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Structural basis for the non-catalytic functions of protein kinases

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Summary

Protein kinases are known primarily for their ability to phosphorylate protein substrates, which constitutes an essential biological process. Recently, compelling evidence has accumulated that the functions of many protein kinases extend beyond phosphorylation and include an impressive spectrum of non-catalytic roles, such as scaffolding, allosteric regulation, or even protein-DNA interactions. How the conserved kinase fold shared by all metazoan protein kinases can accomplish these diverse tasks in a specific and regulated manner is poorly understood. In this review, we analyze the molecular mechanisms supporting phosphorylation-independent signaling by kinases and attempt to identify common and unique structural characteristics that enable kinases to perform non-catalytic functions. We also discuss how post-translational modifications, protein-protein interactions, and small molecules modulate these non-canonical kinase functions. Finally, we highlight current efforts in the targeted design of small molecule modulators of non-catalytic kinase functions – a new pharmacological challenge for which structural considerations are more important than ever.

Introduction

By catalyzing phosphorylation, protein kinases play a central role in regulating cellular homeostasis. This enzymatic function is mediated by the conserved kinase domain fold, which is highly preserved among all protein kinases. Despite their structural conservation, protein kinases exhibit remarkable diversity in their ability to recognize unique sets of substrates, as well as other binding partners that might serve as activators or inhibitors. The specificity of these interactions is often encoded in unique binding sites within the kinase domain itself. A kinase domain therefore skillfully couples its role as a catalytic enzyme with a role as a protein scaffold in order to orchestrate an efficient and specific phosphotransfer.

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Studies over the past decades have revealed that scaffolding functions of kinases extend beyond a mere supporting role in phosphorylation. This was initially suggested by observations that inhibition of kinase function can result in fundamentally different biological outputs depending on the experimental approach used to inhibit the enzyme. For example, genetic knockout of the epidermal growth factor receptor (EGFR) results in animal death soon after birth (Miettinen et al., 1995), while loss of EGFR activity through a "kinase-dead" V743G mutation produces only mild epithelial defects (Luetteke et al., 1994). Similarly, there are significant phenotypic discrepancies between Parkinson's disease manifestations observed in mice in which leucine-rich repeat kinase 2 (LRRK2) is knocked out versus inactivated by a D1994S (D166 in Protein Kinase A (PKA)) mutation (Herzig et al., 2011). Although these discrepancies might be partially attributed to the fact that mutations or inhibitor treatment, as opposed to gene knockdown, might still preserve a low level of kinase activity, they also suggest an alternative, more exciting, possibility that the functions of kinases are not limited to catalyzing phosphorylation but extend to other roles that are independent of enzymatic activity. These divergent outcomes of kinase inactivation have been found for an increasing number of kinases, indicating that the roles of kinases in cellular signaling are considerably more complex than we think (Figure 1, Table 1).

The potential of the kinase domain fold to signal through an alternate non-catalytic mechanism is perhaps best exemplified by a subset of protein kinases that have seemingly evolved to lack catalytic activity and have thus been termed "pseudokinases". Pseudokinases are defined by the presence of mutations in critical catalytic residues that prevent catalysis of phosphorylation, while preserving the overall structure of the kinase domain (Manning et al., 2002). Although initially thought of as evolutionary remnants that lack biological function, pseudokinases are often highly conserved in evolution and play essential roles in signaling. As such, pseudokinases represent ~10% of all human kinases (Figure 1), underscoring the importance of the non-catalytic utility of the kinase scaffold for cellular signaling. They also provide elegant case studies to investigate the molecular basis for non-catalytic kinase functions since these functions have likely evolved in pseudokinases in isolation from the constraints that catalysis imposes on the structure of the kinase domain.

With our increasing appreciation for the phosphorylation-independent roles of kinases in cellular signaling, several important questions emerge. First, what are the structural components within the kinase domain that mediate these non-catalytic functions, and what are the molecular mechanisms of their regulation? Second, can we modulate the non-catalytic functions of kinases using small molecules in a manner analogous to the ways in which their catalytic activity can be regulated? Lastly, and perhaps the most critical from a therapeutic standpoint, how do existing inhibitors of kinase catalytic functions affect kinase non-catalytic functions? In this review, we analyze the molecular mechanisms supporting phosphorylation-independent signaling in kinases and attempt to identify common structural characteristics that enable kinases to perform these non-catalytic functions. We then discuss how these non-canonical functions could be modulated using small molecules and examine current progress toward the development of compounds that modulate non-enzymatic activities of kinases.

Known non-catalytic functions of kinases

Kinases interact with a surprisingly wide range of binding partners given the high degree of conservation of the kinase domain fold. This versatility enables kinases to perform a diverse array of non-catalytic functions, including allosterically regulating other kinases and/or unrelated enzymes, acting as molecular scaffolds to coordinate interactions between different components of signaling pathways, and regulating transcription through interactions with transcription factors or by binding directly to DNA. Structural and functional studies in recent years have shown that each of these novel kinase functions utilizes a unique molecular mechanism, highlighting an impressive degree of adaptation.

Allosteric regulation of other kinases

Several kinases and pseudokinases allosterically regulate the catalytic activity of other kinases through dimerization (Figure 2A). The human epidermal growth factor receptor (HER/EGFR) family of receptor tyrosine kinases is one example of such regulation. The HER family consists of three catalytically active kinases: EGFR, HER2 and HER4, and one catalytically impaired pseudokinase HER3. Although HER3 retains very weak kinase activity *in vitro* (Shi et al., 2010), this activity does not seem to be necessary for HER3 signaling (Mendrola et al., 2013). The canonical model of receptor tyrosine kinase activation postulates that trans-phosphorylation of kinases in response to ligand-induced receptor dimerization is necessary for kinase activation and, therefore, is contingent on both receptors being catalytically active. However, the catalytically active HER receptors (EGFR, HER2 and HER4) readily form signaling-competent heterodimeric complexes with the catalytically impaired HER3, suggesting that only one kinase in HER receptor dimers needs to be catalytically active. Comparisons of crystal structures of the EGFR kinase domain in inactive and active states revealed that, instead of trans-phosphorylation, EGFR activation relies on asymmetric dimerization between kinase domains where one kinase allosterically activates the other (Brewer et al., 2009; Jura et al., 2009a; Zhang et al., 2006). This allosteric activator function is independent of kinase catalytic activity but reliant upon the structure of the kinase domain (Zhang et al., 2006). Consequently, it can be carried out by both the catalytically active and catalytically impaired HER kinases, enabling HER3 to form functional signaling complexes with other HER receptors (Jura et al., 2009b; Littlefield et al., 2014; Monsey et al., 2010).

The RAF family of kinases (A-RAF, B-RAF, C-RAF) also relies on catalysis-independent allosteric activation between two kinase domains, yet through a very different mechanism. Analysis of RAF mutations found in human cancers has identified several substitutions in B-RAF (G466E, G466V, G596R) that impair its catalytic activity but still stimulate cell proliferation by activating C-RAF through heterodimerization (Garnett et al., 2005; Wan et al., 2004). Additionally, at subsaturating conditions, certain B-RAF inhibitors paradoxically activate MAPK signaling in cells by promoting dimerization of inhibited B-RAF molecules with uninhibited B-RAF and C-RAF (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). These observations have revealed that RAF kinases become activated through the formation of symmetric “side-to-side” dimers in which one RAF molecule allosterically activates the other in a manner independent from catalytic activity

(Figure 2A). The activating effect of mutations and inhibitors is a result of inducing a conformational change that renders the inactivated kinase able to allosterically activate other RAF molecules. Through this dimeric interaction, B-RAF was also shown to allosterically activate kinase suppressor of Ras (KSR), a closely related kinase previously thought to be a bona fide catalytically inactive pseudokinase (Brennan et al., 2011; Hu et al., 2011).

The Janus kinase (JAK) family, which includes JAK1, JAK2, JAK3, and TYK2, provides an example of how the interaction between two kinases can be inhibitory. JAK kinases are cytoplasmic receptor-associated kinases that become activated upon binding of extracellular ligands to their cognate receptors. Each of the JAKs possesses both a pseudokinase domain and an active kinase domain that are adjacent to each other on one polypeptide chain. In the absence of stimulation, the pseudokinase domain interacts with and inhibits the active kinase domain (Figure 2A). Only recently, structural studies have identified the interaction interface between the pseudokinase and kinase domains and showed that many disease-related mutations that activate JAK kinases map to this interface (Lupardus et al., 2014; Shan et al., 2014). Other recent studies suggest that the inhibitory pseudokinase/kinase interaction might be occurring in trans between two different JAK kinases that pre-associate with the receptor in the absence of an activating ligand (Brooks et al., 2014; Varghese et al., 2014). This interaction could contribute to stabilization of an inactive receptor complex, underscoring the multi-layered role of JAK kinase domain-mediated interactions in receptor activation.

