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## **The PEAK family of Pseudokinases, their role in cell signalling and cancer**

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### **Abbreviations:**

PEAK, pseudopodium enriched atypical kinase; SgK, Sugen kinase; NACK, Notch activation complex kinase; PEST, proline, glutamate, serine, threonine; Csk, C-terminal Src kinase; CrkII, **This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/FEBS.15087](https://doi.org/10.1111/FEBS.15087)**

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CT-10 regulator of kinase; Grb2, Growth factor receptor-bound protein; Shc1, Src homology 2 domain-containing transforming protein; CagA, cytotoxin-associated gene A; PINK1, PTEN-induced putative kinase 1; ATP, Adenosine 5' triphosphate; KSR2, Kinase suppressor of Ras 2; MEK, Mitogen activated protein kinase; Abl, Abelson tyrosine kinase; Erk, extracellular signal-regulated kinase; Rnd2, Rho family GTPase 2; GTP, Guanosine 5' triphosphate; SHP2, Src homology 2 domain containing protein-tyrosine phosphatase; Maml1, Mastermind Like Transcriptional Coactivator 1; p130Cas, Cas family scaffolding protein; KRAS, Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; TGF $\beta$ , Transforming growth factor beta 1; MAPK, Mitogen-activated protein kinase; EGFR, Epidermal growth factor receptor; Stat3, Signal transducer and activator of transcription 3; PPP1CA, Serine/threonine protein phosphatase 1 alpha subunit; PPP1CC, Serine/threonine protein phosphatase 1 gamma catalytic subunit; protein phosphatase 1 regulatory subunit (PPP1R12B).

## **Abstract**

The study of pseudokinases has uncovered that catalysis-independent functions play a critical role in cell signalling regulation. However, how pseudokinases dynamically assemble and regulate oncogenic signalling pathways remains, in most cases, unclear due to a limited knowledge of the structural determinants that are critical for their functions. Here, we review the recent progress made to unravel the role of the PEAK family of pseudokinases, which comprises SgK269, SgK223 and the recently identified PEAK3, in assembling specific oncogenic signalling pathways that contribute to the progression of several aggressive cancers. We focus on recent structural advances revealing that SgK269 and SgK223 can homo- and hetero-associate via a unique dimerization domain, comprising conserved regulatory helices directly surrounding the pseudokinase domain, which is also conserved in PEAK3. We also highlight a potential oligomerization mechanism driven by the pseudokinase domain. While it is likely that homo/hetero dimerization and oligomerization mechanisms contribute to the assembly of complex signalling hubs and provide a means to spatially and temporally modulate and diversify signalling outputs, the exact role that these oncogenic scaffolds play in regulating cell migration, invasion and morphology remains unclear. Here, we attempt to link their structural characteristics to their cellular functions by providing a thorough analysis of the signalling transduction pathways they are known to modulate.

## Introduction

Hyperactivity and malfunction through mutations or overexpression of protein kinases are often hallmarks of a vast range of diseases, including many cancers. Aside from their well-known catalytic functions, which govern appropriate spatio-temporal cellular activation of specific signalling pathways via phosphorylation of downstream effectors, protein kinases employ an array of non-catalytic functions to drive and modulate their signalling potential [1, 2]. The critical role that these non-catalytic functions play in the establishment of disease states are today best exemplified with the study of a subset of the kinome, called pseudokinases. Despite exhibiting low to no catalytic activity, pseudokinases, which make 10% of the human kinome, are now known to be an integral signalling components of kinase signalling pathways [3-5]. Recent studies have showcased pseudokinases as allosteric regulators/molecular switches that propagate/regulate kinase signal output [2, 6, 7] or as rigid scaffolds that nucleate the assembly of signalling complexes [8-12], clearly demonstrating the remarkable signalling adaptability of the kinase fold. Each pseudokinase seems to have evolved a unique mode of regulation to enable signal integration, diversity and specificity. Thus, understanding the molecular signatures that dictate pseudokinase signalling functions is critical to decipher how they dynamically assemble and regulate kinase signalling pathways and how their deregulation contributes to the disease state.

The PEAK family of proteins, which comprises Sugen kinase 269 (Pseudopodium-enriched atypical kinase 1, PEAK1) [13], SgK223, an orthologue of rat Pragmin [14] and mouse Notch activation complex kinase (NACK) [15], and the recently identified PEAK3 (C19orf35) [16, 17] are all classified as pseudokinases [18]. SgK269 and SgK223 are defined as oncogenic pseudokinase scaffolds that mainly regulate cytoskeleton reorganisation, cell migration and invasion [19-21]. Whilst the function of PEAK3 is less understood, it has recently been shown to interact with CrkII in regulating cell motility [16]. Each are thought to recruit a distinct set of signalling molecules contributing to signal diversity in a spatially and temporally regulated manner. However, the mechanistic insight into how these signalling scaffolds are assembled and regulated and how they contribute to cancer progression is unknown.

Here, we review the recent progress made in understanding the scaffolding functions of the PEAK family of pseudokinases from a structural biology perspective and discuss the biological

implications of their ability to form homo- and hetero- dimers and oligomeric complexes by outlining their expression levels in cancer and the signalling pathways they are known to modulate.

### **SgK223 and SgK269: a structural and functional overview**

SgK223 and SgK269 are 149 kDa and 193 kDa, respectively, and share a unique domain organisation that comprises a N-terminal domain of around 200 residues (residues 1-216 in SgK223), a large unstructured PEST linker (residues 217-931 in SgK223) and a C-terminal pseudokinase domain flanked by helical domains (residues 932-1406 in SgK223) (Fig. 1A) [13, 14]. PEAK3, in contrast, is much smaller (53 kDa), lacks the N-terminal region and has a significantly shorter PEST linker [22]. The ~200-residue N-terminal region displays some level of conservation between SgK223 and SgK269, but does not share sequence similarity to any known protein and its function remains uncharacterised. The unstructured PEST linker harbours a number of tyrosine phosphorylation sites and proline rich motifs, therefore contributing to their classification as scaffolds by recruiting SH2/SH3-containing adaptor proteins, such as C-terminal Src kinase (Csk), CT-10 regulator of kinase (CrkII), Growth factor receptor-bound protein (Grb2) and Src homology 2 domain-containing transforming protein (Shc1) (Fig. 1A) [13, 23-25]. These adaptor proteins dictate signal transduction pathways by assembling specific signalling complexes involved in cytoskeleton reorganisation, cell migration and invasion. The N-terminal region and the PEST linker are dispensable for an elongated morphology and their deletion do not result in increased cell migration, as typically seen during SgK223 and SgK269 overexpression [10]. However, both of these regions are predicted to play a role in the localization of the proteins to focal adhesion and actin cytoskeleton. An actin targeting region was mapped to residues 338-727 in the PEST linker of SgK269 [13]. The PEST linker of SgK223 and SgK269 harbours an EPIYA motif (EPTYA in SgK269), that once phosphorylated acts as a docking site for adaptor proteins, such as the C-terminal Src-kinase Csk for SgK223 [24], therefore linking SgK223 to focal adhesion regulation. Interestingly, this motif is under-represented in the human proteome but commonly found in a number of pathogenic bacterial proteins, including the *Helicobacter pylori* CagA oncoprotein, with its phosphorylation leading to host signalling perturbation [26]. Further research is required to fully unravel the functional regulation of the EPYIA motif and decipher the exact role that the N-terminal region and PEST linker play in the overall function and localization of SgK223 and SgK269. Additionally, SgK223 and SgK269 harbour a CrkII N-SH3 binding site (residues 812-819 in SgK223) within their PEST linkers, a feature conserved in PEAK3 despite a significantly shorter PEST linker (Fig. 1B) [16].

