwen Dai1,2, Joshua M. Hardy1,2, Lung-Yu Liang1,2, Thomas R. Cotton1,2, Bernhard C. Lechtenberg1,2, Toby A. Dite1,2, Xiuquan Ma3,4, Roger J. Daly3,4, Onisha Patel1,2* and Isabelle S. Lucet1,2,*,#

1. The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia.
2. Department of Medical Biology, University of Melbourne, Parkville, VIC 3052, Australia.
3. Cancer Program, Biomedicine Discovery Institute, Monash University, Melbourne, VIC 3800, Australia.
4. Department of Biochemistry and Molecular Biology, Monash University, Melbourne, VIC 3800, Australia.
* Co-last authors
# Correspondence: roy@wehi.edu.au, lucet.i@wehi.edu.au.

Running Title: Mapping structural interactions of oncogenic PEAK pseudokinases
Abstract

PEAK pseudokinases regulate cell migration, invasion and proliferation by recruiting key signaling proteins to the cytoskeleton. Despite lacking catalytic activity, alteration in their expression level is associated with several aggressive cancers. Here, we elucidate new molecular details of key PEAK signaling interactions with the adapter proteins CrkII and Grb2 and the scaffold protein 14-3-3. Our findings rationalize why the dimerization of PEAK proteins has a crucial function in signal transduction and provide biophysical and structural data to unravel binding specificity within the PEAK interactome. We identify a conserved high affinity 14-3-3 motif on PEAK3 and demonstrate its role as a molecular switch to regulate CrkII binding. Together, our studies provide a detailed structural snapshot of PEAK interaction networks and further elucidate how PEAK proteins, especially PEAK3, act as dynamic scaffolds that exploit adapter proteins to control signal transduction in cell growth/motility and cancer.

Keywords: PEAK Pseudokinases, scaffolds, signaling network, oligomerization, dimerization, CrkII, 14-3-3, Grb2, cancer.
INTRODUCTION

The pseudopodium enriched atypical kinase (PEAK) family of proteins, which comprises Sugen kinase 269 (Pseudopodium-enriched atypical kinase 1, PEAK1), SgK223 (PEAK2), an ortholog of rat Pragmin and mouse Notch activation complex kinase (NACK), and the recently identified PEAK3 (C19orf35), play critical roles in actin cytoskeleton remodeling, influencing cell migration and invasion in normal and cancer cells. Abnormal expression of PEAK proteins alters cell morphology and confers enhanced migratory and invasive characteristics to cells, implying a role of PEAKs in the spatio- and temporal assembly of signaling hubs at focal adhesions (FAs) and with the actin cytoskeleton.

PEAK proteins are a unique group of pseudokinase (PsK) scaffolds that can self-assemble regulatory networks. Their scaffolding activities rely on dimerization, post-translational modifications and recruitment of modular adapter proteins. PEAK family proteins homo- and/or heterodimerize via a unique alpha-helical domain, the split helical dimerization (SHED) domain which flanks the PsK domain (Fig. 1), as recently elucidated by the structures of PEAK2 and PEAK1.

In addition to the conserved PsK and SHED domains, all PEAK proteins have an N-terminal intrinsically disordered region (IDR) (Fig. 1) replete with short linear motifs (SLiMs) – conserved sequence motifs that can that form dynamically regulated docking sites to recruit interactors involved in PEAK signalling. To date, these IDRs have only been partially characterized. Several tyrosine phosphorylation sites have been identified within the large IDRs of PEAK1 and PEAK2 (IDR length ~1200 and ~900 residues, respectively) that regulate cell proliferation, migration and invasion, and in some cases the recruited proteins with Src Homology 2 (SH2) and phosphotyrosine binding (PTB) domains have been characterized. In PEAK1, Tyr is phosphorylated by Src, linking PEAK1 to the Src-p130Cas-Crk-paxillin pathway that regulates FA dynamics. Additionally, phosphorylation of Tyr by Lyn creates a binding site for the SH2 domain of the adapter protein Grb2, which leads to the activation of the Ras/Raf/Erk signaling pathway that controls cell proliferation and invasion. Both PEAK1 and PEAK2 are linked to the signaling adapter Shc1 and share a conserved ‘EPIYA’ phosphotyrosine motif (PEAK2 Y; PEAK1 Y), which serves as a docking site for the SH2 domain of the C-terminal Src kinase (Csk). This interaction brings Csk to FAs where it likely regulates cell morphology and cell motility by modulating the activity of Src family kinases (SFKs).

In contrast, the role of PEAK3 in signaling is poorly understood. PEAK3 has a more restricted expression profile than PEAK1/PEAK2; predominantly expressed in granulocytes and monocytes (Human Protein Atlas, proteinatlas.org) suggesting a specialized function. PEAK3’s IDR is significantly smaller than PEAK1/PEAK2 (PEAK3 IDR length ~130 residues), but still harbors...
various SLiMs, some of which are conserved in PEAK1 and PEAK2. Almost all reported cellular interactions of PEAK3 with binding partners appear to require PEAK3 dimerization\textsuperscript{4,14,23}. Amongst PEAK3 interactors identified from proteomic/cellular studies are the adapter proteins CrkII/CrkL\textsuperscript{24}. CrkII/CrkL are highly similar but non-identical proteins, with roles in focal adhesion signaling, cell migration, and cancer\textsuperscript{24}. They both share the identical domain architecture (SH2-NSH3-CSH3) (Fig. 1d) and identical binding preferences in SH2 and NSH3 domains but are reported to have a distinct structural architecture and interactome\textsuperscript{24}. PEAK proteins have been shown to interact with CrkII via a conserved proline-rich motif (PRM) present in the N-terminal IDR of all PEAK proteins that binds the CrkII NSH3 domain (CrkII\textsuperscript{NSH3}; consensus sequence PxLPxK) (Fig. 1d)\textsuperscript{4,23}. Additionally, for PEAK3, and likely also PEAK1/2, CrkII binding requires PsK dimerization via the SHED domain and is impacted by mutations which disrupt the PsK domain conformation and dimerization\textsuperscript{4,14}. Beyond this, two recent studies have identified further PEAK3 interactors, including: adapter protein Grb2; the E3 ubiquitin ligase Cbl; proline-rich tyrosine kinase 2 (Pyk2); and Arf GTPase-activating protein 1 (ASAP1)\textsuperscript{14,23}. Grb2 (domain organization NSH3-SH2-CSH3) is involved in multiple cellular signal transduction pathways, most prominently downstream of the epidermal growth factor (EGF) receptor to the mitogen-activated protein kinase (MAPK) signalling cascade (via Grb2/Sos complex)\textsuperscript{25} but also survival (phosphatidylinositol 3-kinase 3-kinase (PI3K)/AKT) signaling (via Grb2-associated binder 1, Gab1)\textsuperscript{26}. Other PEAK3 interactors identified include 14-3-3 proteins\textsuperscript{4}, ubiquitous dimeric regulatory/scaffold proteins, of which there are seven isoforms in humans (β, γ, ε, η, σ, τ, and ζ). 14-3-3 proteins\textsuperscript{27,28} recognize specific phosphoserine/threonine motifs in partner proteins, including kinases, often with regulatory roles in cellular pathways, such as to alter conformation, activity or sub-cellular localization\textsuperscript{29-31}. In this study, we use an integrated bioinformatic, biochemical, and structural biology approach to further characterize the interactions and scaffolding activity of PEAK proteins. We identify highly conserved protein docking motifs within the N-terminal IDR of PEAK proteins and structurally characterize the phospho-dependent interaction of PEAK1/PEAK3 with the adapter protein Grb2. We provide new molecular details of PEAK interactions with CrkII and identify a role for the scaffold protein 14-3-3 as a new regulator of PEAK3 signaling. Lastly, we demonstrate that phosphorylation at PEAK3 S69 generates a high affinity 14-3-3 binding site. Binding of 14-3-3 to PEAK3 pS69 generates a highly stable PEAK3:14-3-3 dimer:dimer resulting in markedly reduced binding of CrkII to PEAK3 dimers. These findings contextualize why dimerization of PEAKs has a crucial function in signal transduction and demonstrate how signal specificity amongst the family is achieved. We demonstrate a 14-3-3-mediated molecular switch mechanism involving PEAK3/CrkII, providing new insights into the dynamic role of PEAKs in cell migration and invasion.
RESULTS