Allosteric regulation of other enzymes

This ability of kinases to act as allosteric regulators of signaling also extends to modulation of the activity of unrelated enzymes (Figure 2B). The mitogen-activated protein (MAP) kinase ERK2 (extracellular-regulated kinase 2), for instance, regulates several different enzymes through allostery. ERK2 plays a critical role in regulation of the activity of the dual specificity phosphatase MKP3 (DUSP6), which inactivates ERK2 by dephosphorylation. By binding directly to MKP3, ERK2 triggers a conformational change in the MKP3 catalytic domain that markedly enhances MKP3 phosphatase activity. Active wild type ERK2 and a kinase-dead ERK2 K52A (K72 in PKA) mutant activate MKP3 to the same extent, suggesting that this function of ERK2 is not dependent on kinase activity (Camps et al., 1998). Similarly, ERK2 allosterically activates topoisomerase IIα and poly (ADP-ribose) polymerase-1 (PARP1) through mechanisms that are independent of its catalytic activity (Cohen-Armon et al., 2007; Shapiro et al., 1999).

Another kinase implicated in the regulation of phosphatase activity in a phosphorylation-independent manner is vacinnia-related kinase 3 (VRK3), a catalytically inactive pseudokinase in the VRK family (Figure 2B). The crystal structure of VRK3 was the first structure of a pseudokinase to be solved and revealed that the putative active site of VRK3 is highly distorted and likely incompatible with nucleotide binding. Biochemical studies confirmed that VRK3 is indeed catalytically inactive (Scheeff et al., 2009). Despite lacking activity, VRK3 directly binds and stimulates the activity of vaccinia H1-related (VHR) phosphatase, which is involved in dephosphorylation of ERK (Kang and Kim, 2006).

Assembly of signaling components through scaffolding

Scaffold proteins play critical roles in cellular signaling by binding to multiple components within a signaling pathway and facilitating their interactions (Langeberg and Scott, 2015). Such scaffolds often consist of several modular protein interaction domains, such as SH2 and PDZ domains. Although singular kinase domains lack typical characteristics of protein scaffolds, accumulating evidence suggests that kinase domains can likewise promote interactions between distinct components within signaling pathways (Figure 2C).

Several pseudokinases have documented roles as scaffold proteins and strictly rely on the kinase domain to perform this function. The pseudokinases STRADα and STRADβ (STE20-related kinase adaptor) are involved in activation of liver kinase B1 (LKB1), which in turn activates AMP-activated kinase (AMPK) to inhibit cell proliferation when cellular ATP levels are low. In order to be activated, LKB1 needs to bind to the adaptor protein Mo25, and STRAD pseudokinases serve as scaffolds that facilitate this interaction (Baas et al., 2003; Boudeau et al., 2003). In this complex, both STRAD and Mo25 cooperate to stabilize the active conformation of LKB1 and stimulate its catalytic activity (Zeqiraj et al., 2009a; 2009b). STRAD therefore not only acts as a passive scaffold, but also plays an active role in allosteric regulation of the scaffolded components.

The well-studied scaffold KSR plays a critical role in regulating the Ras/MAPK pathway by coordinating assembly of the RAF/MEK/ERK signaling module (Kolch, 2005). These functions are dependent on the kinase domain of KSR, but not on its catalytic activity. In response to growth factor stimulation, KSR localizes to the plasma membrane and interacts with MEK and RAF to promote MEK phosphorylation. KSR also interacts with ERK, which then phosphorylates RAF and KSR to negatively regulate the MAPK pathway (McKay et al., 2011). Like STRADα, KSR is not a passive scaffold but is involved in allosteric regulation of its binding partners. KSR allosterically activates B-RAF through a “side-to-side” heterodimerization mechanism similar to that observed in heterodimers between different RAF isoforms (Hu et al., 2013; Rajakulendran et al., 2009).

Perhaps least understood is the scaffolding role of the Tribbles (Trb) family of pseudokinases composed of three closely related proteins: Trb1, Trb2 and Trb3. Genetic and biochemical studies have suggested that Trbs exhibit a diverse range of scaffolding functions. Like KSR, Trbs facilitate interactions between kinases in the MAPK cascade to modulate phosphorylation of MAPKs by interacting directly with MAPKKs (Kiss-Toth et al., 2004; Yokoyama et al., 2010). Trbs have also been found to regulate proteasomal degradation by interacting with ubiquitin ligases, including COP1 and βTrCP, and their substrates to promote ubiquitination. This function of Trbs has been documented to regulate the stability of a number of different proteins, such as acetyl CoA carboxylase, C/EBP transcription factors, and β-catenin (Keeshan et al., 2006; Li et al., 2009; Xu et al., 2014). At present, the molecular basis for these diverse scaffolding functions of Trb pseudokinases is poorly understood. Recently, crystal structures of the Trb1 pseudokinase domain revealed that the putative active site of Trb1 deviates markedly from that of canonical active kinases and is incompatible with ATP-binding or catalysis (Murphy et al., 2015). The structures suggest that the interaction between the C-terminal tail of Trb1 and its pseudokinase domain regulates its ability to recruit COP1.

Like pseudokinases, catalytically active kinases can also act as scaffolds independently of their catalytic activity. The bifunctional enzyme Ire1 possesses a kinase domain fused C-terminally to an RNase domain and utilizes its kinase domain as a scaffold to mediate oligomerization and activation of the RNase domain. This results in the cleavage of the HAC/XBP1 mRNA to stimulate the unfolded protein response activated by endoplasmic reticulum (ER) stress. The ability of Ire1 to oligomerize and subsequently activate the RNase domain is regulated through a conformational change in the kinase domain, and not directly by kinase activation (Korennykh et al., 2009; Papa et al., 2003). Another related enzyme, RNase L, is also composed of a kinase domain C-terminally fused to an RNase domain and uses an oligomerization mechanism analogous to Ire1 for RNase activation. In contrast to Ire1, the kinase of RNase L was found to be a catalytically inactive pseudokinase, underscoring that RNase activation can be achieved even in the absence of kinase catalytic activity (Dong and Silverman, 1999; Han et al., 2014; Huang et al., 2014).

RIPK1 and RIPK3 (receptor-interacting protein kinases 1 and 3) are critical regulators of inflammation and cell death that have scaffolding roles in apoptosis that are independent of their catalytic activity. Genetic and biochemical studies have revealed very distinct properties of these kinases. While knockout of RIPK1 in mice is lethal due to extensive apoptosis in several tissues (Kelliher et al., 1998), mice that express kinase-dead RIPK1 K45A (K72 in PKA) or D138N (D166 in PKA) mutants are viable and mature normally into adults, suggesting that non-catalytic functions of RIPK1 are required for survival (Berger et al., 2014; Newton et al., 2014). In contrast, RIPK3-deficient mice are healthy, but expression of kinase-dead RIPK3 mutants has differential effects depending on the mutation used to inactivate the kinase. While mice expressing catalytically inactive RIPK3 K51A (K72 in PKA) are viable, expression of RIPK3 D161N (D166 in PKA) is lethal through promotion of Caspase 8-dependent apoptosis (Mandal et al., 2014). The D161N mutation likely augments the ability of RIPK3 to recruit apoptotic regulators, including $cFLIP_L$, FADD, and Casp8, to form an apoptosis-inducing complex and demonstrates that this scaffolding function of RIPK3 does not depend on its catalytic activity (Mandal et al., 2014). The discrepancies between the effects of the K51A and D161N mutations suggest that a specific conformation of the kinase domain, favored by the D161N mutant, rather than loss of catalytic activity is responsible for promoting this scaffolding function of RIPK3 in apoptosis.

DYRK2 (dual-specificity tyrosine phosphorylation-regulated kinase 2) utilizes both catalytic and non-catalytic functions to regulate ubiquitination. DYRK2 is required for assembly of the complex between the EDD ubiquitin ligase and the adaptor proteins DDB1 and VPRBP that together form the EDVP complex to mediate ubiquitination of substrates, like katanin p60. While DYRK2 kinase activity is required for the phosphorylation and subsequent degradation of katanin p60, it is dispensable for forming the EDVP complex (Maddika and Chen, 2009). DDB1 and VPRBP also serve as components of a different ubiquitin ligase complex, Cul4-Roc1, where the cullin Cul4, instead of DYRK2, mediates interactions between DDB1-VPRBP and the ubiquitin ligase Roc1. Although the structural basis for the role of DYRK2 in the formation of the EDVP complex remains unknown, it is intriguing to speculate that DYRK2 and Cul4 might act in an analogous manner in assembling E3 ligases with other components of ubiquitination complexes.

Regulation of transcription

Although the repertoire of non-catalytic kinase functions spans many diverse processes, their involvement in regulation of transcription, though less well understood, is worthwhile highlighting (Figure 2D). Kinases, such as CDK6 and CDK9, have been found to regulate transcription by interacting with transcription factors like STAT3, c-Jun, and B-Myb in a manner that does not depend on catalytic activity (De Falco et al., 2000; Kollmann et al., 2013). A recent study profiling interactions between human proteins and DNA revealed that another group of kinases, including ERK2, RIPK3, and MAP4K2, appear to regulate transcription by binding directly to DNA (Hu et al., 2009). In the case of ERK2, a cluster of positively charged residues in the C-lobe of the kinase domain mediates ERK2 interactions with the promoters of several genes to repress transcription. A kinase-inactivating K54R (K72 in PKA) mutation and chemical inhibition had no effect on the inhibitory role of ERK2 in transcription, indicating that this function is independent of kinase activity (Hu et al., 2009).