The C-terminal tail, which consists of helices  $\alpha$ J,  $\alpha$ K,  $\alpha$ L and  $\alpha$ M are well conserved among SgK223, SgK269 and PEAK3 (Fig. 1C). The recent structures of the pseudokinase domain and the

surrounding N- and C-terminal helices of human and rat SgK223 [12], [16] and human SgK269 [27] have provided valuable insights into the unique structural features of these pseudokinases. We summarize below some of these unique features.

### **Identification of a novel dimerization domain: a key scaffolding element**

To gain insight into the structural basis of the PEAK family, our group solved the first crystal structure of human SgK223 and identified a novel mechanism of dimerization involving the N- and C-terminal helices that directly surround the pseudokinase domain (residues K948-F974 and residues G1331-L1406 respectively) which bridges the two monomers and leaves the pseudokinase domains (residues F984-W1330) exposed at the periphery (Fig. 2A). This dimerization domain, which has not been previously identified among the protein kinase family, consists of a novel helix bundle that forms a 'XL' shaped fold in each monomer and nucleates the assembly of high affinity 'XX' symmetrical dimer interface (Fig. 2A) [12]. Following our study, Ha *et al.* published the structure of human SgK269, which was shown to also dimerize through the same N- and C-terminal helices. This dimerization domain was then referred to as split helical dimerization (SHED) domain (Fig. 2B) [27]. The same dimerization mechanism was also later reported in rat SgK223 (Fig. 2C) [16]. Alignment of 111 SgK269 sequences from various species further suggests this dimerization domain to be evolutionary conserved [27]. Whilst the crystal structure of PEAK3 is currently unknown, sequence analysis corresponding to the dimerization domain clearly suggests a similar mechanism of dimerization compared to SgK223 and SgK269 (Fig. 1C). Additionally, PEAK3 homo-dimerization was recently demonstrated using differently tagged versions of PEAK3 that co-immunoprecipitated when expressed in HEK293 cells [17]. In the crystal structures solved of PEAK family members, dimerization occurs through a large buried surface area (1600-4200Å<sup>2</sup>) and biochemical/biophysical studies using AUC, SAXS and MALS have confirmed that SgK223 and SgK269 form stable high affinity homo-dimers in solution [12, 16, 27] and in cells [10] [16]. Surprisingly, the structural fold of the C-terminal helices in human SgK223, rat SgK223 and human SgK269 have high similarity to the C-terminal helices of PTEN-induced putative kinase 1 (PINK1), a mitochondrial-associated kinase that regulates mitophagy. While there is a clear link between disruption of PINK1 kinase activity and neurodegenerative diseases, the importance of this C-terminal region in the regulation PINK1 kinase activity and/or dimerization remains to be elucidated [28, 29].

The overall structural organisation of human and rat SgK223 [12, 16] and human SgK269 [27] is highly conserved (overall rmsd is 1.66 between human SgK223 and human SgK269 over 534 C $\alpha$  aligned atoms and 1.1 between human SgK223 and rat SgK223 over 541 C $\alpha$  aligned atoms) (Fig.

2). It is worth noting the presence of an additional helix preceding the  $\alpha$ N1 helix that was able to be modelled in the rat SgK223 structure, within one molecule of the dimer (Fig. 2C). While this helix is not directly involved in dimerization, it packs against the  $\alpha$ N1 helix of the same protomer and the  $\alpha$ J and  $\alpha$ K of the related dimer molecule, further locking the dimerization domain through interactions between the N- and C-regulatory helices. The dimeric interface is dominated by hydrophobic interactions mediated by residues that are conserved in SgK223 and SgK269 (Fig. 1C and Fig. 3A). Extensive alanine scanning mutagenesis studies performed on SgK223 have identified hot spot residues L955 and L966, located within the  $\alpha$ N1 helix and conserved across the PEAK family, to form the core of the dimeric interface [12]. The intramolecular network that connects the N- and C- regulatory helices to the pseudokinase domain comprises polar and non-polar interactions, and alanine mutations of conserved residues F1366 in  $\alpha$ K and W1382 in  $\alpha$ L had the greatest impact in disrupting SgK223 dimerization (Fig. 3B). Transfection of these mutants into SgK223 knock-out MCF-10A cells, failed to enhance cell migration, contrary to what is typically observed upon overexpression of the wild-type SgK223. In addition, these mutants also failed to co-immunoprecipitate endogenous SgK269 suggesting that SgK223-SgK269 could exist as a signalling hetero-dimer or hetero-oligomer in cells. Introduction of the A1329E dimer interface mutant into rat SgK223 led to an overall reduction in cellular protein tyrosine phosphorylation, likely due to dimer disruption. This included a reduction in Csk-mediated phosphorylation of rat SgK223 at position Y391, highlighting the effect of dimer disruption on Csk activity [14]. Whether SgK223 and SgK269 exist as homo- or hetero-dimer in cells will depend on many factors, such as their cellular expression, localisation and response to signalling inputs [12]. Recent studies in PEAK3 showed that mutation of key residues within the N- and C-terminal helices or deletion of one of the  $\alpha$ N1,  $\alpha$ J or  $\alpha$ K helices not only disrupted PEAK3 dimers, as evidenced by co-immunoprecipitation data, but also altered its ability to interact with CrkII, which underscores the importance of the conserved dimerization interface for proper signalling outputs [17]. In summary, the recent structural and functional studies of the PEAK family have provided the first steps in understanding the role of SgK223, SgK269 and PEAK3 as scaffolds and how signal integration, amplification and diversification could be achieved through homo- and hetero-dimeric complex formation.