Sequence analysis of the PEAK3 N-terminal IDR identifies conserved interaction motifs

The N-terminal IDRs of PEAK family proteins contain numerous predicted Slims; several have confirmed roles in PEAK1/2 signalling\(^4,9,14,21,23,32,33\), but it is likely that other important interaction sites remain to be validated, in particular those critical to PEAK3 signalling for which less is known.

To further study the functionally relevant interactors of PEAK3, we first conducted an extensive bioinformatic analysis of the PEAK3 N-terminal IDR, which is comparatively shorter than those of PEAK1/2. We compared PEAK3 orthologs and direct conservation of this region with PEAK1 or PEAK2 (Fig. 1). This analysis highlighted two regions of interest. The first was a tyrosine/SH2 motif (YSNL; PEAK3\(^{Y24}/PEAK1^{Y1107}\)) that is highly conserved in PEAK3 and PEAK1 vertebrate orthologs (and absent in PEAK2 vertebrate orthologs) that matches the known phosphotyrosine consensus motif for the Grb2 SH2 domain (Grb2\(^{SH2}\); consensus motif: pYxNx) and the CrkII SH2 domain (CrkII\(^{SH2}\); consensus motif: pYxx(P/L), proline typically preferred) (Fig. 1a-d and Extended Data Fig. 1)\(^{14,34}\).

The second region we identified was a ‘tandem site’ that comprises two directly adjacent motifs that were both conserved: a PRM known to bind CrkII\(^{NSH3}\) (consensus motif: PxLPxK)\(^4,14\); and a putative binding site for 14-3-3 proteins (mode 1 consensus motif: Rxx(pS/T)xP) (Fig. 1a-d). This tandem site was found to be conserved not only in vertebrate orthologs of PEAK3, but also in vertebrate orthologs across the PEAK family (Fig. 1c and Extended Data Fig. 1). While cellular studies have shown that PEAK3 interaction with Grb2 and CrkII\(^4,14\) are functionally important, neither have been directly characterized structurally or biophysically, nor have there been reports of a functional role for 14-3-3 with PEAK proteins.

The conserved PEAK3\(^{Y24}/PEAK1^{Y1107}\) SH2 motif is phosphorylated by Src and interacts with Grb2\(^{SH2}\)

We first focused on the molecular characterization of the conserved PEAK3\(^{Y24}/PEAK1^{Y1107}\) SH2 motif with Grb2. We recently identified phosphorylation of the PEAK3 TYSNL (pY24) motif in cells and showed this is required for recruitment of Grb2, and possibly CrkII and ASAP1\(^{14}\), an Arf GTPase-activating protein that regulates cytoskeletal remodeling and is associated with tumor progression and invasiveness\(^{33,35,36}\). In MCF-10A cells, phosphorylation of PEAK3 at this site is blocked by treatment with either the SFK inhibitor eCF506 or the SFK/Abl inhibitor dasatanib, suggesting that this site is dependent on SFK activity\(^{14}\). We recently also described the expression and purification of full length recombinant PEAK3 (PEAK3\(^{FL}\)) and N-terminally truncated forms of PEAK1 (PEAK1\(^{IDR1}\) that includes PEAK1\(^{Y1107}\) site, residues 1082-1746) and PEAK2 (PEAK2\(^{IDR1}\), residues 802-1406) from insect cells (refer to online methods)\(^37\) – all of which also include the tandem motif in the IDR in addition to SHED/PsK domains. Building on this, we performed an in vitro kinase assay with purified...
recombinant PEAK3\textsuperscript{FL} and PEAK1\textsuperscript{IDR1} proteins followed by tandem mass spectrometry (MS/MS) analysis of tryptic peptides. We found that Src, but not Abl, can indeed phosphorylate this conserved SH2 motif on PEAK3\textsuperscript{FL} (pY24) and PEAK1\textsuperscript{IDR1} (pY1107) corroborating our recently reported cellular data\textsuperscript{14} (Fig. 2a).

We next sought to obtain structural data to better understand the mode of interaction of PEAK3 with Grb2. To do this we utilized a synthetic 7-mer pY phospho-peptide encompassing this SH2 motif (T\textsuperscript{pY}S\textsuperscript{N}L\textsuperscript{G}Q; corresponding to PEAK3\textsuperscript{23-29}-pY24 and PEAK1\textsuperscript{1106-1112}-pY1107), hereafter ‘SH2-pY peptide’. We have previously shown by isothermal titration calorimetry (ITC) that this SH2-pY peptide binds to both full-length Grb2 (Grb2\textsuperscript{FL}, \(K_D\) 2.6 \(\mu\)M) and CrkII (CrkII\textsuperscript{FL}, \(K_D\) 7.8 \(\mu\)M)\textsuperscript{14}.

Using an approach developed for apo-Grb2\textsuperscript{FL}\textsuperscript{38}, we succeeded in crystallizing a complex of human Grb2\textsuperscript{FL}:PEAK SH2-pY peptide and solved an X-ray structure to a resolution of 2.7 Å (Table 1 and Fig. 2b-d). In our structure, Grb2\textsuperscript{FL} is present as a dimer as seen in the structure of apo-Grb2\textsuperscript{FL} (Protein Data Bank (PDB) ID: 1GRI)\textsuperscript{38}, with the SH2-pY peptide bound to the SH2 domain of only one copy of Grb2 (chain B) (Fig. 2). Close crystal packing within the asymmetric unit leaves only this SH2 site (chain B) available for phosphopeptide binding (Fig. 2b-c). Our structure confirms that the PEAK3/PEAK1 SH2-pY peptide adopts a characteristic β-turn, with the expected recognition of the phosphotyrosine moiety and the specificity-determining (+2) N residue forming three important hydrogen bonds to the Grb2 backbone (R86, H107 and L109), consistent with other Grb2\textsuperscript{SH2}:pY\textsuperscript{XNX} peptide structures (eg. PDB ID: 1TZE)\textsuperscript{39}. Whilst no density was apparent for the C-terminal (-GQ) residues of the peptide, we noticed a potential surface groove directly adjacent on Grb2\textsuperscript{SH2}. In our orthologue multiple sequence alignment (MSA) data there is an extended region of high sequence conservation in PEAK3 and PEAK1 C-terminal to this SH2 motif (Fig. 2e). This corresponded to a predicted helical structure in AlphaFold2 (AF2)\textsuperscript{40,41} models of PEAK3 and PEAK1 (Extended Data Fig. 2a-b). Based on this, we spliced the peptide structure from our Grb2\textsuperscript{FL}: PEAK SH2-pY peptide crystal structure with the AF2 model coordinates of the adjacent helical region (PEAK3\textsuperscript{28-38}) to form an extended pY peptide model (PEAK3\textsuperscript{23-38}-pY24) and undertook docking of this spliced peptide model into Grb2\textsuperscript{FL} using HADDOCK\textsuperscript{42}. The resulting model of Grb2\textsuperscript{FL}:PEAK3\textsuperscript{23-38}-pY24 (Fig. 2e) reveals a compelling extended helix/groove interaction of the longer PEAK3 phosphopeptide with Grb2\textsuperscript{FL}, with significant shape complementarity. Interestingly, whilst some of the highly conserved residues on PEAK3 are buried in the Grb2\textsuperscript{SH2} interface, others project into solvent presenting a new interaction surface. We speculate this creates a new conserved surface that might be involved in recruitment of an additional interactor to the Grb2:PEAK3-pY24 complex (and similarly for PEAK1) and may potentially also enhance selectivity for Grb2 over CrkII\textsuperscript{SH2}. 
PEAK proteins bind CrkII\textsuperscript{NSH3} with similar affinity (low micromolar range)