“Repurposing” the kinase domain for non-catalytic functions

The strategic placement of kinases in critical cellular signaling networks likely explains why they evolved to carry not one but several alternative functions. However, the versatility with which the kinase domain can be used to support so many unrelated functions in a cell poses an obvious structural puzzle. How does the primary, or so we think, function of kinases to catalyze phosphorylation co-exist with these alternative roles, and what physical features of the kinase have evolved the capability to serve these new functions?

Structural transitions underlying catalytic function of kinases

Kinases exhibit a highly conserved fold with the active site located between two lobes, the N- and C-lobes, connected via the hinge region (Figure 3A). The motion of the hinge determines the relative orientation of the N- and C-lobes during the catalytic cycle. To conduct catalysis, kinases need to be dynamic to allow nucleotide binding, hydrolysis, and release in a sequential fashion, as well as binding and release of their substrates. A set of defined conformational transitions accompanies these steps and has been well characterized through decades of insightful structural and biophysical studies (Huse and Kuriyan, 2002; Taylor and Kornev, 2011).

In contrast to the N-lobe, which is composed primarily of β-strands and undergoes significant structural rearrangements during catalysis, the C-lobe, which consists mostly of α helices, is more rigid and possesses less conformational flexibility except for one conserved structural element called the activation loop. The activation loop starts with a highly conserved Asp-Phe-Gly (DFG) motif and extends to include one or more regulatory phosphorylation sites present in the majority of kinases. The activation loop undergoes significant conformational changes during catalysis that are intimately tied to kinase activation. In most kinases, once phosphorylated, the activation loop adopts a fully extended conformation that stabilizes the active state of the enzyme and is important for binding substrates. In this conformation, the DFG aspartate points into the active site to coordinate Mg^{2+}-ATP, while the adjacent phenylalanine points away from the catalytic cleft (Figure

3B). This position of the DFG motif is referred to as the “DFG-in” state. The aspartate and phenylalanine can exchange positions (“DFG-out”), positioning the bulky phenylalanine side chain to prevent binding of the nucleotide to the active site. This “DFG-out” state locks the kinase in the inactive conformation and is often accompanied by significant changes in the conformation of the activation loop (Figure 3B).

Conformational changes of the activation loop are tightly coupled to the movements of helix αC, a conserved structural element located in the N-lobe. In the active conformation, helix αC is rotated toward the active site, allowing a conserved lysine residue in the β3 strand within the N-lobe to form a salt bridge with a conserved glutamate in helix αC (Figure 3A). Helix αC also plays a critical role in stabilization of an inactive conformation of the kinase domain termed the Src/CDK-like inactive conformation, which is distinct from the “DFG-out” inactive conformation (Figure 3B). This conformation was first observed in structures of cyclin-dependent kinases (CDKs) and Src family kinases, and subsequently, in several other unrelated protein kinases, pointing to its potentially important role in the kinase catalytic cycle (Jura et al., 2011). In the Src/CDK-like inactive state, helix αC is rotated away from the active site such that the glutamate residue in this helix can no longer interact with the β3 lysine. The DFG motif adopts a DFG-in conformation, but the remainder of the activation loop folds into a short helix rather than adopting the extended conformation seen in the active state.

The relevance of conformational changes in kinases for their non-catalytic functions

While the structural steps of kinase-mediated catalysis have been studied extensively, the structural underpinnings of non-catalytic kinase functions are poorly understood. Much of the pioneering work on this aspect of kinase signaling suggests that the structural transitions associated with the kinase catalytic cycle are indeed also supporting the non-catalytic functions of kinases. Since the three structurally well-characterized kinase conformations: the active state, the inactive DFG-out state, and the Src/CDK-like inactive state, are associated with significant changes on the kinase domain surface (Figure 3B), it is therefore plausible that non-catalytic functions of kinases that rely on protein-protein interaction interfaces could be regulated by these conformational changes.

Activation loop conformation and the DFG-out state—In several kinases, the conformation of the activation loop is coupled to their ability to act as allosteric regulators or as scaffolds. In the case of Ire1, crystal structures revealed that the Ire1 kinase domain dimerizes in a “back-to-back” orientation in which the kinases adopt an active conformation (Korennykh et al., 2009; Lee et al., 2008) (Figure 4A). Ire1 dimerization and subsequent oligomerization of the back-to-back dimers are critical for activation of the adjacent RNase domain. These findings show that, even though it is possible to activate the RNase function of Ire1 in the absence of Ire1 kinase catalytic activity, this scaffolding function of Ire1 requires that the kinase adopt an active conformation. Similarly, although the pseudokinase domain of RNase L is catalytically inactive, its RNase activity also seems to rely on the “active”, DFG-in, conformation of the kinase domain to form a back-to-back dimer reminiscent of that observed for Ire1 (Han et al., 2014; Huang et al., 2014).

The mixed lineage kinase domain-like (MLKL) pseudokinase provides a striking example of how the activation loop can adopt a non-canonical conformation to regulate non-catalytic functions of a kinase. Crystal structures of the MLKL pseudokinase domain reveal an unusual conformation in which a portion of the activation loop forms a helix, which takes the position normally occupied by helix αC (Murphy et al., 2013a; Xie et al., 2013) (Figure 4B). A glutamine residue, Q343, in the activation loop helix of MLKL forms a hydrogen bond with the β3 lysine K219 (K72 in PKA), mimicking the salt bridge formed between the β3 lysine and the αC helix glutamate in the active conformation of conventional kinases. Mutations, such as K219M and Q343A, that disrupt this non-canonical interaction result in constitutive activation of necroptosis by MLKL, suggesting that the unusual conformation of its activation loop represents an autoinhibited state of MLKL and that MLKL activation relies on a conformational change of the activation loop to release this inhibition (Murphy et al., 2013a).

Helix αC conformation and the Src/CDK-like inactive state—A significant change in the position of helix αC relative to the active site, as well as associated changes in the conformation of the activation loop, are hallmarks of the inactive, Src/CDK-like, conformation (Figure 3B). The non-catalytic functions of several kinases might be controlled by cycling between the active and Src/CDK-like inactive state. In the case of RAF kinases, only the active kinase conformation is compatible with allosteric trans-activation of kinases in RAF dimers (Figure 4C). Hence, inhibitors of B-RAF that stabilize the active state of the kinase domain promote formation of B-RAF/C-RAF dimers, in which the inhibitor-bound B-RAF allosterically activates C-RAF (Hatzivassiliou et al., 2010). Similarly, mutations in B-RAF, C-RAF, and KSR that stabilize the active state of the kinase domain also promote dimerization (Hu et al., 2011). In contrast, inhibitors like vemurafenib that favor a Src/CDK-like inactive conformation of B-RAF fail to promote B-RAF/C-RAF heterodimerization (Hatzivassiliou et al., 2010; Thevakumaran et al., 2015).

The Src/CDK-like inactive conformation might also play a key role in the function of the pseudokinase STRADα. Even though STRADα lacks catalytic activity, the crystal structure of the STRADα-Mo25-LKB1 ternary complex reveals that STRADα adopts a conformation resembling the active conformation of catalytically active kinases (Zeqiraj et al., 2009a; 2009b) (Figure 4D). In the structure, the “active” conformation of STRADα is stabilized through binding of Mo25 to the αC helix of STRADα. This interaction is reminiscent of the mechanism by which cyclin binding stabilizes the active conformation of CDKs and asymmetric dimerization of HER kinases activates one of the kinases in the asymmetric dimer. In both CDK and HER kinases, disruption of these interactions promotes adoption of the Src/CDK-like inactive conformation and kinase inactivation. Although it is not known whether mutation of the STRADα/Mo25 binding interface, which abrogates STRADα-mediated activation of LKB1, shifts STRADα into the Src/CDK-like inactive conformation, one could speculate that, like CDK and EGFR, STRADα resides in this conformation when not bound to Mo25.

The αC helix of the pseudokinase Trb1 adopts an unusual conformation, in which the αC helix is truncated and bent relative to that of conventional active kinases (Figure 4E). Intriguingly, this distorted conformation of helix αC appears to be important for regulating

Trb1 function by creating a docking site for the C-terminal tail of Trb1 and thereby sequestering a motif required for Tribbles pseudokinases to recruit the ubiquitin ligase COP1 (Murphy et al., 2015). This example along with that of the non-canonical activation loop of MLKL illustrate how conserved kinase structural elements can be adapted and repurposed by pseudokinases to regulate non-catalytic functions.

Cases in which there is no apparent conformational change—In several kinases, no conformational transitions regulating their non-enzymatic functions have been observed. In these cases, relatively rigid segments of the C-lobe are primarily responsible for those non-catalytic functions. An example of this type of non-catalytic kinase signaling is the activation of HER receptors through formation of an asymmetric kinase dimer. In this dimer, a hydrophobic surface on the C-lobe of one kinase is essential for binding and allosteric activation of another kinase in the dimer (Figure 4F). The C-lobe-localized interaction interface remains the same in crystal structures of HER kinases regardless of the overall conformation adopted by the allosteric activator kinase (Aertgeerts et al., 2011; Brewer et al., 2009; Littlefield et al., 2014; Qiu et al., 2008; Stamos et al., 2002) (Figure 4F). It remains possible that the C-lobe interface is subject to some degree of conformational control and that non-static biophysical approaches that inform on dynamic properties of proteins are necessary to capture these transitions.