### **Conformational rearrangement of the pseudokinase domain**

Each monomer within the dimer maintains a conserved kinase catalytic fold with  $\beta$ -sheets dominating the N-lobe and  $\alpha$ -helices dominating the C-lobe (Fig. 2A). Despite the presence of insertion loop of varying lengths in between the secondary structure elements, the pseudokinase domains adopts a conserved fold with a rmsd of 0.72 and 0.59 between human SgK223 and human

SgK269 (over 247 C $\alpha$  atoms) and rat SgK223 (over 259 C $\alpha$  atoms), respectively. The  $\alpha$ C helix in human and rat SgK223 crystal structures is disordered, however was able to be partly modelled (residues H1373-S1380) in the structure of SgK269 (Fig. 2B). However, the residues connecting the  $\beta$ 3 strand and the  $\alpha$ C helix are disordered in all three structures, suggesting flexibility in conformation in this region, which may be due to the lack of the conserved  $\alpha$ C helix glutamate that usually forms the canonical  $\beta$ 3 lysine- $\alpha$ C glutamate salt bridge of an active kinase conformation akin to the K72-E91 salt bridge of the exemplar kinase, protein kinase A [30]. Instead, in all three structures, the  $\beta$ 3 lysine (1024 in SgK223) forms an H-bond interaction with a glutamine (1048 in SgK223) located in the loop preceding the  $\beta$ 4 strand (Fig. 3B). This unusual interaction locks the orientation of the N-lobe of the pseudokinase domain with respect to the C-lobe, influencing the relative positions of the key residues that define the regulatory and catalytic spines of a conventional protein kinase [31]. Both the  $\beta$ 3 lysine and the glutamine participating in this interaction are conserved in PEAK3 suggesting that the N-lobe of the pseudokinase domain of PEAK3 will adopt a similar orientation. In all three structures, the N-lobe is further stabilised by a triad interaction between F1045 from the  $\beta$ 3- $\beta$ 4 loop, F974 of  $\alpha$ N1 helix and W1382 of  $\alpha$ L helix (Fig. 3B), anchoring the dimerization domain to the pseudokinase domain.

**An occluded ATP binding site:** In all three structures, the ATP binding site is inaccessible and occluded by the side chains of conserved residues Y1008, C1001, Q1048 and L1152 (numbering from human SgK223) (Fig. 3C). Of these residues, Y1008, Q1048 and L1152 are absolutely conserved in PEAK3. Considering the strong contribution of these residues to blocking the ATP binding site and the conservation of residues within the anchoring pseudokinase-dimerization domain, it is likely that the ATP binding site of PEAK3 will be similarly occluded. These structures together have therefore confirmed that the members of the PEAK family are likely devoid of any catalytic activity, as previously demonstrated by nucleotide binding experiments [18].

**The conformation of the activation loop highlights a regulatory mechanism:** In all three structures the majority of the activation loop is not resolved, highlighting the inherent flexibility of this region, a property known to contribute to the mechanism of activation of active kinases. However, both the start and the end of the activation loop are visible, which provides some indication of the various conformations that it can adopt (Fig. 3D). Although we cannot rule out that some of the differences seen in these structures could be due to crystal packing interactions, interesting features are evident. The phenylalanine sidechain from the SNF motif (corresponding to the canonical DFG motif) in SgK223 and SgK269 structures is buried under the  $\alpha$ C helix and the serine and asparagine side-chains are pointing towards the pseudo-ATP binding site, therefore adopting a conformation akin to an active kinase conformation. As a result, the conserved motif

AKQK at the start of the activation loop, is resolved in both rat SgK223 and human SgK269 and is packed close to the  $\alpha$ C and  $\alpha$ E helices. In contrast to the start of the activation loop, the conformation of the C-terminal end of SgK269 activation loop (residues R1572-A1584) greatly diverges with that of SgK223. In SgK223, the C-terminal end of the activation loop is extended and solvent exposed, making an interaction with the symmetry-related molecule within the crystal lattice. Interestingly, in SgK269 the same region snuggles between the pseudokinase domain  $\alpha$ F and  $\alpha$ G helices and is stabilised by a salt bridge interaction between activation loop residue R1576 and  $\alpha$ F residue E1601 (Fig. 3D). The glutamate and arginine are conserved within the PEAK family, suggesting that the activation loop of SgK223 and PEAK3 could readily adopt this conformation. Potential phosphorylation sites within the activation loop, namely T1227 in SgK223 [32, 33] and S1563 in SgK269 [34] have been reported in phosphoproteomics studies, however additional studies will be required to unravel if phosphorylation regulates the activation loop conformation of SgK223 and SgK269 and determine the biological implications of these phosphorylation events. Our structural and mutagenesis studies have shown that the region occupied by the end of the activation loop in SgK223 is also a site for oligomerization, as discussed in more detail in the next section [12]. In contrast to SgK223 and SgK269, PEAK3 has a degraded activation loop despite retaining the canonical DFG motif (residues 330-332) typical of active protein kinases (Fig. 3D).

### **Oligomerization: implication for signalling**

In addition to forming high affinity dimers *in vitro*, SgK223 and SgK269 have the ability to form higher order molecular assemblies through either homo- or hetero-association mechanisms [10]. Analytical ultracentrifugation analysis on human SgK223 suggested a concentration-dependent higher order oligomerization mechanism [12]. The crystal structure of the SgK223 dimer identified an additional interface formed through a symmetry related dimer that buries 824Å<sup>2</sup> surface area formed through interaction of pseudokinase domain  $\alpha$ G/activation loop of one dimer with the N-lobe of the pseudokinase domain of the symmetry related dimer (Fig. 4A). Alanine substitution of either I1243 of the activation loop, or F1271 or Y1282 of the  $\alpha$ G helix, was sufficient to disrupt higher order oligomers. Interestingly, these mutants, introduced in either SgK269 or SgK223, were unable to form higher order heterotypic oligomers [12], suggesting a critical role for the pseudokinase domain  $\alpha$ G/activation loop in driving heterotypic association. When these mutants were transfected in cells, Y1282A SgK223 showed reduced self-association, however to a lesser extent than the mutants at the dimerization interface, suggesting that the oligomerization interface is of low affinity.

Several studies have reported the role of  $\alpha$ G helix/activation loop in mediating interaction between kinase and pseudokinase domains and between kinase domains and their substrates/regulators [35] [36]. One such example is the interaction of the human Kinase suppressor of Ras 2 (KSR2) pseudokinase domain with Mitogen-activated protein kinase (MEK) solely mediated by an  $\alpha$ G helix/activation loop interaction [37]. This face-to-face interaction prevents MEK1 phosphorylation by retaining MEK1 in an inactive state. A conformational change in KSR2 induced by ATP binding leads to MEK activation and phosphorylation (Fig. 4B). Considering the points of differences noted around the  $\alpha$ G helix/activation loop within the PEAK family (as described above), it is likely that this interface is a critical regulatory element, however the biological signalling relevance of  $\alpha$ G/activation loop oligomerization is yet to be demonstrated .