Having made significant inroads to structurally and biophysically elucidate Grb2 binding at the PEAK3 SH2 site, we next turned our attention to the conserved ‘tandem site’ we identified on PEAKs, containing a CrkII\textsuperscript{NSH3} PRM and putative 14-3-3 binding site. Cellular studies show qualitatively that all PEAKs can interact with CrkII, requiring: (i) the conserved PRM within the ‘tandem site’ (matching the consensus for CrkII\textsuperscript{NSH3}; PxLPxK); as well as (ii) SHED-mediated PEAK dimerization\textsuperscript{4,14,32}. However, PEAK family members have non-identical sequence at this tandem site and to our knowledge no study has yet assessed the relative affinity of each for the CrkII\textsuperscript{NSH3}, which is important to contextualize interaction hierarchy.

We therefore addressed this by generating a series of synthetic peptides that encompass the PRM from the tandem site of each PEAK protein - PEAK3 (residues 54-66), PEAK1 (residues 1150-1162), PEAK2 (residues 809-821) – as well as another PRM in PEAK2 similar to the consensus sequence, PEAK2 (residues 709-721), and determined their affinity towards the immobilized CrkII\textsuperscript{NSH3} domain by Surface Plasmon Resonance (SPR) (Fig. 3a and Extended Data Fig. 3a, Supplementary Information). We confirm that PEAK1\textsuperscript{1150-1162}, PEAK2\textsuperscript{809-821} and PEAK3\textsuperscript{54-66} peptides all bind CrkII\textsuperscript{NSH3} with comparable affinity and fast on/off kinetics (measured dissociation constant, $K_D$ 0.9 – 2.4 µM). Whilst the affinity of PRM/SH3 interactions can vary (1-100 µM range)\textsuperscript{43}, this is comparable to CrkII\textsuperscript{NSH3} affinity for other PRMs that also closely match the CrkII\textsuperscript{NSH3} consensus sequence, such as the Abl\textsuperscript{758} PRM (reported $K_D$ 1.7 µM).\textsuperscript{44} We also show that PEAK2\textsuperscript{709-721} represents a potential second, lower affinity binding site for CrkII\textsuperscript{NSH3} on PEAK2 ($K_D$: 13 µM) (Fig. 3a and Supplementary Information). We observed no binding to CrkII\textsuperscript{CSH3} (data not shown). Comparison of the PEAK3\textsuperscript{54-66} peptide sequence to the crystal structure of the Abl PRM\textsuperscript{758} with CrkII\textsuperscript{NSH3} (PDB: 5IH2)\textsuperscript{44} (Fig. 3b) highlights the role that the Lys62 residue (-3 position) and the complementary electrostatic potential play in determining CrkII\textsuperscript{NSH3} specificity, as previously described for Abl\textsuperscript{44,45}.

Avidity enables stable PEAK interactions with CrkII

We next looked to examine the role of dimerization in CrkII binding to PEAKs. As our initial SPR studies had utilized isolated peptides and only the CrkII\textsuperscript{NSH3} domain, we shifted to utilize recombinant CrkII\textsuperscript{FL} and dimeric PEAK1, PEAK2 and PEAK3. CrkII\textsuperscript{FL} was expressed and purified from E.coli as described previously\textsuperscript{14}. Interestingly, whilst the majority of CrkII\textsuperscript{FL} is monomeric, approximately 10% of the total isolated protein purifies as a stable dimer (Extended Data Fig. 3b-c). Dimerization has been described for other adapters such as Grb2\textsuperscript{46} and the related protein CrkL (mediated by the C-terminal SH3 domain\textsuperscript{47}), however to our knowledge this has not been reported to date for CrkII. We confirm that, in contrast to CrkL, the dimerization of CrkII\textsuperscript{FL} we observe is mediated by the SH2...
domain, as recombinant C-terminally truncated constructs CrkII\(^{\Delta\text{CSH3}}\) (SH2-NSH3) and CrkII\(^{\text{SH2}}\) each form a similar proportion of dimer (Extended Data Fig. 3b-c). As MS analysis of both monomeric and dimeric CrkII\(^{\text{FL}}\) shows no PTMs (See Source Data) we believe the dimer we observed to be most likely due to a domain-swap within the SH2 domain.

We next focused on PEAK1 and PEAK2, as the SHED-PsK domains of these have previously been purified and crystallised\(^{18,37}\) and designed longer constructs, PEAK1\(^{\text{IDR1}}\) and PEAK2\(^{\text{IDR1}}\), harboring the ‘tandem motif’\(^{37}\). We recently reported the expression/purification of these longer constructs from insect cells\(^{37}\). Consistent with our previous findings for PEAK1/2\(^{18}\), PEAK1\(^{\text{IDR1}}\) and PEAK2\(^{\text{IDR1}}\) each purify as a homodimer (Fig. 3c-d and Extended Data Fig. 3d). Purified PEAK1\(^{\text{IDR1}}\) and PEAK2\(^{\text{IDR1}}\) were then analyzed for their ability to interact with either monomeric or dimeric CrkII\(^{\text{FL}}\) on SEC followed by SDS-PAGE (Fig. 3c-d, Extended Data Fig. 2). Strikingly, whilst the PEAK2\(^{\text{IDR1}}\) dimer:CrkII\(^{\text{FL}}\) monomer complex eluted as two peaks at the expected elution volume for each component, consistent with the anticipated low-affinity complex (micromolar range with fast on/off kinetics), PEAK2\(^{\text{IDR1}}\) dimer:CrkII\(^{\text{FL}}\) dimer complex showed a marked peak shift to an earlier elution volume, indicative of a stable interaction (Fig. 3c). SDS-PAGE confirmed that this complex contained both PEAK2\(^{\text{IDR1}}\) and CrkII\(^{\text{FL}}\) dimers (Fig. 3c). Similar results were obtained for PEAK1\(^{\text{IDR1}}\) dimer:CrkII\(^{\text{FL}}\) dimer (Extended Data Fig. 3d). SEC coupled to Multi-Angle Light Scattering (SEC-MALS) analysis of the purified PEAK2\(^{\text{IDR1}}\) dimer:CrkII\(^{\text{FL}}\) dimer complex estimated a mass of 192 kDa, close to the expected mass of 198 kDa for a stoichiometric (2:2) PEAK2\(^{\text{IDR1}}\) dimer:CrkII\(^{\text{FL}}\) dimer complex (Extended Data Fig. 3e).