If the allosteric interface in HER kinases is indeed independent of conformational changes within the kinase domain, then how is the allosteric activator function of these kinases regulated? One potential mechanism by which HER activation could be regulated is through occlusion of the hydrophobic C-lobe interface by binding of other proteins or through intramolecular interactions with adjacent domains. The former of these mechanisms has been described for EGFR and relies on the function of the inhibitory protein, MIG6 (Zhang et al., 2007). The MIG6 binding site on EGFR overlaps with the allosteric activator interface on the C-lobe of EGFR, and MIG6 was shown to block formation of the asymmetric EGFR kinase dimer.

Like the HER receptors, there is no evidence of conformational changes playing a role in the inhibition of the human GTPase IRGa6 by the *Toxoplasma gondii* pseudokinase ROP5B. ROP5B stabilizes the inactive form of IRGa6 by interacting with it through the C-lobe of the kinase domain (Figure 4G) (Fleckenstein et al., 2012; Reese et al., 2014). The conformation of the pseudokinase domain in the ROP5B/IRGa6 structure is nearly identical to that observed in structures of apo ROP5B and ATP-bound ROP5B, suggesting that the pseudokinase domain does not need to undergo significant conformational changes in order to engage with IRGa6 (Reese and Boothroyd, 2011; Reese et al., 2014). It is therefore likely that interaction of ROP5B with IRGa6 is regulated through a different mechanism. As with the HER kinases, a potential mode of regulation could be occlusion of the IRGa6 binding site on ROP5B by other proteins. Since ROP5B binds to the GDP-bound inactive form of IRGa6, it is also possible that the nucleotide state of IRG6a regulates the strength of the IRGa6/ROP5B interaction.

Regulation of conformational transitions involved in non-catalytic functions of kinases

Post-translational modifications

Kinases are frequently phosphorylated on their activation loop or in regulatory regions outside the kinase domain. These phosphorylation events often induce conformational changes that underlie transitions of a kinase between active and inactive states. While we conventionally think of this type of phosphorylation as a means to activate the catalytic activity of a kinase, it has become apparent that phosphorylation-induced conformational changes can also regulate the non-catalytic functions of kinases.

Phosphorylation of the activation loop usually stabilizes it in an extended state, and this conformational change seems to be particularly relevant for non-catalytic functions of kinases. For example, in order to activate PARP1 and topoisomerase IIa in a catalysis-independent manner, ERK2 needs to be phosphorylated on its activation loop by another kinase, MEK (Cohen-Armon et al., 2007; Shapiro et al., 1999). This suggests that the activation loop of ERK2 must adopt an extended conformation for ERK2 to function as an allosteric regulator.

Activation loop phosphorylation is also critical for triggering oligomerization of the pseudokinase MLKL and its translocation to the plasma membrane and intracellular membrane compartments to form membrane-disrupting pores resulting in necroptosis (Wang et al., 2014) (Figure 5A). This effect of phosphorylation is similar to that of previously discussed necroptosis-inducing mutations (K219M and Q343A) in MLKL that disrupt the interaction between the β3 lysine and activation loop helix glutamine (Murphy et al., 2013a; Wang et al., 2014). These observations support a model in which phosphorylation of the MLKL activation loop by RIPK3 kinase induces a unique conformational change in the pseudokinase domain that allows MLKL to promote necroptosis (Figure 4B).

Phosphorylation of an N-terminal acidic (NtA) motif in RAF kinases is important for their allosteric activation by dimerization (Figure 5A). NtA phoshorylation is thought to stabilize an active conformation of RAF kinases, which is essential for formation of active RAF dimers. In a RAF dimer, only the kinase that is the allosteric activator of the other requires NtA phosphorylation. The NtA motif of B-RAF is constitutively phosphorylated, which may explain why even kinase-dead mutants of B-RAF can activate other RAF isoforms, such as C-RAF (Hu et al., 2013). The molecular mechanism by which NtA phosphorylation stabilizes the active conformation remains to be understood.

Occupancy of the nucleotide-binding pocket

Binding of nucleotides and ATP-competitive inhibitors that stabilize the active conformation of the Ire1 kinase domain, was shown to be sufficient to induce activation of the RNase domain through oligomerization (Korennykh et al., 2009; Papa et al., 2003) (Figure 5B). Small molecule binding to the ATP pocket triggers a conformational rearrangement of the αC helix from a position similar to that observed in the Src/CDK-like conformation into an active conformation that supports oligomerization of the Ire1 kinase (Ali et al., 2011;

Korennykh et al., 2011). Interestingly, ADP or its mimic ADPβS are more potent activators of Ire1 RNase activity than ATP because they bind preferentially to the active conformation of the kinase domain, while ATP is believed to bind readily even when helix αC is in an inactive position (Korennykh et al., 2011). However, ADP and ADPβS fail to stimulate RNase activity in the absence of thiophilic metal ions like Mn^{2+} and Cd^{2+} that are likely necessary to properly position the β-phosphate of ADP to enable movement of helix αC into the active position. Uncharged, synthetic ATP-competitive small molecules can bypass the requirement for a metal cation by inserting bulky moieties into the β-phosphate position (Korennykh et al., 2011).

The role of nucleotide binding in activation of the RNase domain of RNase L is slightly different (Figure 5B). In contrast to Ire1, the first step of RNase L oligomerization is regulated by a second messenger 2-5A (2′,5′-oligoadenylate), which binds to a pocket formed by the ankyrin-repeat and pseudokinase domains of RNase L. Binding of 2-5A is sufficient to induce dimerization of the pseudokinase domain even in the absence of nucleotide, but insufficient for activating the RNase domain, for which nucleotide binding to the pseudokinase domain is required (Dong and Silverman, 1999; Huang et al., 2014). Unlike Ire1, RNase L is activated equally well by ADP, ATP, and the non-hydrolyzable ATP analog AMP-PNP. In existing crystal structures, the pseudokinase domain of RNase L adopts a DFG-in conformation resembling the active conformation of canonical kinases regardless of the nucleotide-bound state, making the role of nucleotide binding unclear (Han et al., 2014; Huang et al., 2014).

Protein-protein interactions

The ability of the pseudokinase STRADα to allosterically activate LKB1 is regulated by both nucleotide binding and interaction with a protein partner, Mo25. STRADα requires binding of both ATP and Mo25 to activate LKB1 (Baas et al., 2003; Boudeau et al., 2003; Zeqiraj et al., 2009b) (Figure 5C). Interaction with Mo25 enhances the ability of STRADα to bind ATP. Similarly, ATP increases the affinity of STRADα for Mo25, likely by stabilizing the conformation of STRADα that is recognized by Mo25.

Exploiting the dynamic nature of the kinase domain to regulate non-catalytic functions of protein kinases

The remarkable progress in kinase inhibitor discovery over recent years has led to the development of numerous small molecules targeting kinases, the majority of which compete with ATP for binding to the nucleotide-binding site. In addition to their task of blocking ATP binding, these inhibitors also tend to preferentially stabilize kinases in different conformational states. Most of these ATP-competitive inhibitors bind to the active DFG-in conformation of the kinase domain and have been termed Type I inhibitors. In contrast, Type II kinase inhibitors are also ATP-competitive, but selectively bind to the inactive DFG-out conformation. As it turns out, this conformational selection makes some inhibitors potent regulators of the non-catalytic functions of their target kinases.

Serendipitous discoveries of small molecules that modulate non-catalytic functions of kinases

The observation that several Type I B-RAF inhibitors, such as GDC-0879, paradoxically activate MAPK signaling in cells while potently inhibiting B-RAF kinase activity *in vitro* was puzzling. Now, we know that their potential to stabilize the active kinase conformation allows these inhibitors to activate signaling at subsaturating concentrations where inhibitor-bound RAF molecules can dimerize with and allosterically activate apo RAF molecules, resulting in activation of downstream signaling (Hatzivassiliou et al., 2010; McKay et al., 2011; Poulikakos et al., 2010) (Figure 6A). In contrast, inhibitors like vemurafenib, which stabilizes the Src/CDK-like inactive conformation of RAF (Thevakumaran et al., 2015; Wenglowsky et al., 2011), fail to promote kinase dimerization (Hatzivassiliou et al., 2010) (Figure 6B). Surprisingly, despite this, vemurafenib does induce paradoxical activation of MAPK signaling in cells expressing wild type B-RAF. The mechanism underlying this remains poorly understood, but might be due to vemurafenib's ability to disrupt interaction of B-RAF with inactive MEK (Haling et al., 2014).

Activation of PERK (PKR-like ER kinase) by the ATP-competitive inhibitor IPA appears to proceed through a mechanism similar to that of B-RAF, in which IPA drives PERK oligomerization and stimulates PERK activity at low doses by stabilizing the active conformation of the PERK kinase domain (Mendez et al., 2015). Likewise, the RNase activity of Ire1 can be induced by the addition of ATP-competitive inhibitors that selectively stabilize the active conformation of the Ire1 kinase domain (Korennykh et al., 2009; Wang et al., 2012). These observations suggest that activation of the non-catalytic functions of kinases as a result of drug-induced conformational change may be a more widespread phenomenon associated with kinases that are activated through oligomerization.