### **PEAK family: expression in cancer and mutations**

Several studies over the last decade have reported a strong association of increased levels of SgK269 and SgK223 with tumor progression. SgK269, which was first identified indirectly using a proteomics strategy aimed at uncovering the proteins enriched in the pseudopodia of cells, was found to be amplified in >80% of colon cancer patients [13]. In patients with colorectal cancer, elevated SgK269 levels were found to be associated with poor survival in colon cancer but not rectal cancer [13, 38]. In pancreatic ductal adenocarcinoma (PDAC) and pancreatic intraepithelial neoplasia (PanIN), SgK269 levels were reported to be increased due to KRas-induced activation of Src kinase activity and SgK269 was identified as an early biomarker for PDAC development and progression [39, 40]. In breast cancer, SgK269 over-expression was reported in a subset of basal, HER2-positive and luminal cancers [23] while in lung cancer, overexpression of SgK269 was found to be associated with tumour metastasis [41, 42]. In contrast to SgK269, there are fewer studies reported to date that link SgK223 expression levels with cancer progression. One of the first such reports utilised quantitative phosphoproteomics to analyse Src signaling pathways in colon carcinoma cells and identified SgK223 as a substrate for tyrosine phosphorylation[43]. Following this, Tactacan *et al.* provided the first evidence of increased expression of SgK223 in PDAC tumors and cell lines [25]. Similarly, in non-small cell lung cancer (NSCLC) tumors, SgK223 expression was found to be significantly increased and associated with poor survival prognosis [41, 42]. It is interesting to note that even though the majority of these studies indicate a strong link between elevated expression levels of SgK269 and SgK223 and tumor progression, there are a few recent studies have reported the opposite. Guo *et al.* reported a decrease in SgK269 expression in gastric cancer and demonstrated that loss of SgK269 is associated with tumor growth and invasion[44]. More recently, Ding *et al.* also reported downregulation of SgK269 in colorectal cancer, while its overexpression resulted in suppression of cell growth and metastasis suggesting a role as a tumor

suppressor [45]. These studies suggest that the effects of SgK269 (and more broadly PEAK family proteins) on signalling outcomes are context dependent and may be either oncogenic or tumour suppressive depending on the type of cancer and the stage of its progression. This nuanced effect is perhaps unsurprising given the established role of these proteins as scaffolds that regulate complex protein-protein interaction networks.

In addition to a variation in expression levels, a total of 244, 202 and 34 missense mutations have been reported across various cancer types for SgK269, SgK223 and PEAK3, respectively (cBioPortal, [46, 47]). These mutations are distributed across the N-terminal region, the PEST linker region and the pseudokinase domain. The biological significance of these mutations in altering PEAK family function await future studies.

### **PEAK family: localisation and signalling**

Cytoskeleton-associated proteins play a critical role in cell spreading, migration and invasion, all of which are hallmarks of cancer progression. SgK223 and SgK269 are thought to contribute to the regulation of the cytoskeletal network through their non-catalytic scaffolding functions, and deregulation of these pathways have been implicated in human cancer. The signal integrating functions of SgK223/SgK269 within these pathways depends on site-specific phosphorylation of the PEST linker by kinases such as Csk and SFKs for SgK223 (Y411) [24] and Abl (Y797), Src (Y665), Lyn (Y635) and Erk (S779, T783) for SgK269 [13] and the recruitment of specific signalling adaptor molecules such as Shc1 for SgK223 [25] and CrkII, Grb2 and Shc1 for SgK269 [13, 25] (Fig. 5). However, the molecular mechanisms by which SgK269/SgK223 modulates these signalling outputs is not completely understood.

Rat SgK223 was first isolated through its interaction with Rnd2, a Rho family GTP-binding protein predominantly expressed in neurons and whose function is to antagonise RhoA signalling [48]. Rnd2-SgK223 interaction stimulates RhoA activity and induces cell contraction through the Rho-kinase pathway. The interaction between SgK223 and Rnd2, demonstrated through pulldown assays, was GTP dependent and mediated by the C-terminal region of rat SgK223 (residues 830-1368) while the N-terminal region of rat SgK223 (res 1-829) was responsible for RhoA activation and inhibition of neurite outgrowth [14]. It is interesting to note that this is the only study that highlights a role for the C-terminal pseudokinase domain in mediating interaction with adaptor proteins. However, in addition to proline rich motifs identified in the N-terminal and PEST regions of SgK223 and SgK269, such as those corresponding to the canonical binding motif for the CrkII SH3 domain [13], a small number of proline rich motifs are also predicted to be present within flexible regions of the C-terminal pseudokinase domain. The potential for these proline rich motifs to provide docking sites for adaptors is yet to be validated [49, 50]. Many regulatory

phosphorylation sites located outside the pseudokinase domain core have been identified. This include the tyrosine within the EPIYA motif of the PEST linker region identified in rat SgK223 (Y391), which is conserved in human SgK223 (Y411) and SgK269 (Y616) [24, 26]. In *Helicobacter pylori* CagA protein, the EPIYA motif is a site for phosphorylation by SFKs or c-Abl kinase, and once phosphorylated serves as a promiscuous docking site for a number of host SH2-containing proteins, such as the pro-oncogenic tyrosine phosphatase SHP2 and the SFK regulator Csk. These host-pathogen interactions are known to subvert host cell signalling pathways to the benefit of the pathogen. However, unlike the high functional versatility of the bacterial EPIYA motifs [51], rat SgK223 was shown to be specifically phosphorylated on the EPIYA motif at Y391 (Y411 in human) by Csk (Fig. 1A and Fig. 5A) [24, 26], highlighting a critical role for SgK223 in controlling Csk localisation through the formation of a SgK223-Csk complex. This cytoplasmic complex, which prevents translocation of Csk from the cytoplasm to the membrane, is thought to result in an increased SFK activity [26], linking SgK223 to cell invasion and migration pathways. Bacterial effector proteins containing EPIYA, such as CagA, were elegantly shown to inhibit SgK223-Csk formation when co-transfected with Sgk223 and Csk [26] demonstrating a possible mechanism for the bacteria to evade host immune response. More recently, the SgK223-Csk interaction was shown to enhance Csk kinase activity, resulting in increased phosphorylation of SgK223 EPIYA motif in addition to increased phosphorylation at the distal sites, Y238 and Y343. While the functional relevance associated with SgK223-Csk complex formation is yet to be fully unravelled, SgK223 co-immunoprecipitated with vinculin, a focal adhesion marker, demonstrating that the SgK223-Csk complex colocalizes to focal adhesions, where it likely regulates cell morphology and cell motility [24]. While SgK223-Csk complex formation may promote the recruitment of Csk to focal adhesions, additional experiments that examine whether the complex arises from a direct interaction in cells are required. With the advance in super-resolution techniques including single-molecule fluorescence microscopy or single-molecule localisation microscopy, visualising these interactions within cells will now be possible.