The marked differences in complex stability observed between binding of dimeric and monomeric CrkII\(^{\text{FL}}\) to dimeric PEAK1 and PEAK2 reveal an important role for avidity for stable recruitment to PEAKs, helping to rationalize the dimerization requirement observed in cellular studies\(^{48}\).

**PEAK3 can bind 14-3-3 via the tandem site 14-3-3 motif (pS69)**

We next wanted to extend these studies to recombinant PEAK3\(^{\text{FL}}\). Interestingly, PEAK3\(^{\text{FL}}\) from insect cells was purified as a stable heteromeric complex with two additional interactors, identified by MS as insect cell derived 14-3-3 proteins (14-3-3\(\varepsilon,\zeta\) heterodimer) (Fig. 4a and Source Data)\(^{37}\). This was consistent with our SLiM identification of a highly conserved putative 14-3-3 motif present in the PEAK3 IDR. Co-purification of 14-3-3 from insect cells has been observed for other kinases with 14-3-3 binding sites (e.g. BRAF, LRKK2)\(^{29,49-51}\). Proteomic analysis of the purified PEAK3\(^{\text{FL}}\):14-3-3\(\varepsilon,\zeta\) complex confirmed that recombinant PEAK3 purified from insect cells was phosphorylated only at the 14-3-3 motif (pS69) within the tandem site (Extended Data Fig 4a). Despite apparent conservation of this tandem 14-3-3 motif across the PEAK family, we observed differences in both
phosphorylation and 14-3-3 interaction at this motif between PEAK family members. Proteomic analysis of insect cell derived PEAK2\textsuperscript{IDR1} showed phosphorylation of the tandem site 14-3-3 motif (RAASSP), whereas phosphorylation at this motif was not observed for PEAK1\textsuperscript{IDR1} (RANTEP), moreover co-purification with 14-3-3 was only observed for PEAK3\textsuperscript{FL} (Extended Data Fig. 4a). To complement these data and further characterize the contribution of PEAK3 pS69 in recruiting 14-3-3 in cells, we performed anti-HA IPs from HEK293 cells expressing HA-tagged PEAK3\textsuperscript{FL} WT (HA/PEAK3\textsuperscript{FL}) and PEAK3\textsuperscript{FL}-S69A mutant (HA/PEAK3\textsuperscript{FL}-S69A; Fig. 4b and Extended Data Fig. 4b). These experiments confirmed that while HA-tagged PEAK3\textsuperscript{FL} WT can co-immunoprecipitate endogenous 14-3-3, this interaction was abolished with HA-tagged PEAK3\textsuperscript{FL}-S69A, indicating that S69 is the primary cellular 14-3-3 interaction site for PEAK3.

**PEAK3:14-3-3 form a stable dimer:dimer heterocomplex**

We recently showed that PEAK3 can form homodimers in a cellular context\textsuperscript{14}. To study the effect of PEAK3 dimerization on 14-3-3 binding in cells, we co-expressed in HEK293 cells Flag-tagged PEAK3\textsuperscript{FL} WT with HA-tagged versions of PEAK3\textsuperscript{FL} WT or PEAK3\textsuperscript{FL} dimerization mutants (L146E, A436E, C453E)\textsuperscript{14,18}. Immunoprecipitation and western blotting experiments confirmed that the inability of these mutants to undergo homodimerization is associated with a lack of recruitment of 14-3-3 (Extended Data Fig. 4c), demonstrating that 14-3-3 association to PEAK3 is also dependent on PEAK3 dimerization. These findings build on our previous data\textsuperscript{14} (and reports from the Jura laboratory\textsuperscript{4}) demonstrating the dimerization dependent recruitment of other interactors such as CrkII and ASAP1 to PEAK3.

We next used SEC-MALS to confirm the stoichiometry of our recombinant PEAK3\textsuperscript{FL}:14-3-3ε,ζ heteromeric complex, as homo or heterodimers of 14-3-3 will contain two separate phosphopeptide binding sites. A single symmetrical peak with an experimental mass of 153 kDa was observed, suggesting a stable PEAK3\textsuperscript{FL}:14-3-3ε,ζ dimer:dimer complex (expected mass 155 kDa) (Fig. 4c). We next performed SEC interaction studies of the PEAK3\textsuperscript{FL}:14-3-3ε,ζ complex with the CrkII\textsuperscript{FL} dimer. Interestingly, while CrkII\textsuperscript{FL} complexes with PEAK1\textsuperscript{IDR1} or PEAK2\textsuperscript{IDR1} (Fig. 3c-d), the PEAK3\textsuperscript{FL}:14-3-3ε,ζ complex showed no complex formation with CrkII\textsuperscript{FL}, despite the presence of the CrkII\textsuperscript{NSH3} binding motif in the PEAK3\textsuperscript{FL} construct (Fig. 4d).

**PEAK3 shows a high affinity phosphodependent interaction with 14-3-3 at the tandem site**

Given the proximity of the 14-3-3 motif and CrkII\textsuperscript{NSH3} motifs within the tandem site, we next wanted to compare binding affinity of 14-3-3 and CrkII\textsuperscript{NSH3} at each respective site (Fig. 5 and Extended Data Fig. 5). We generated peptides of PEAK3, PEAK1 or PEAK2 that encompass the tandem site (CrkII\textsuperscript{NSH3} and 14-3-3 motif), phosphorylated or non-phosphorylated at the 14-3-3 motif and
conducted biophysical interaction studies by SPR with purified 14-3-3 and CrkII proteins (Extended Data Fig. 5a and Supplementary Information). We selected, as an initial subset, four human 14-3-3 isoforms (γ, ε, η, σ) out of the 7 human isoforms, as those were the most highly represented in proteomic interaction analysis with PEAK proteins in cells\(^4,20\). SPR analysis of PEAK3 tandem peptides confirmed that phosphorylated PEAK3\(^{\text{tandem}-pS69}\) peptide binds with high affinity to immobilized 14-3-3\(\gamma\) (SPR dissociation constant, \(K_D = 0.19\) µM), whereas the corresponding non-phosphorylated PEAK3\(^{\text{tandem}-S69}\) peptide shows negligible binding (\(K_D > 10\) µM) (Fig. 5a).

We next compared SPR binding of PEAK3, PEAK1 and PEAK2 tandem peptides to human 14-3-3\(\gamma\), 14-3-3\(\varepsilon\), 14-3-3\(\eta\) and 14-3-3\(\sigma\). For each isoform, phosphodependent binding was observed and the PEAK3\(^{\text{tandem}-pS69}\) phosphopeptide consistently showed approximately 10 fold tighter affinity (\(K_D \sim 0.16-0.78\) µM) than those of PEAK1\(^{\text{tandem}-pT1165}\) or PEAK2\(^{\text{tandem}-pS826}\) (\(K_D \sim 1-10\) µM) (Extended Data Fig. 5c). This difference was also apparent in the comparatively slower dissociation kinetics for 14-3-3:PEAK3\(^{\text{tandem}-pS69}\) complexes relative to PEAK1 or PEAK2 tandem peptides (Extended Data Fig. 5c and Supplementary Information).