Another example of inhibitors that unexpectedly unveiled a novel mode of non-catalytic kinase signaling are RIPK3-specific inhibitors, which were initially shown to block RIPK3-dependent necroptosis when apoptosis is blocked via caspase inhibition (Kaiser et al., 2013; Mandal et al., 2014). Surprisingly, in the absence of caspase inhibition, these inhibitors display unexpectedly high cytotoxicity by triggering caspase activation and apoptotic cell death by promoting RIPK3 oligomerization and RIPK3-dependent formation of a large apoptotic signaling complex. This outcome is reminiscent of the effect that the kinase-inactivating D161N mutation has on RIPK3 signaling (Mandal et al., 2014; Newton et al., 2014), underscoring the innate potential of RIPK3 to promote apoptosis in an oligomerization-dependent manner that likely relies on a specific conformation of the kinase domain that is induced by both the D161N mutation and the inhibitors.

Successful design of inhibitors targeting non-catalytic functions of protein kinases

Since type I Ire1 kinase inhibitors that stabilize the active conformation of the kinase domain result in activation of the RNase domain, the hypothesis was made that type II inhibitors that bind to the inactive DFG-out conformation would inhibit RNase activity (Wang et al., 2012). A subset of these compounds, termed KIRAs (kinase-inhibiting RNase attenuators), was identified and predicted by molecular modeling to stabilize the DFG-out conformation of the kinase domain (Ghosh et al., 2014; Wang et al., 2012). Comparison of

KIRAs with the type I inhibitor APY29 confirmed their differential effect on Ire1 oligomerization and activation. Although both types of compounds inhibit Ire1 autophosphorylation, APY29 enhances oligomerization and stimulates RNase activity, while KIRAs disrupt oligomerization and block RNase activity (Ghosh et al., 2014; Wang et al., 2012) (Figure 6A and B).

Aurora A kinase, which is a catalytically active kinase, is frequently overexpressed in cancers and is a target of several ATP-competitive inhibitors. Aurora A was found to interact with the transcription factor N-Myc and the ubiquitin ligase SCF^{Fbxw7} to prevent N-Myc degradation in neuroblastomas where *MYCN*, the gene encoding N-Myc, is amplified (Otto et al., 2009). This function of Aurora A is preserved in the presence of kinase-inactivating mutations, such as K162R (K72 in PKA) and D274N (D184 in PKA), as well as some ATP-competitive inhibitors, such as hesperadin, indicating that this function does not depend on Aurora A kinase activity (Otto et al., 2009). A different class of ATP-competitive inhibitors has shown promise in inhibiting Aurora A-mediated stabilization of N-Myc and was further developed into potent compounds, including MLN8054, MLN8237, and CD532, that induce degradation of N-Myc by disrupting the interaction between Aurora A and N-Myc (Figure 6C). Unlike hesperadin, these inhibitors stabilize an unusual inactive conformation of Aurora A in which the phenylalanine of the DFG motif is oriented such that helix αC is further away from the active site than is typically observed in kinase structures (Brockmann et al., 2013; Gustafson et al., 2014). The potency of these compounds seems to be related to the degree with which they displace helix αC (Gustafson et al., 2014). Although these conformational transitions might represent a structural artifact of drug binding to Aurora A, it is exciting to speculate that they might also constitute a physiologically-relevant regulatory step in controlling N-Myc degradation.

In an effort to use ATP-competitive small molecules to modulate the non-catalytic interactions of MAP kinases with their binding partners, Hari and colleagues found that inhibitors that bind to the DFG-in and DFG-out conformations of ERK2 have distinct effects on interactions of ERK2 with upstream activators (Hari et al., 2014). While a type II inhibitor that stabilizes the DFG-out conformation markedly reduced phosphorylation of the activation loop of ERK2 by MEK2, type I inhibitors that bind in a DFG-in state had only a weak effect. The type I and type II inhibitors also had differential effects on the ability of ERK2 to allosterically activate MKP3. The type II, DFG-out ERK2 inhibitor decreased the rate of dephosphorylation of ERK2 by MKP3, whereas type I DFG-in inhibitors enhanced dephosphorylation (Figure 6A and B). Interestingly, the DFG-out inhibitor blocked the ability of ERK2 to activate MKP3 without affecting their mutual binding (Hari et al., 2014). These findings further underscore the potential for modulation of non-enzymatic kinase functions through design of conformation-specific inhibitors.

The catalytically inactive pseudokinase MLKL exemplifies another successful effort to identify an inhibitor of a non-enzymatic kinase function. A screen of known kinase inhibitors identified a compound that binds to the nucleotide-binding pocket of MLKL. This compound enhances phosphorylation of the MLKL activation loop by RIPK3, but it prevents translocation of MLKL to membrane compartments and therefore blocks necroptosis, the physiological consequence of MLKL activation loop phosphorylation

(Hildebrand et al., 2014). These findings suggest that the compound induces only a partial conformational change that enables activation loop phosphorylation of MLKL but not signal propagation.

Conclusions

Structural studies of kinases have highlighted a wide spectrum of conformational states achieved by the kinase domain that can uniquely contribute to cellular signaling independently of kinase enzymatic activity. Interestingly, it is an active kinase conformation that most frequently seems to be essential for the non-catalytic functionalities of kinases (Figure 6A). In the case of catalytically active kinases, this conformational preference might simply reflect the fact that catalytic and non-catalytic kinase functions are coupled to the same activating input. In the case of pseudokinases, such as RNase L, the functional importance of the active conformation is also not entirely surprising since the function of the RNase pseudokinase closely mimics the mechanism by which the catalytically active Ire1 kinase regulates activity of the adjacent RNase domain. More intriguing is the fact that pseudokinases, such as STRADα, also rely on a conformation resembling the active DFG-in conformation observed for active kinases to form a functional signaling complex with Mo25 and the catalytically active LKB1 kinase. This widespread relevance of the active DFG-in conformation in regulation of the non-catalytic functions might reflect the fact that this conformation carries several recognizable structural features that can easily plug into protein-protein interaction networks trained to recognize active kinases.

Although many non-catalytic functions of kinases are misregulated in disease, so far only a limited set of small molecules that can target these functions has been discovered. The examples discussed in this review demonstrate that it is possible to modulate the non-catalytic functions of both active kinases and pseudokinases by leveraging our knowledge of small molecule design for targeting the nucleotide-binding pocket. Since many non-catalytic functions of kinases appear to be tied to the active DFG-in conformation of the kinase domain, inhibitors that select for inactive conformations like the DFG-out conformation or Src/CDK-like conformation could be used to disrupt non-enzymatic functions, as demonstrated for Ire1 and ERK2. This is also particularly important to consider in light of examples where kinase inhibitors can induce paradoxical activation of their targets, as shown for B-RAF (Hatzivassiliou et al., 2010; McKay et al., 2011; Poulikakos et al., 2010) and, more recently, PERK (Mendez et al., 2015). At the same time, as illustrated by the Aurora A inhibitors MLN8054, MLN8237, and CD532 (Brockmann et al., 2013; Gustafson et al., 2014), these small molecules need not stabilize ‘native’ conformations of the kinase domain, but any conformation that deviates from the one relevant for signaling.

While progress is being made in designing ATP-competitive inhibitors of non-catalytic kinase functions, the potential of utilizing other small molecules should not be ignored. For example, molecules that bind to allosteric regulatory sites in kinases could stabilize the kinase domain in unique conformational states and be particularly useful in drugging pseudokinases, many of whom possess putative nucleotide binding pockets that are impaired in binding nucleotide analogs (Murphy et al., 2013b). Alternatively, as recently shown for a catalytically impaired PINK1 G309D disease mutant, kinases whose active sites are poor

binders of ATP, can sometimes accommodate other molecules in their active site pockets (Hertz et al., 2013). This observation opens up the possibility that more kinases could bind non-canonical small molecules in their active sites as a means of regulating their non-catalytic functions. This also shows that we should be open-minded about therapeutic strategies aiming to pharmacologically regulate these emerging kinase functions.

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The functions of protein kinases are not limited to phosphorylation, and many kinases signal through non-enzymatic mechanisms. Kung and Jura review known non-catalytic functions of kinases, analyze the molecular mechanisms underlying these functions, and discuss efforts to design small molecule modulators of non-catalytic kinase signaling.