Recent studies by Capobianco *et al.* [15] implicated SgK223 as the Notch activation complex kinase (NACK), a binding partner of the Notch1 receptor, suggesting SgK223 serves as a regulator of nuclear function. The Notch1-SgK223 interaction is required for both Notch target gene expression and Notch-driven tumorigenesis [15, 52]. SgK223 is recruited to the Notch1 receptor via interactions with the Notch intracellular domain and Mastermind like transcriptional coactivator 1 (Mam1), suggesting that it may play a role in modulating the strength and duration of the transcriptional activity of Notch complexes. Mam1 is acetylated on lysines 188 and 189 by the

transcriptional co-activator protein p300, which drives the recruitment of SgK223 to the Notch transcription complex and subsequent transcription initiation (Fig. 5B) [52].

SgK269, first identified as a protein enriched in pseudopodia of cells, localises to the actin cytoskeleton and focal adhesions via elements harboured within the PEST linker region (residues 338-727) [13]. SgK269 is a known integral component of the Src-p130Cas-Crk-Paxillin signalling pathway and its expression in cells alters the phosphorylation of cytoskeleton associated proteins. Integrin and growth factors activate Src kinase activity, leading Src to phosphorylate p130Cas at Y249, which then acts as a docking site for the SH2 domains of signalling adaptor protein, CrkII. The SH3 domain of CrkII binds SgK269 and is recruited to the p130Cas/Crk scaffold where it is phosphorylated by Src kinase at Y665 [13, 53] (Fig. 5C). Phosphorylation of Y665 is critical for SgK269 localization and its role in regulating focal adhesions dynamics. Inhibitory (Phe) or activating (Glu) mutations of SgK269 Y665 perturbed cell migration and led to shorter focal adhesion lifetimes and assembly [54]. Given that SgK269 localises to focal adhesion, SgK269 could serve as a scaffold that transports Src-p130Cas-Crk-scaffold to focal adhesion via the strong association of SgK269 with the actin cytoskeleton. However, the exact molecular mechanism that drives this complex assembly at focal adhesion is yet to be unravelled. Interestingly, in *KRAS* driven pancreatic ductal adenocarcinoma, elevated Src activity drives upregulation of SgK269 expression, which modulates the activity for ErbB2 to drive tumorigenesis [39]. SgK269 has also been shown to regulate TGF $\beta$  signalling in breast cancer cells by switching its role as a tumor suppressor through Smad2/3 pathway to tumour promoter through Src and MAPK signalling pathway [55], whilst in lung cancer SgK269 overexpression is associated with tumour metastasis through the regulation of Erk1/2 and Jak2 pathways (Fig. 5D) [41].

SgK269 also functions via the Ras/Raf/Erk signalling pathway to control cell proliferation and invasion. This is dependent on phosphorylation of Y635 by Lyn kinase, which facilitates Grb2 recruitment (Fig. 5E). High expression of the Src family kinase Lyn has been observed in basal breast cancer cells [56]. Phosphoproteomic profiling of BT549 and MDA-MB-231 cell lines following *Lyn*-knockdown identified Y635 of SgK269 as a target of Lyn phosphorylation [23]. Phosphorylation of Y635 is required for SgK269 to promote activation of Erk and Stat3 in 3D culture, cell invasion and acinar growth [23]. How Y635 phosphorylation leads to Stat3 activation is unclear. One hypothesis is that sequestration of Grb2 by SgK269 leads to enhanced recruitment and phosphorylation of Stat3 by EGFR, which could occur because Grb2 and Stat3 compete for the same binding site on EGFR (Fig. 5E).

SgK269 and SgK223 are linked to the signalling adaptor Shc1 [25]. Upon EGF stimulation, EGFR phosphorylates Shc1, which rapidly binds Grb2-associated proteins that stimulate mitogenic and survival pathways. SgK269/SgK223 were found to be critical scaffolds for the assembly of the late phase Shc1 complex that promote cytoskeletal reorganisation, trafficking and downregulation of pro-mitogenic pathways. Phosphorylation of SgK269 Y1188 within the NPXY motif is important for the phosphotyrosine binding domain of Shc1. Serine/threonine protein phosphatase 1 alpha (PPP1CA) and gamma catalytic (PPP1CC) subunits of protein phosphatase 1 are recruited to Shc1 through their association with SgK269, but not SgK223 (Fig. 5F). Matrigel assays using MCF-10A cells overexpressing SgK269 generated acini with increased diameter and a multi-lobular morphology, thereby confirming the role of a late phase Shc1-SgK269 complex in regulating acinar morphogenesis [25]. Interestingly, SgK269 downregulation in colorectal cancer inhibits the Grb2/PI3K/Akt pathway and hence tumorigenesis by regulating protein phosphatase 1 regulatory subunit (PPP1R12B) expression and Shc binding, which implicates SgK269 as a tumour suppressor in colorectal cancers [45]. This, however, contradicts previous reports of SgK269 acting in colorectal cancers as a tumour promoter as a result of the activation of EGFR/KRas signalling [38], and highlights the need to carefully delineate the PEAK family signalling pathways in cancer. The role of PEAK3 in signalling is currently less understood and awaits future investigation. Recent studies showing its role in regulating cell motility by preventing CrkII-dependent membrane ruffling offers some promising insights into how it may function distinctly from SgK223 and SgK269 [17]. Much of our understanding of PEAK family member signalling has arisen from overexpression studies. Consequently, these examples most likely represent functions of homo-dimerised PEAK proteins; however, our understanding of signal diversification through hetero-dimerisation /oligomerisation is currently limited and awaits further research. Understanding which specific adaptors are brought together through specific hetero-dimerization/oligomerisation of SgK223, SgK269 or PEAK3 will provide insights into the signalling pathways they modulate. One such example is the recent demonstration by Liu *et al.* using Grb2, an adaptor of SgK269, also co-immunoprecipitated with SgK223, suggesting that SgK269 might act as a bridge to mediate SgK223 and Grb2 interactions [10].