To gain molecular details of the PEAK3 interaction with 14-3-3, we determined the crystal structure of a 14-3-3\(\varepsilon\):PEAK3\(^{\text{tandem}-pS69}\) complex to 2.5 Å resolution (Table 1). This complex crystallized with two 14-3-3\(\varepsilon\) dimers in the asymmetric unit (ASU), each bound to a single copy of the PEAK3\(^{\text{tandem}-pS69}\) peptide. In each symmetric 14-3-3\(\varepsilon\) dimer, the PEAK3\(^{\text{tandem}-pS69}\) peptide is present in the peptide binding groove in an overall antiparallel arrangement (Fig. 5b), with clear unbiased electron density for the central phosphoserine residue (PEAK3 pS69) recognized by 14-3-3\(\varepsilon\) as well as adjacent residues of the PEAK3 motif (approximately 7 residues in total). Notably, interactions typical of such 14-3-3 complexes are observed: the PEAK3 pS69 phosphate group is coordinated by 14-3-3\(\varepsilon\) residues K50, R57, R130 and Y131 and 14-3-3\(\varepsilon\) D127 and N176 interact to enable N176 to form a hydrogen bond to the backbone amide -NH of PEAK3 L70. Each copy of the PEAK3\(^{\text{tandem}-pS69}\) peptide within the ASU adopts a similar conformation (Fig. 5c-e, Extended Data Fig. 5b and Table 1). We also solved two crystal structures of 14-3-3\(\varepsilon\): PEAK1\(^{\text{tandem}-pT1165}\) and 14-3-3\(\varepsilon\):PEAK2\(^{\text{tandem}-pS826}\) complexes, which were lower resolution (3.1Å) but similarly confirmed phosphorecognition and a canonical mode of binding. In each of the three 14-3-3:tandem peptide structures, no clear electron density was observed for the adjacent CrkII\(^{\text{NSH3}}\) PRM likely due to flexibility and crystallographic averaging.

**The 14-3-3 site on PEAK3 represents a molecular switch for CrkII binding.**
To biophysically evaluate potential positive or negative cooperativity between these two adjacent binding sites in the PEAK3 tandem motif, we turned to sequential binding studies by ITC (Fig. 6a-b and Extended Data Fig. 6a-d). In these experiments, the PEAK3 tandem peptide (either phosphorylated or non-phosphorylated) was loaded in the cell and protein (either 14-3-3γ or CrkII<sup>NSH3</sup>) in the syringe. First a titration of 14-3-3γ was measured, to saturate the first site on the peptide (binary experiment). The tandem peptide/14-3-3γ complex was retained in the cell and a second titration measured for CrkII<sup>NSH3</sup> in the presence of 14-3-3γ (ternary experiment) (Fig. 6).

Corresponding sequential titrations were also conducted using buffer (first injection) followed by CrkII<sup>NSH3</sup> (second injection) (binary experiment). Direct SPR binding studies for each PEAK3<sup>tandem</sup> peptide to immobilized CrkII<sup>NSH3</sup> were also undertaken. The results for ITC and SPR binding experiments for the PEAK3<sup>tandem</sup> peptides are summarized in Table 2.

For the non-phosphorylated PEAK3<sup>tandem</sup> peptide, both SPR and ITC yield an affinity ($K_D$) of 0.60 µM for binding to CrkII<sup>NSH3</sup>, whilst no appreciable binding of this peptide to 14-3-3γ is observed. Similarly, the affinity of this peptide for CrkII<sup>NSH3</sup> was unaffected by first pre-saturating with 14-3-3γ (ITC $K_D$ 0.70 µM). In contrast, phosphorylated PEAK3<sup>tandem-pS69</sup> peptide showed strong binary binding to 14-3-3γ by either method (SPR $K_D$ 0.19 µM, ITC $K_D$ 0.13 µM), but modestly weaker binary binding to CrkII<sup>NSH3</sup> relative to the non-phosphorylated peptide (SPR $K_D$ 2.2 µM, ITC $K_D$ 1.1 µM). It is possible that the phosphate group reduces electrostatic complementarity to the adjacent negatively charged patch on the CrkII<sup>NSH3</sup> binding cleft<sup>52</sup>. When the phosphorylated PEAK3<sup>tandem-pS69</sup> peptide was first pre-complexed with 14-3-3γ, binding of CrkII<sup>NSH3</sup> was further weakened (ITC $K_D$ 3.6 µM). This represents a 6-fold loss in affinity for the PEAK3 tandem peptide towards CrkII<sup>NSH3</sup> following pS69 phosphorylation and 14-3-3 binding, demonstrating negative cooperativity in these interactions at the tandem site. In the context of a dimeric scaffold (dimer:dimer of PEAK3:14-3-3ε,ζ) it is expected this negative cooperativity will be further pronounced due to avidity effects, which supports the inability of the CrkII<sup>FL</sup> dimer to show measurable complex formation with PEAK3:14-3-3ε,ζ via SEC.

Consistent with these biochemical and biophysical data, in our HEK293 cellular immunoprecipitation experiments in using wildtype HA-tagged PEAK3<sup>FL</sup> and PEAK3<sup>FL-S69A</sup> mutant, we observed that alongside the complete loss of 14-3-3 association for the PEAK3<sup>FL-S69A</sup> mutant was a modest but significant increase in level of associated CrkII, relative to PEAK3<sup>FL-WT</sup> (Extended Data Fig. 4b). Importantly, this is despite the level of CrkII associated with PEAK3<sup>FL-WT</sup> (or S69A) in these experiments likely being a composite of a number of multivalent CrkII interactions with multiple partners, including potentially interaction of CrkII with heterodimeric PEAK3/PEAK1 or
PEAK3/PEAK2 via the CrkII$^{NSH3}$ site, PEAK3-pY24 motif via the CrkII$^{SH2}$ site and possible bridging via other adapters such as Grb2, as we have shown previously$^{14}$.

Building a model for PEAK3/CrkII regulation via the tandem site 14-3-3 motif

Taken together, our data provide the molecular basis for a model of PEAK3 regulation, in which the ‘tandem site’ we identified within the relatively short IDR of PEAK3 acts a molecular switch for CrkII binding (Fig. 7a), acting via 14-3-3. PEAK3, PEAK1 and PEAK2 (as homo- or heterodimers) can all bind CrkII at the conserved tandem site ($K_D \sim 1-2 \mu M$ for each 1:1 interaction with CrkII$^{NSH3}$ domain, but higher effective $K_D$ of PEAK dimer for clustered CrkII to avidity) (Fig. 7a(i)).