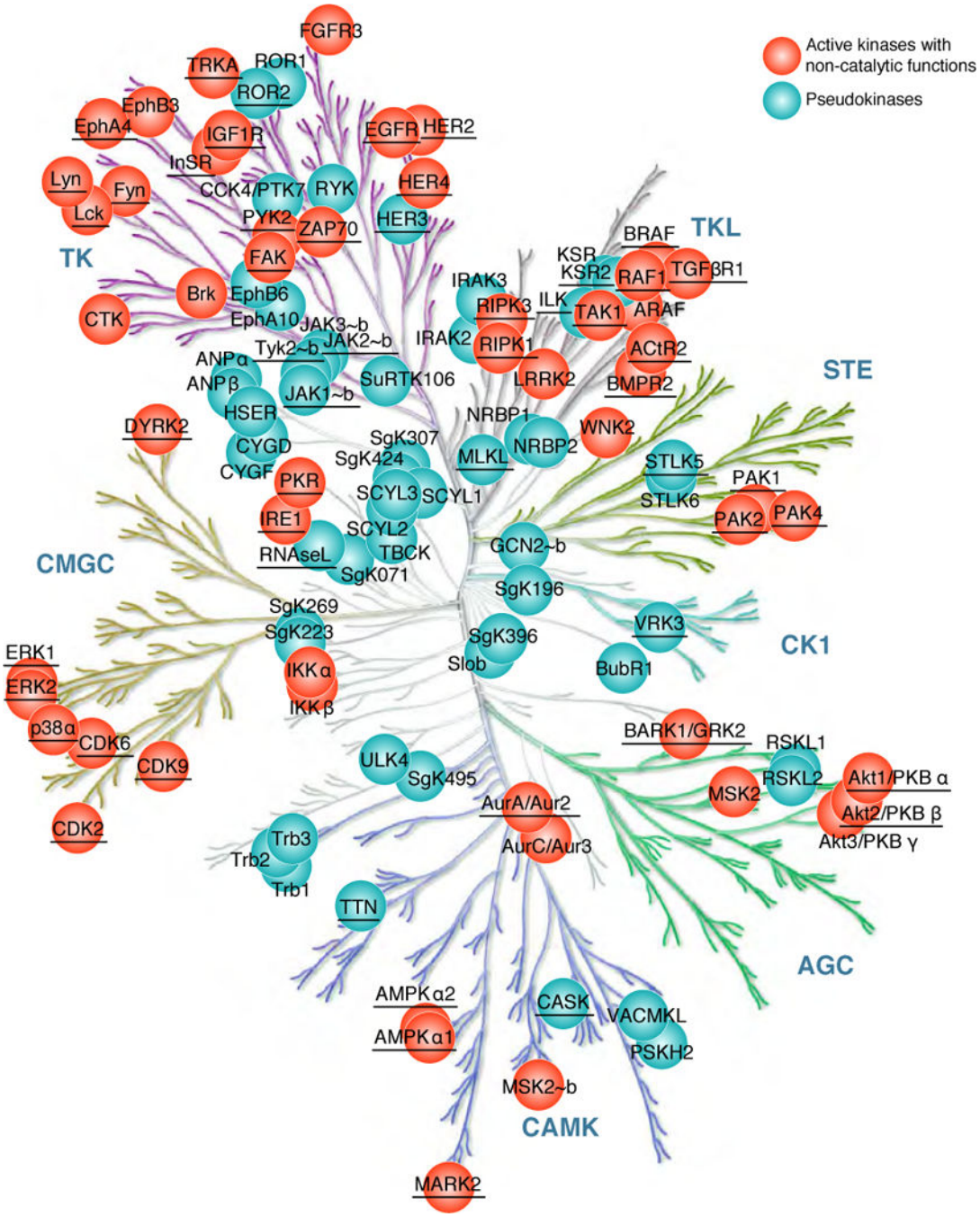

Figure 1. Kinases reported to have non-catalytic functions

Active kinases (red circles) and pseudokinases (blue circles) that have been reported to perform non-catalytic functions are marked on the human kinome tree. The names of kinases whose crystal structures are available are underlined.

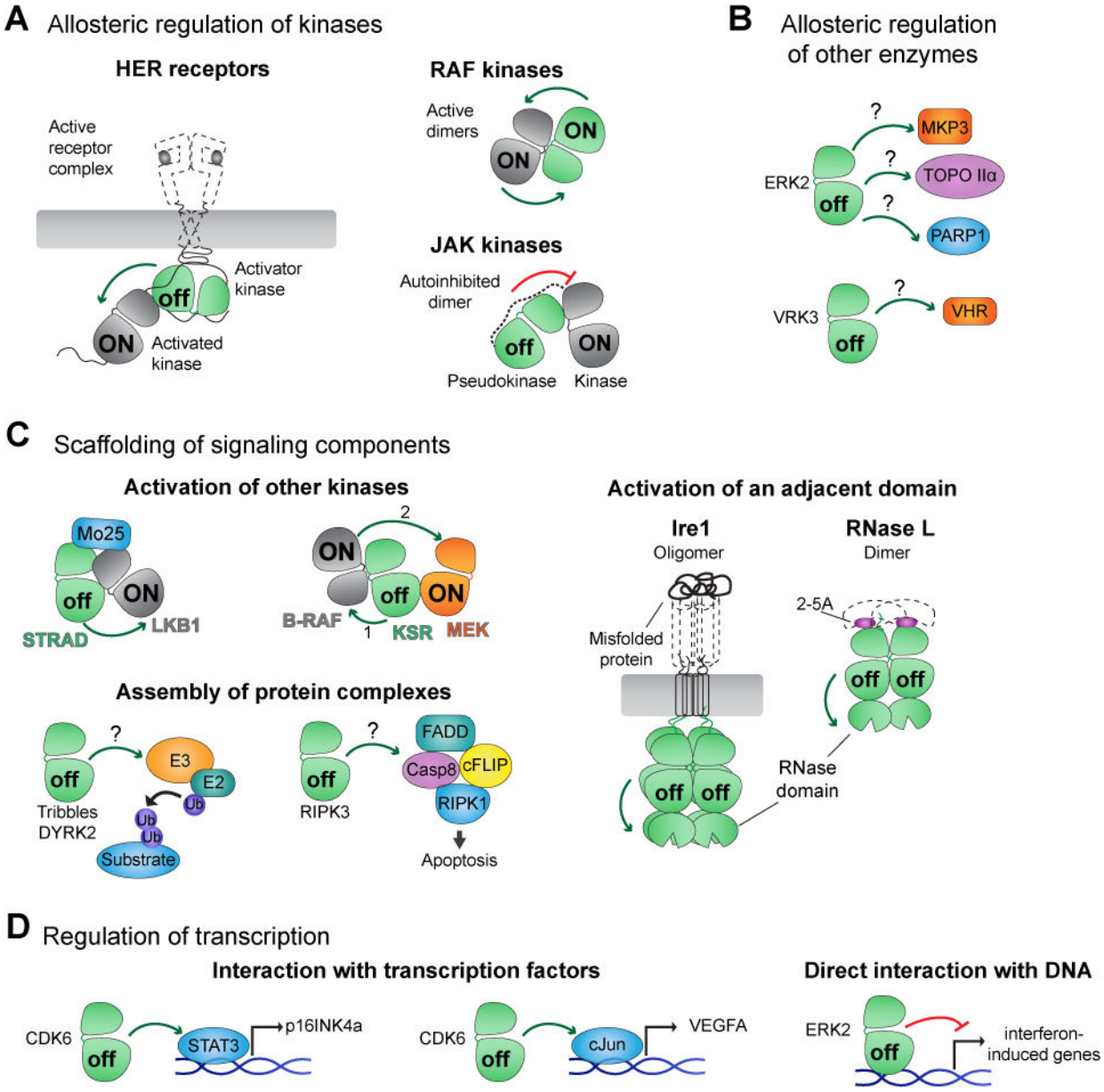

Figure 2. Non-catalytic functions of protein kinases

(A) Dimerization-induced conformational changes distal to the active site result in activation of HER and RAF family kinases. In JAK kinases, in which both kinase domains are located on the same polypeptide chain, the N-terminal pseudokinase domain inhibits the adjacent kinase domain.

(B) ERK2 allosterically activates diverse enzymes, including MAP kinase phosphatase 3 (MKP3), topoisomerase IIα (TOPO IIα), and poly (ADP-ribose) polymerase-1 (PARP1). VRK3 allosterically activates vaccinia H1-related (VHR) phosphatase.

(C) STRAD interacts with Mo25 to activate LKB1. KSR activates B-RAF through dimerization (1) and brings it to MEK (2). Tribbles and DYRK2 are thought to form complexes with ubiquitin ligases to promote ubiquitination. RIPK3 recruits RIPK1, Casp8,

FADD, and cFLIP to form a complex that induces apoptosis. Ire1 and RNase L use their kinase domains as scaffolds for oligomerization to activate their adjacent RNase domains. (D) CDK6 interacts with transcription factors to promote transcription of their targets. ERK2 binds directly to promoter sequences to repress transcription.