## Conclusion

The PEAK family of pseudokinases are an emerging group of oncogenic scaffolds that regulate many signalling pathways to drive cancer growth and progression. In this review, we have highlighted recent advances in the structural and biophysical characterisation of this family that have provided some novel insights into their scaffolding function. The novel dimerization domain identified and the re-orientation of the pseudokinase N-lobe leading to an occluded ATP binding

site are among the conserved features, while a conformational flexibility within the activation loop indicates a likely regulatory mechanism reminiscent of active kinases. The scaffolding properties of the PEAK family dictated by their ability to dimerise and oligomerise thus highlight a possible signalling mechanism to enable recruitment of different effectors to regulate cell migration and invasion pathways. However, many outstanding biological questions still remain. These include examining whether higher order oligomers form in cells, dissecting the mechanisms that drive the localisation of the PEAK pseudokinases to focal adhesions or nucleus, characterising their adaptor binding sites and subsequent signalling outcomes and characterising the critical phosphorylation sites that may drive conformational changes and impact the assembly of higher order oligomers. In addition, based on the reported data, it is anticipated that heterotypic association within the PEAK family promote the cross-talk of signalling pathways and regulate these pseudokinases spatially and temporally in response to signalling stimuli. Insights into the molecular basis of these mechanisms will be crucial to understand their deregulation in cancer. Technological developments in single molecule microscopy and cryo-electron microscopy will allow visualisation of these complex assemblies and their interaction partners to advance our understanding of the signalling pathways mediated by this intriguing pseudokinase family. Additionally, given the suggested role of PEAK family of pseudokinases in cancer, they may represent novel targets for cancer therapy. Most pseudokinases that are currently viewed as promising drug targets, including HER3[57], MLKL[32], KSR [58], JAK2 [59] and TYK2 [60, 61], have an accessible ATP binding site, which can accommodate ATP competitive molecules. These may be capable of modulating the non-catalytic functions of these proteins through small molecule induced conformational changes [62]. A significant challenge with respect to the PEAK family proteins as drug targets is that their ATP binding site is largely occluded; it remains to be seen whether small molecule ligands targeting the PEAK family can be generated. If so, an innovative and emerging strategy might be to utilise small molecules to target these proteins for degradation, such as the proteolytic targeting chimera (PROTAC) approach. This strategy has previously been successfully utilised to target proteins with scaffolding functions (including active kinases)[63] and challenging proteins using ligandable sites that are non-essential for function[64]. Evaluating the true potential of PEAK family proteins as therapeutic targets will require a deeper functional understanding of these proteins, including the mechanisms that drive their allosteric regulation, an exploration of their scaffolding functions through the characterisation of their main interacting partners and how the resulting signalling complexes are spatially and temporally regulated.

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## Figure legends

Fig. 1. Schematic diagrams and sequence alignment of the PEAK family. (A) Schematic representation of SgK269, SgK223 and PEAK3 domain organisation highlighting phosphorylation and adaptor proteins binding sites. (B) Alignment of CrkII binding site within the PEAK family. (C) Alignment of the N- and C-terminal helices  $\alpha$ N1,  $\alpha$ J,  $\alpha$ K,  $\alpha$ L and  $\alpha$ M within the PEAK family. Hot spot residues (red), identical residues (\*) and conserved residues (:) are as indicated. The sequence alignment was carried out using Clustal Omega [65]. Accession number for human SgK223, human SgK269 and rat SgK223 are Q86YV5, Q9H792 and NP\_001100785, respectively.

Fig. 2. Structure of the pseudokinase domain and surrounding helices of the PEAK family. (A) Structure of SgK223 monomer and surrounding helices highlighting the 'XL' helix bundle made by  $\alpha$ N1,  $\alpha$ J,  $\alpha$ K,  $\alpha$ L and  $\alpha$ M and the corresponding dimer highlighting the 'XX' helix bundle (PDB: 5VE6). (B) Structure of SgK269 monomer and the corresponding dimer mediated through the SHED domain using the same arrangement of  $\alpha$ N1,  $\alpha$ J,  $\alpha$ K,  $\alpha$ L and  $\alpha$ M helices observed in SgK223 (PDB: 6BHC). (C) Structure of rat SgK223 monomer and the corresponding dimer (PDB: 6EWX). The additional helix preceding the  $\alpha$ N1 that is resolved in one monomer is highlighted. Human SgK223, grey; rat SgK223, green; human SgK269, orange;  $\alpha$ C helix, pink; additional helix resolved in rat SgK223 in magenta. Structure figures were generated using PyMOL (The PyMOL Molecular Graphics System Version 1.7.4.0, Schrödinger, LLC).

Fig. 3. The unique structural features with the PEAK family. (A) Close up of the dimer interface in human SgK223 structure mediated by interaction of the  $\alpha$ N1 and  $\alpha$ K helices from the two monomers (PDB: 5VE6). (B) Interaction between the N-lobe of human SgK223 with  $\alpha$ N1,  $\alpha$ K and  $\alpha$ L helices, highlighting the  $\beta$ 3 lysine (1024)- glutamine (1048 in SgK223) interaction and the triad interaction between F1045 from the  $\beta$ 3- $\beta$ 4 loop, F974 of  $\alpha$ N1 helix and W1382 of  $\alpha$ L helix. Human SgK223, grey;  $\alpha$ N1 cyan;  $\alpha$ K salmon;  $\alpha$ L purple and  $\alpha$ M brown; hotspots residues L955, L966, F1366 and W1382 yellow. (C) ATP binding site in SgK223. Residues occupying the site where ATP would normally bind in a conventional protein kinase are shown in red. (D) Activation loop/ $\alpha$ G overlap with PEAK family proteins highlighting the different conformations of the A-loop. Human SgK223 (PDB: 5VE6), grey; rat SgK223 (PDB: 6EWX), green; human SgK269 (PDB: 6BHC), orange;  $\alpha$ C helix, pink; H-bonds and salt-bridge interactions in black dashed lines and van der Waals interactions in red dashed lines. Structure figures were generated using PyMOL (The PyMOL Molecular Graphics System Version 1.7.4.0, Schrödinger, LLC).

Fig. 4. SgK223 oligomer interface and sequence alignment of the  $\alpha$ G helix (PDB: 5VE6) . Close up view of the oligomeric interface shows the interaction of the activation loop/ $\alpha$ G of one monomer with the N-lobe of the pseudokinase domain of the symmetry related monomer.  $\alpha$ G shown in green. (B) Interaction between the human Kinase suppressor of Ras 2 (KSR2) pseudokinase domain with Mitogen-activated protein kinase (MEK) mediated by the activation loop/ $\alpha$ G helix (PDB: 2Y4I). KSR2 pale green, MEK1 grey. Structure figures were generated using PyMOL (The PyMOL Molecular Graphics System Version 1.7.4.0, Schrödinger, LLC).