Phosphorylation at PEAK3 S69 (tandem site) generates a high affinity binding site for dimeric 14-3-3 proteins ($K_D \sim 0.1-1 \mu M$ for at each site, further enhanced by avidity due to dimer/dimer interaction). This enables formation of a highly stable PEAK3:14-3-3 dimer:dimer complex (Fig. 7a(ii),b). Due to negative cooperativity between 14-3-3 and CrkII$^{NSH3}$ binding at each tandem site, the PEAK3 interaction with CrkII is destabilized (Fig. 7a(iii)). 14-3-3 thus appears to be a key regulator of PEAK3 activity and modulates signalling via CrkII, with the crucial switching event being phosphorylation at the PEAK3 tandem site S69 motif. As an intact PEAK3 binding site for CrkII$^{NSH3}$ is also shown to be crucial for Src/SFK-mediated phosphorylation of PEAK3 and binding of Grb2/ASAP1 at the adjacent Y$^{24}$ site, this may also implicate 14-3-3 binding in regulation of this process (Fig. 7a(iv)).

DISCUSSION

Our studies illuminate several aspects of the dynamic regulation of PEAK scaffolding functions, particularly in the context of integrin- or EGFR-mediated signaling (Fig. 7, Extended Data Fig. 7). We apply a combination of analytical techniques to characterize PEAK3 interactions with Grb2, CrkII and 14-3-3 via its N-terminal IDR, and the role of phospho-regulation within the PEAK3 IDR. We demonstrate how dimerization-induced avidity effects and molecular crowding can each modulate SH3/PRM interactions, including how these mechanisms of interaction are conserved or differ across the PEAK family.

Firstly, we provide new molecular insights to contextualize the interaction of PEAK3 and PEAK1 with Grb2/ASAP1$^{14,23}$. We confirm that active Src, but not Abl, phosphorylates the conserved TYSNL SH2-motif (PEAK3$^{Y24}$, PEAK1$^{Y1107}$), supporting previous cellular studies,$^{14}$ and thereby provides a binding site on either PEAK3 or PEAK1 for Grb2$^{SH2}$ or possibly CrkII$^{SH2}$ (Fig. 2). We provide a high-resolution structure of Grb2$^{FL}$ characterizing the binding of PEAK1/PEAK3 at this
site and identify a conserved interface mediated by the PEAK3 peptide when bound to Grb2 that provides a possible docking site for a PEAK/Grb2 interactor. ASAP1 is a possible candidate, as PEAK3 pY24 phosphorylation is required for both ASAP1 and Grb2 interaction with PEAK3\textsuperscript{14} and the conserved solvent-exposed residues of PEAK3 predicted from the Grb2\textsuperscript{FL} docking model (K\textsuperscript{38}, P\textsuperscript{36}, L\textsuperscript{35}, R\textsuperscript{31}) also resemble the linear consensus motif for the ASAP1 SH3 domain (KPxR)\textsuperscript{43} (Fig. 2). The observation that PEAK3 dimerization is required for Y\textsubscript{24} phosphorylation (and Grb2 association)\textsuperscript{14} is also consistent with a model in which clustered CrkII assists to colocalize dimeric PEAK3 with an active kinase (e.g. Src) for efficient Y\textsubscript{24} phosphorylation. Grb2 is also thought to undergo dynamic switching between dimeric (inactive) and monomeric (active, Sos-binding) states; a feature reported to be pivotal for Grb2 signalling in cancer\textsuperscript{46}. Further studies are needed to examine whether PEAK dimers can impact this process also.

Secondly, we examine how NSH3/PRM interactions and avidity together mediate PEAK3 interaction with CrkII, a key player in FA turnover. Our work clearly places PEAKs as recruited scaffolds within this context at FAs (Fig. 7a,b), together with PYK2 and active Src, other important downstream mediators of integrin signaling\textsuperscript{54,55}. We show that the conserved PRM in the PEAK IDR tandem site alone confers modest affinity for CrkII\textsuperscript{NSH3} (SPR $K_D \sim 1 \mu$M with a rapid off-rate; Fig. 3), which is essentially equivalent for all PEAKs. However, we demonstrate using monomeric and dimeric forms of CrkII\textsuperscript{FL} that SHED-dependent homodimerization (and by implication also hetero-dimerization of PEAKs)\textsuperscript{4,17-20} enables enhanced stability of PEAK complexes with dimeric CrkII due to avidity (Fig. 3; Extended Fig. 7b). These findings further support and rationalize data from this work and other reports\textsuperscript{4,14} demonstrating that mutation of key residues of the PEAK3 dimer interface disrupts PEAK3/CrkII interactions in cells, which would impact avidity of these complexes rather than the PRM/SH3 interaction.

We therefore propose that high avidity binding between the CrkII\textsuperscript{NSH3} motif and the conserved tandem site PRM on PEAK dimers is a general feature of PEAKs that enhances localization to FAs or other sites of CrkII clustering (Fig. 7a; Extended Fig. 7)\textsuperscript{32,56}. Whilst we identify an SH2-mediated dimeric form of CrkII\textsuperscript{FL} from overexpression in \textit{E. coli}, further work will be required to confirm whether this exists in a cellular context. However, as multivalency is a general feature of both CrkII and CrkL signaling complexes, our work has broader implications. A notable example is cell adhesion-mediated CrkII/CrkL interactions with the Cas family of scaffolds (p130Cas or NEDD9), each of which harbor flexible central substrate domains with multiple repeats (15 repeats in p130Cas) of the YxxP motif; a consensus binding sequence for SH2- and PTB-containing proteins such as CrkL, Nck, and SHIP2. Integrin-mediated cell adhesion allows Cas to activate Src/SFKs, which
processively phosphorylate the Cas substrate domain following mechanosensitive changes in its accessibility, ultimately enabling multivalent CrkII recruitment\textsuperscript{57,58}. In this manner, CrkII cooperates with p130Cas/NEDD9 in dynamic integrin signaling at FAs\textsuperscript{59}. Bound and clustered CrkII then recruits other adapters/effectors of other pathways to amplify and diversify the signal including Dock180/Rac (regulation of migration) and C3G/Rap1 (regulation of cellular morphology and adhesion)\textsuperscript{60}. Likewise, AXL-mediated NEDD9 phosphorylation is reported to recruit CrkII and thereby PEAK1, leading to altered FA dynamics in breast cancer cells\textsuperscript{32}. This is consistent with roles for p130Cas/NEDD9 in tumor progression through regulating cytoskeletal dynamics\textsuperscript{61}. Thus, although not specifically examined in our work, our findings with CrkII likely also have implications for PEAK interactions with CrkL, which interestingly can additionally undergo CSH3-mediated homodimerization in cells.\textsuperscript{47} Notably, the SH2-mediated localization of CrkL at FAs is reported to be important for correct co-recruitment of ASAP1 (via the ASAP1 SH3 domain) to FAs in platelets\textsuperscript{62}. Further studies would be needed to confirm whether PEAK3 has a role in this process also.

Finally, whilst SH3/PRM interactions do not typically require PTMs for binding, they are by no means static, and we exemplify two mechanisms by which they can be dynamically regulated: via clustering/avidity, as described above; and via accessibility, which can be modulated by the phosphorylation of an adjacent site. We identify a conserved tandem motif (CrkII\textsuperscript{NSH3} site / 14-3-3 site) present on all PEAKs (Fig. 5) and define a role for 14-3-3 as a potential negative regulator of PEAK3/Crk interactions at FAs (Fig. 7 and Extended Data Fig. 7a). We demonstrate that PEAK3\textsubscript{FL} forms a highly stable dimer:dimer complex with 14-3-3, an interaction that prevents stable PEAK3 binding to dimeric CrkII\textsubscript{FL} (Fig. 5). Our ITC competition studies with PEAK3 tandem peptides confirm that the CrkII\textsuperscript{NSH3} and 14-3-3 directly exhibit negative cooperativity for binding to the PEAK3 tandem site, providing a mechanistic explanation for the observed effect on PEAK3/Crk interaction with full-length proteins (Fig.6).