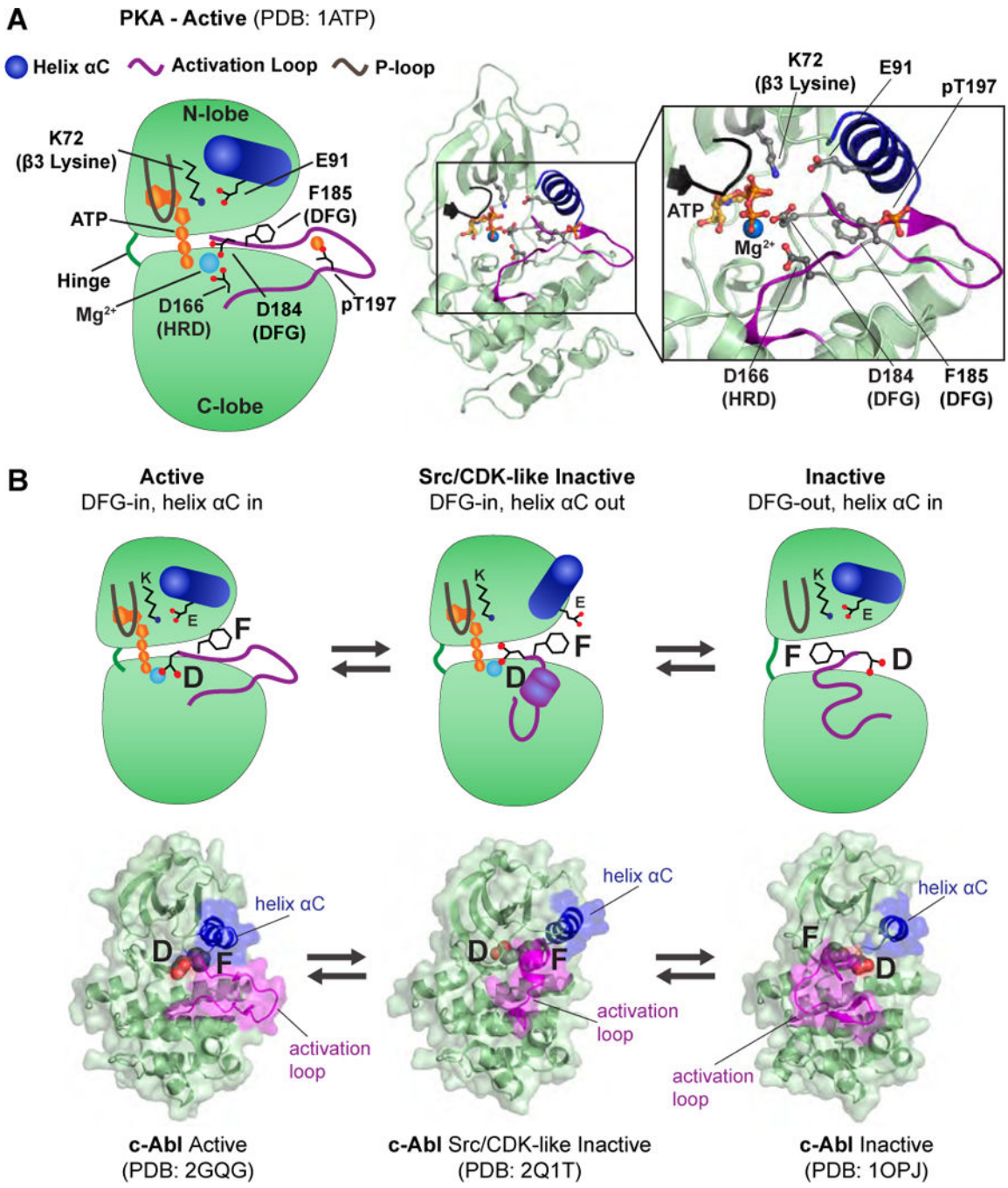

Figure 3. Conformational transitions linked to kinase catalysis

(A) Cartoon representation and crystal structure (PDB: 1ATP) of the active conformation of Protein Kinase A (PKA). The inset displays a close-up view of the active site, highlighting structural elements that are critical for catalysis.

(B) Cartoon representations (top) and crystal structures (bottom) illustrating the structural changes that occur as the c-Abl kinase domain transitions between different conformational states.

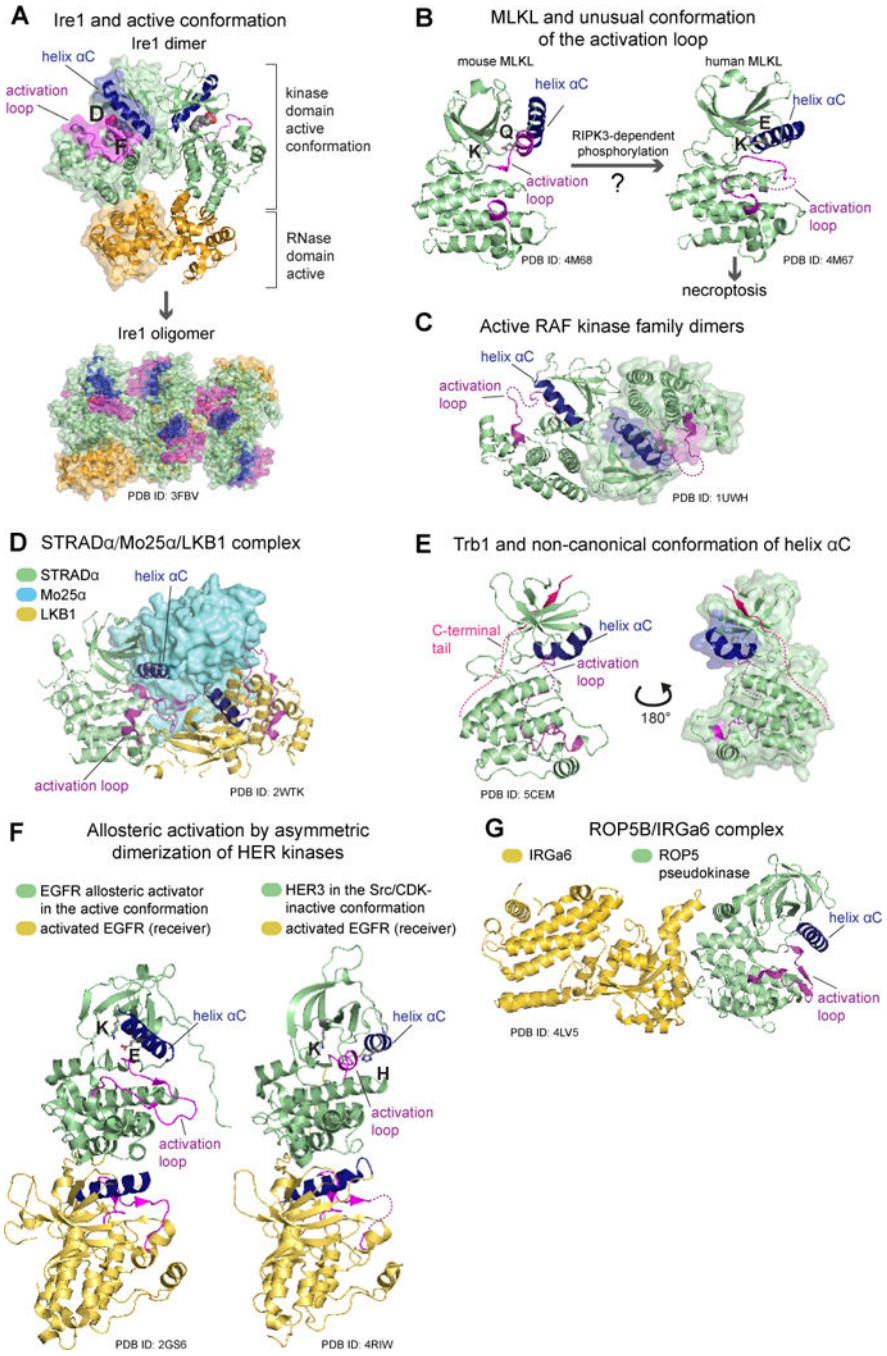

Figure 4. Significance of kinase conformation for non-catalytic functions

(A) Active conformation of the Ire1 kinase domain induces formation of a back-to-back dimer and higher order oligomers of Ire1 kinase domains that activate the adjacent RNase domain (PDB: 3FBV).

(B) Structures of mouse MLKL pseudokinase (PDB: 4M68) (left) show its activation loop in an unusual conformation, which is hypothesized to rearrange upon RIPK3-dependent phosphorylation, triggering MLKL oligomerization and necroptosis. In contrast, structures of human MLKL (PDB: 4M67) (right) show the activation loop adopting an extended

conformation, possibly the conformation that results from RIPK3-dependent phosphorylation.

(C) The active conformation of the RAF kinase domain is required to transactivate a dimerization partner in a side-to-side dimer (PDB: 1UWH).

(D) Mo25 stabilizes STRADα pseudokinase in an “active” DFG-in conformation, which is necessary for LKB1 activation (PDB: 2WTK).

(E) Structures of the pseudokinase Trb1 reveal a non-canonical conformation of helix αC that creates a binding site for the C-terminal tail of Trb1 (PDB: 5CEM).

(F) In crystal structures of HER kinase asymmetric dimers (EGFR/EGFR PDB: 2GS6, HER3/EGFR PDB: 4RIW) the conformation of the allosteric activator kinase (active or Src/CDK-like inactive conformation) does not seem to matter for activation of the receiver kinase.

(G) No conformational changes in the *T. gondii* pseudokinase ROP5B have been implicated in regulation of the human GTPase IRGa6 (PDB: 4LV5).