Fig. 5. Schematic of signalling pathways associated with SgK223 and SgK269

### **Acknowledgments**

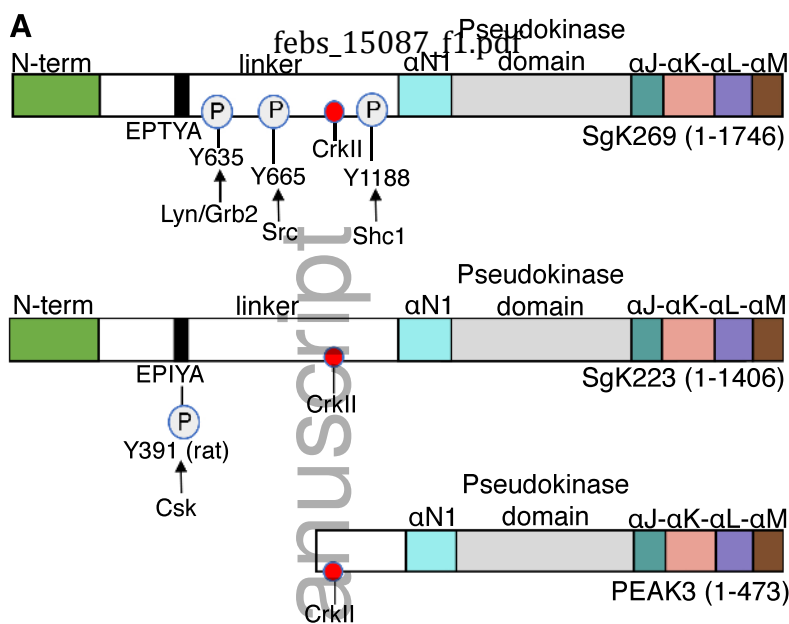
This work was supported by the Australian Cancer Research Foundation (to O.P., M.R and I.S.L.). I.S.L. acknowledges support from the Walter and Eliza Hall Institute. This work was supported by NHMRC Project Grant (1162058), NHMRC IRIISS 9000433 and Victorian Government Operational Infrastructure Support Scheme. J.M.M was supported by NHMRC fellowship (1105754).

### **Author Contributions**

OP created the figures and together with ISL wrote the original draft of the manuscript with input from MR and JMM. All authors participated to the reviewing and editing process.

### **Conflict of interest**

The authors declare no competing financial interests.



**B** CrkII binding site

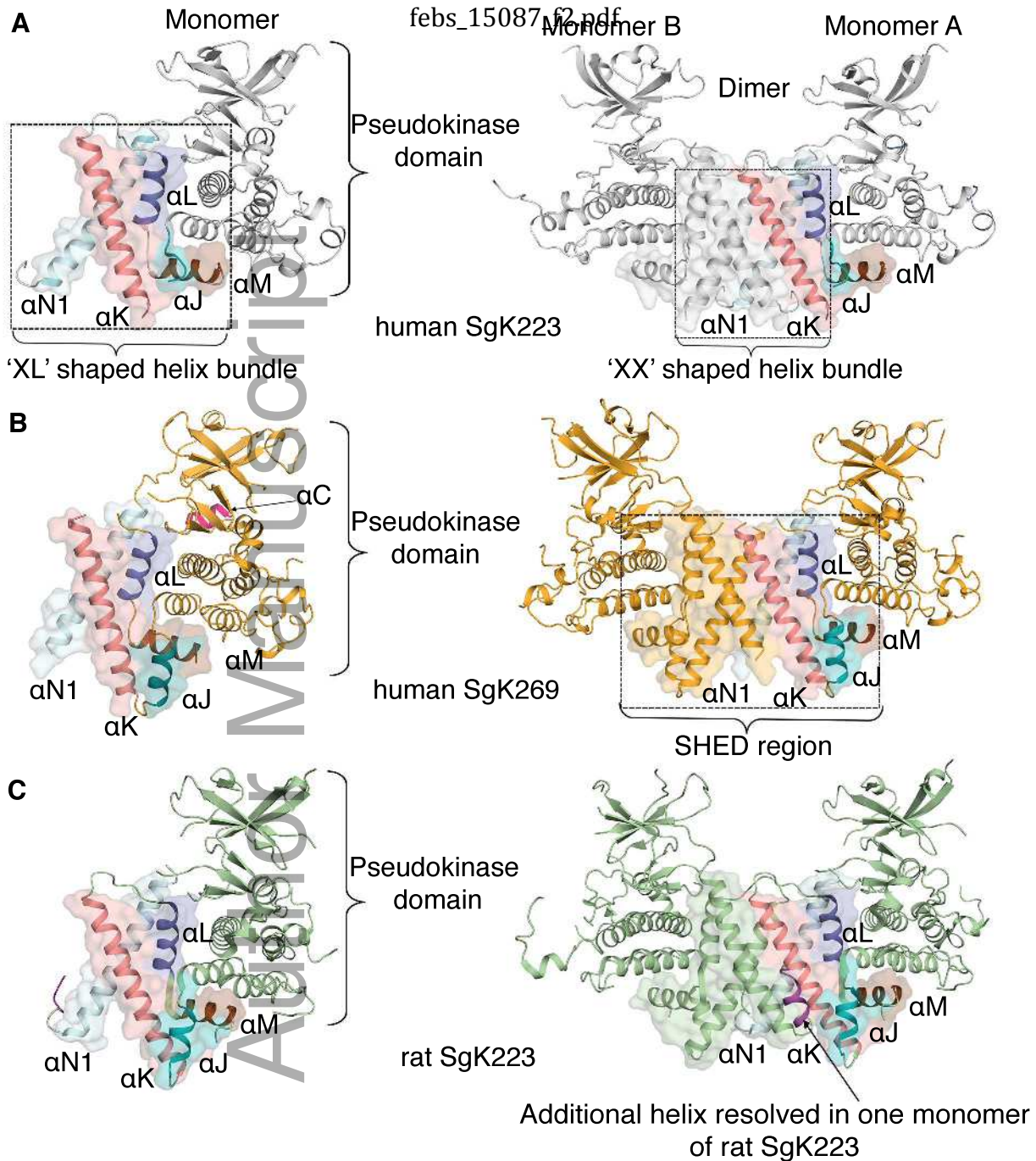
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 SgK269 QPTFPPLPKKMIIRAN  
 SgK223 PQQFPPLPQKKIVSRA  
 \*\*\*\*\*:\*