PEAK3 appears to be unique from PEAK1/2 in its interaction with 14-3-3 at the tandem motif, displaying ~ 10 fold higher affinity for 14-3-3 (PEAK3 pS69), compared to either PEAK1 (pT1165) or PEAK2 (pS826). For PEAK3, the pS69 site is sufficient for stable 14-3-3 recruitment, whereas the longer IDRs of PEAK1/2 are predicted to harbor additional 14-3-3 motifs and different mechanisms may exist. For example, the conserved tandem site 14-3-3 motif might act as a phosphodependent secondary (low-affinity) 14-3-3 site to alter PEAK1/2 activity following initial 14-3-3 recruitment at a distal ‘gatekeeper’ (high-affinity) site\textsuperscript{63,64}.
14-3-3 binding has been reported to provide a regulatory switch in proteins involved in cytoskeletal dynamics (e.g. SSH1L, cortactin and IRSp53) by regulating downstream effector interactions and subcellular localization. For IRSp53, 14-3-3 binding masks SH3-mediated docking with effectors at lamellipodia and filopodia, terminating IRSp53 signaling. As CrkII and Grb2 appear to cooperate in PEAK3 signalling, PEAK3-pS69 phosphorylation and 14-3-3 binding may thus provide an analogous negative feedback loop to terminate PEAK3/Crk/Grb2-mediated signaling at FAs, possibly also by altering PEAK3 subcellular localization (Fig. 7, Extended Data Fig. 7).

Consistent with our findings that pS69A mutation of PEAK3 disrupts 14-3-3 binding and leads to a modest increase in association with CrkII, we therefore predict that this may also enhance PEAK3 pY24 phosphorylation and Grb2/ASAP1 association, possibly in an integrin-dependent manner. We and others have reported that PEAK3 forms complexes in cells with Grb2/PYK2/ASAP1. In THP1 cells in the absence of growth factors, PEAK3 binding was reported to result in PYK2-mediated activation of ASAP1 and activation of PI3K/AKT signalling. We propose that the S69 site on PEAK3 may therefore act as a site of negative regulation of PEAK3 activity in these signaling pathways, via phosphorylation by a basophilic serine/threonine kinase, however further studies will be required to confirm the relevant kinase/s for the PEAK3 S69 site in cells.

**Conclusion**

Together our work highlights distinct mechanisms of regulation and signaling outputs possible from homo/heterodimeric PEAK complexes (Extended Data Fig. 7b). We characterize the role of the conserved IDR motif and dimerisation/avidity in CrkII binding, features expected to be common to all PEAK complexes. We illuminate additional features of an IDR interaction with Grb2 present only in PEAK3/PEAK1. Lastly, we identify a role for the IDR tandem site in mediating PEAK binding to 14-3-3 proteins. For PEAK3, phosphorylation at S69 enables formation of a distinct high affinity PEAK3:14-3-3 complex and we propose a mechanistic role in negative regulation of PEAK3/CrkII signaling. This provides a framework to better understand the contribution of intrinsically disordered regions to signaling of dimeric PEAK family pseudokinase scaffolds in healthy cells and cancer.
Illustrations
All illustrations were generated using PyMOL, UCSF ChimeraX, PRISM, Microsoft Excel or Adobe Illustrator and InDesign.

Reporting Summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data Availability
Coordinates and structure factors for the X-ray crystal structures have been deposited in the PDB with accession codes 8DGO (Grb2FL:PEAK SH2-pY peptide), 8DGP (14-3-3c:PEAK3tandem-pS69), 8DGM (14-3-3c:PEAK1tandem-pT1165) and 8DGN (14-3-3c: PEAK2tandem-pS826).

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Author contributions
Competing interests
The authors declare no competing financial interests.

Additional information
Extended data is available for this paper at ...
Supplementary information is available for this paper at ...
Correspondence and requests for materials should be addressed to M.R. or I.L.

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Table 1. Data collection and refinement statistics.

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<th>Grb2FL:PEAK SH2-pY peptide (PDB 8DGO)</th>
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<th>14-3-3ε: PEAK tandem-pS826 (PDB 8DGNN)</th>
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aData are from one crystal for each structure.

bValues in parentheses are for the highest-resolution shell.
Table 2: Summary of ITC sequential binding studies data

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<th>ITC $K_D$ (µM)</th>
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<th>SPR $K_D$ (µM)</th>
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<td>-</td>
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Refer to Supplementary Information Table (SPR, ITC data)

Values are mean, uncertainties are SD (N = 2) or S.E.M (N ≥ 3), from the listed number of independent experiments (N).
**Figure 1: PEAK domain organization and interaction motifs in N-terminal IDR.**

**a.** Domain organization of the PEAK family and diagram of PEAK homodimer arrangement showing SHED, PsK and IDR motifs identified (boxed).

**b.** Sequence alignment of the N-terminal IDR of human PEAK3 with the corresponding region of PEAK1 and PEAK2.

**c.** Multiple Sequence Alignment (MSA) of PEAK vertebrate orthologues highlighting Short Linear Interaction Motifs (SLiMs) identified in regions of high sequence conservation, including a pY/SH2 motif (Grb2<sub>SH2</sub>/CrkII<sub>SH2</sub>) and the tandem site encompassing a proline rich motif (CrkII<sub>NSH3</sub>) and conserved putative 14-3-3 motif.
**Fig. 2**

**Table**

<table>
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<th>Protein</th>
<th>+ Kinase</th>
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<td>PEAK3FL</td>
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<td>pY112 (SH2 motif)</td>
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<td>CrkII</td>
<td>Abl</td>
<td>pY(SH2 motif)</td>
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**Fig. 2:** Structural and biophysical analysis of PEAK/Grb2SH2 interaction. **a,** TYSNL site of PEAK3FL (Y24) and PEAK1IDRI (Y1107) can be phosphorylated by Src (MS/MS analysis of tryptic peptides following in vitro kinase assay of PEAK3FL and PEAK1IDRI available in Source Data). Both Abl and Src can phosphorylate CrkIIY221, a known Abl phosphorylation site, demonstrating that both Abl and Src kinases are active. **b,** Ribbon representation of the structure of Grb2FL:PEAK SH2-pY peptide of PEAK323-29-pY24/PEAK1106-1112-pY1107 showing dimer arrangement (chain A in light green and chain B in dark green) with cartoon illustrating domain organization. **c,** Zoom in highlighting peptide groove and the PEAK phosphopeptide (yellow) adopting a characteristic β-turn forming three important hydrogen bonds to the Grb2 backbone with residues Arg 86, His 107 and Lys109. Peptide and interacting residues of Grb2 displayed in a stick representation. **d,** Unbiased Fo-Fc omit map (grey mesh, contoured at 3.0 σ) showing peptide density prior to modelling and final modelled PEAK3/PEAK1 phosphopeptide (TpYSNLQ, yellow sticks, underlined residues modelled). **e,** HADDOCK docked model of extended helical PEAK323-38-pY24 peptide bound to Grb2, showing solvent exposed residues of high sequence conservation in PEAK3 orthologues (marked *) that may form a binding site for a PEAK3/Grb2 interactor.
Fig. 3: Structural and biophysical analysis of PEAK/CrkII$^{NSH3}$ interaction. (a) SPR data summarizing measured steady-state binding affinity ($K_D$) of synthetic PEAK PRM peptides towards immobilized CrkII$^{NSH3}$ domain. (b) Depiction of the published CrkII$^{NSH3}$:Abl$^{758}$ structure (PDB: 5IH2), showing key residues within the CrkII$^{NSH3}$ consensus motif in Abl$^{758}$ (peptide shown in gold stick representation) crucial for high affinity binding to CrkII$^{NSH3}$ (main chain light blue; CrkII$^{NSH3}$ surface is coloured by electrostatic surface potential calculated using UCSF Chimera v 1.16; blue = positive, white = hydrophobic, red = negative). Aligned is the sequence of PEAK$^{354-66}$, showing key conserved residues of the CrkII$^{NSH3}$ motif. (c, d) Interaction studies with recombinant
PEAK1\textsuperscript{IRD1}/PEAK2\textsuperscript{IRD1} and CrkII\textsuperscript{FL} underscore the role of avidity for high affinity binding of CrkII\textsuperscript{FL} to PEAK dimers; (c) Incubation of PEAK2\textsuperscript{IRD1} with CrkII\textsuperscript{FL} dimer at 1:1.3 ratio results in a complex formation while (d) incubation with CrkII\textsuperscript{FL} monomer does not result in a complex, as confirmed by SDS-PAGE analysis of SEC eluted fractions. CrkII\textsuperscript{FL} dimer and monomer in light blue; PEAK2\textsuperscript{IRD1} in pink; complex of PEAK2\textsuperscript{IRD1} dimer:CrkII\textsuperscript{FL} dimer in black line (see Extended Data Fig. 3d for PEAK1\textsuperscript{IRD1} dimer:CrkII\textsuperscript{FL} dimer complex).

**Fig. 4**

**PEAK3:14-3-3 forms a stable high affinity heterocomplex.** **a,** Recombinant PEAK3\textsuperscript{FL} from insect cells elutes on Size Exclusion Chromatography (SEC; S200 10/300) as a high affinity stoichiometric complex of dimeric PEAK3 with a 14-3-3\(\varepsilon,\zeta\) heterodimer, results supported by SDS-PAGE analysis of eluted fractions (See Extended Data Fig. 4a, Source Data). **b,** Immunoprecipitation of PEAK3 WT and PEAK3 S69A showing S69 is required for 14-3-3 co-immunoprecipitation from cells (n=3, see Extended Data Fig. 4a for biological repeats). **c,** SEC-MALS analysis of PEAK3\textsuperscript{FL}-14-3-3\(\varepsilon,\zeta\) heterodimer complex confirming the experimentally determined mass closely matches the mass of 155 kDa expected for a stoichiometric dimer:dimer complex. **c,** SEC profile (S200 10/300) of recombinant PEAK3\textsuperscript{FL}-14-3-3\(\varepsilon,\zeta\) heterodimer pre-incubated with CrkII\textsuperscript{FL} dimer in a 1:1.2 molar ratio showing no complex formation.
Fig. 5: Structural and biophysical analysis of PEAK3/14-3-3 interaction. a, Binding of PEAK3\textsuperscript{tandem} peptide: Ac-PLPPLPKKILTQSLPTRLR-NH\textsubscript{2} to 14-3-3\(\gamma\) as measured by SPR. b, Overall structure of 14-3-3\(\epsilon\): PEAK3\textsuperscript{tandem}-pS69 peptide showing 14-3-3 dimer antiparallel arrangement with each monomer (pink cartoon/surface) bound to a single copy of the PEAK3\textsuperscript{tandem}-pS69 peptide (yellow sticks). Chain A of 14-3-3\(\epsilon\) is in dark pink and chain B is in light pink. c, Zoom in highlighting peptide groove and showing the central pS69 residue of the PEAK3 phosphopeptide interacting with K50, R57, Y131 and R130. d, Unbiased Fo-Fc omit map (green mesh, contoured at 3.0 \(\sigma\)) showing peptide density prior to modelling and final modelled PEAK3\textsuperscript{tandem}-pS69 phosphopeptide (yellow sticks). e, Superposition of the peptide modelled in each 14-3-3 monomer (chains A-D, sticks) in the asymmetric unit.
Fig. 6: 14-3-3γ binding to PEAK3\textsuperscript{tandem} peptide reduces the affinity of CrkII\textsuperscript{NSH3} binding to the adjacent CrkII motif. a, b, Sequential ITC binding studies using PEAK3\textsuperscript{tandem}-S69 non-phosphorylated (a) and PEAK3\textsuperscript{tandem}-pS69 phosphorylated (b) peptides, purified 14-3-3γ and purified CrkII\textsuperscript{NSH3} domain. High affinity binding of 14-3-3γ to PEAK3\textsuperscript{tandem}-pS69 peptide (b) reduces the affinity of CrkII\textsuperscript{NSH3} binding to the adjacent CrkII motif at the tandem site (negative cooperativity). See Extended Data Fig. 6a-d.
**Fig. 7: Proposed model for PEAK3/Grb2 and PEAK3/CrkII regulation via the tandem site 14-3-3 motif.**

**a,** (i) Avidity stabilizes PEAK3/CrkII interaction with clustered CrkII (interacting via the CrkII^NSH3 and tandem motif PRM of the PEAK3 dimer). CrkII is likely to be clustered by interaction with other adapters/scaffolds, such as SH2 domain-mediated binding to partner proteins containing multiple adjacent pYxxP motifs (e.g. p130cas/NEDD9, paxillin), but possibly also via dimerization (e.g. SH2-mediated as we identify for recombinant protein) (ii) Phosphorylation of PEAK3 at S69 creates a high-affinity binding site for 14-3-3, leading to the formation of a highly stable PEAK:14-3-3 dimer:dimer. (iii) Phosphorylation/binding of 14-3-3 to PEAK3 destabilizes CrkII binding in the adjacent tandem site (negative cooperativity), leading to disruption of PEAK3/CrkII complex. This may terminate PEAK3/CrkII signaling and/or alter PEAK3 subcellular localization. (iv) Phosphorylation of PEAK3 at Y24 (e.g. by Src kinase) recruits Grb2 via its SH2 domain leading to association with ASAP1 and PYK2 and potential PYK2/ASAP1-mediated activation of the PI3K/AKT pathway. **b,** Illustrative representation of a potential dimeric human PEAK3^FL:14-3-3ε complex to illustrate relative scale and key sites of interaction. Depicted is the 14-3-3ε:PEAK3^SHED-PsK crystal structure (PDB 8DGP) and a structural model of the PEAK3^SHED-PsK dimer prepared from AlphaFold2 multimer modelling of a human PEAK3^FL dimer. Unmodelled IDR linker regions are depicted as dotted lines. The precise arrangement of the PEAK3^FL dimer relative to the 14-3-3 dimer as shown is arbitrary, although some constraint on relative positioning is expected to be imparted by the short length of the PEAK3 IDR and antiparallel arrangement of interaction with 14-3-3.
Supplementary Files

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