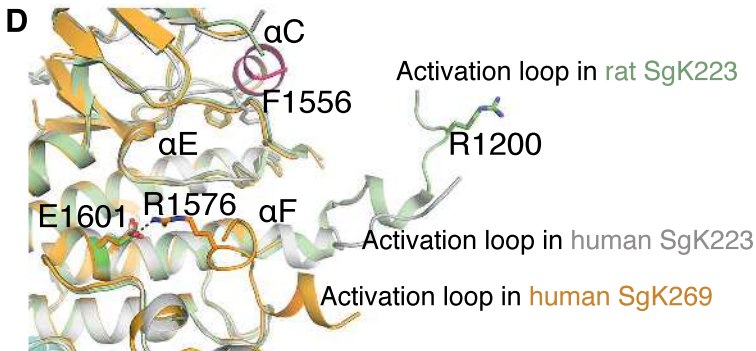
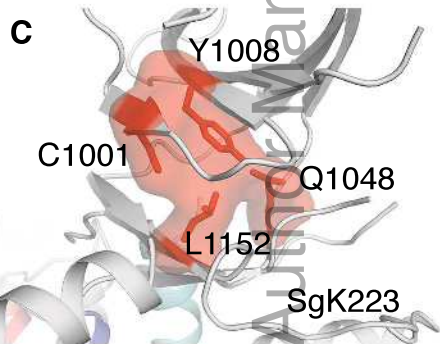
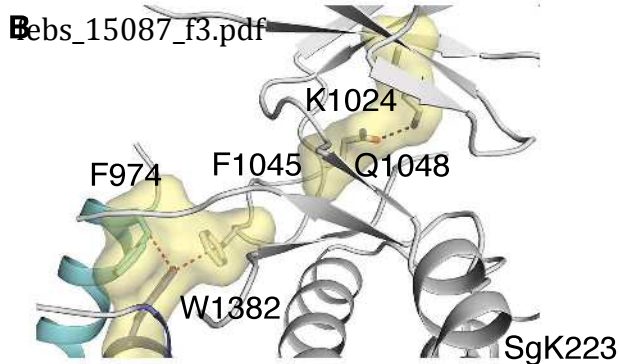
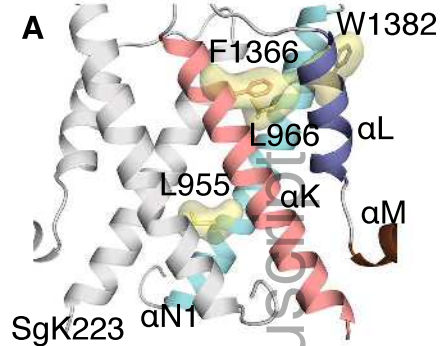
**C** aN1

PEA3 PEAVH<sup>A</sup>IAAARQIQCLR<sup>T</sup>IYARLRARLIMG  
 SgK269 -KEVVGKIRSLH<sup>T</sup>DAIKKIAVKCEIDLFGA  
 SgK223 KEGTYAKLGGLYTQSLARLVAKCEDLFGM  
 \* : : \* : : \*

αJ αK  
 PEA3 PELRGRGA QPWT<sup>R</sup>ALGPWIRVRRGILVLR<sup>I</sup>AKRAA  
 SgK269 EDLFQIFTA VQRNT<sup>L</sup>IQNWIDIKRTILMIKFAEKSL  
 SgK223 RELVQQPGT -ALCGT<sup>L</sup>HNWIDMKRALMMMKFAEKAV  
 :\* \* \* : : \* \* : : : : \*

αL αM  
 PEA3 WLCCEYLA IYFEEYLA  
 SgK269 WLCAYILA DSLSCIVKIL  
 SgK223 WLCQYILA GALLQSLKLL  
 \*\*\* :\*\*\* : : : : \*

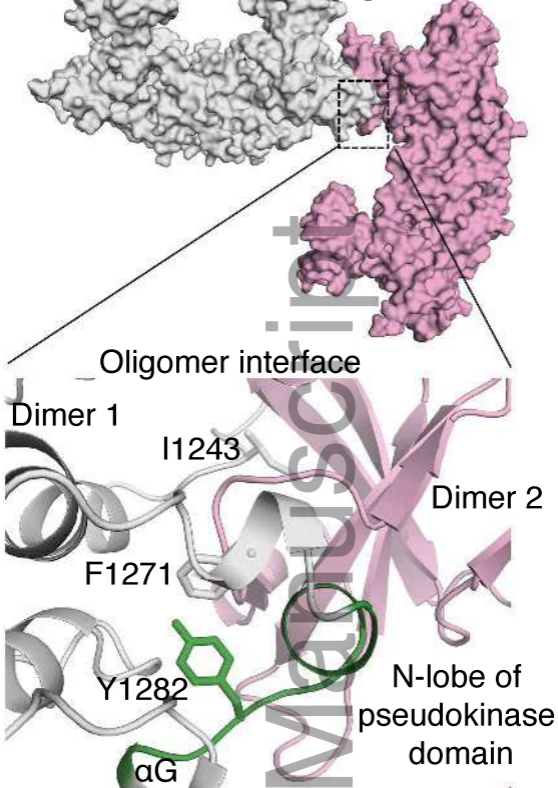




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-----Activation loop-----  
 SgK269 SNFSQAKQKSHL-VDPEILRDQSRRLAPEIITATQYKKCDEFQTGILYIEML  
 SgK223 SNFLKAKQKPGGTPNLQOKKSQARLAPEIVSASQYRKFDEFQTGILYIELL  
 ::\* :. \* . \*

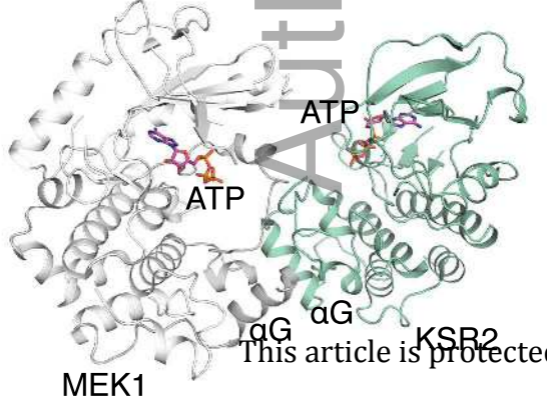
**A** Dimer 1 febs\_15087\_f4.pdf Dimer 2



**αG helix**

SgK269	PNPFIDENPETKEREYTRADLPRLP
raL SgK223	PNPF <del>F</del> EVRAQLRERDYRRREDLPPLP
SgK223	PNPF <del>F</del> EVRAQLRERDYRQEDLPPLP
	****: . :*:**:* : *** :*

**B**



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