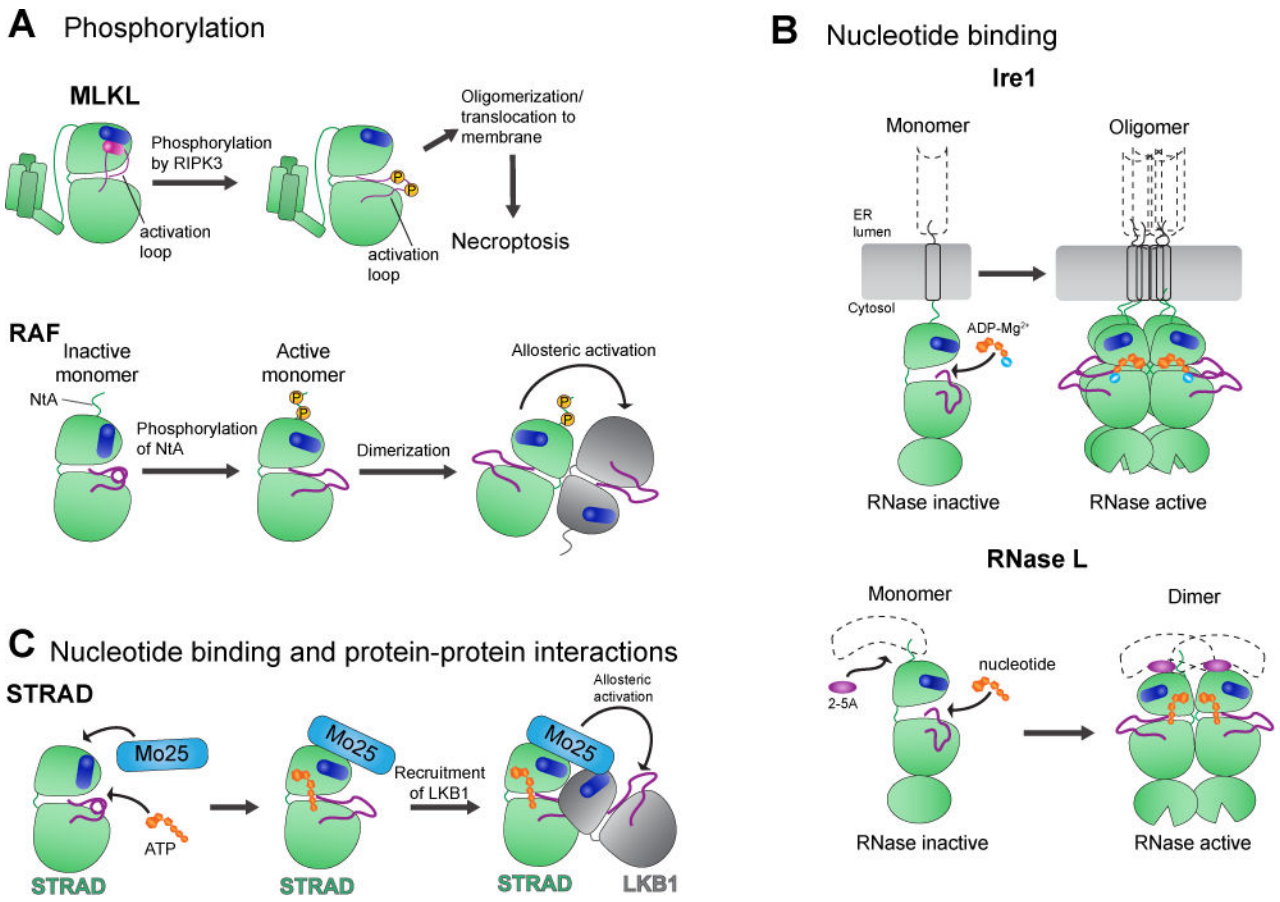

Figure 5. Mechanisms regulating kinase conformational changes involved in non-catalytic functions

(A) Phosphorylation by RIPK3 induces a conformational change in the activation loop of MLKL triggering MLKL oligomerization and necroptosis. Phosphorylation of the N-terminal acidic (NtA) motif in RAF kinases stablizes their active conformation enabling them to allosterically activate another RAF kinase in a side-to-side dimer.

(B) Binding of ADP-Mg^{2+} stabilizes the active conformation of the Ire1 kinase domain, promoting oligomerization and activation of the RNase domain. Similarly, binding of the second messenger 2-5A and a nucleotide to the RNase L pseudokinase domain induces dimerization and activation of the RNase domain.

(C) STRADα requires binding of both Mo25 and ATP in order to be able to allosterically activate LKB1.

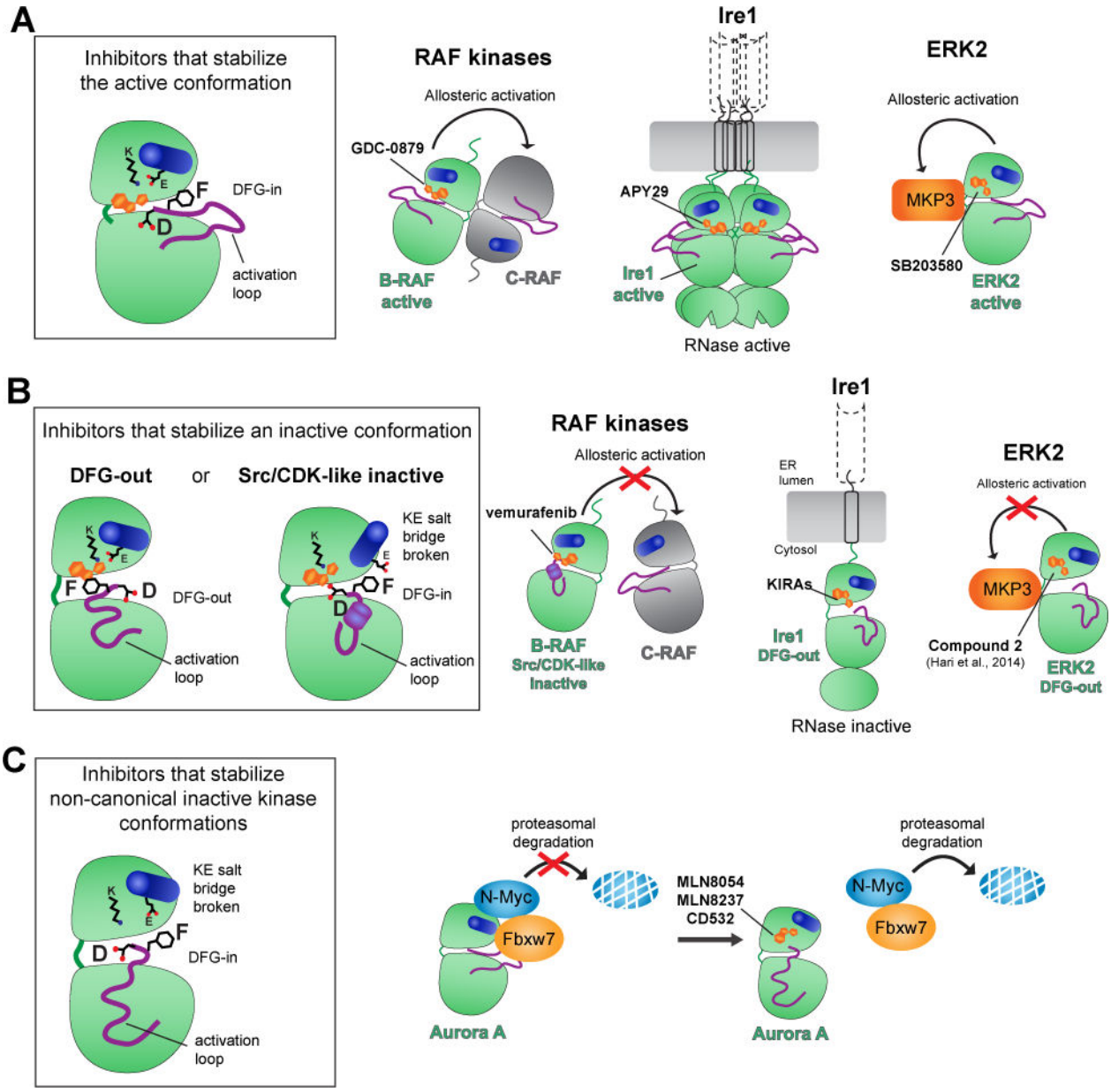

Figure 6. Targeting non-catalytic functions of kinases with small molecules

(A) Examples of inhibitors that stabilize the active conformation of kinases, which in the majority of cases promote their non-catalytic functions.

(B) Examples of inhibitors that stabilize inactive conformations of kinases and their inhibitory effect on non-catalytic kinase functions.

(C) Inhibitors of Aurora A that interfere with its non-catalytic function by stabilizing a non-canonical inactive conformation.

Table 1

Active kinases reported to perform non-catalytic functions.

Kinase	Non-catalytic function(s)	Corresponding residue(s) in the active site of PKA[a]	References
A-RAF, B-RAF, C-RAF (RAF1)	RAF kinases inactivated through mutations in catalytic spines (B-RAF A471F, C-RAF A373F) or the active site (B-RAF G466E, G466V, G596R), or binding of an inhibitor can allosterically activate other RAF kinases through homo- or heterodimerization.	A70, G52, G186	Mooz, J. et al. (2014). Science Signaling 7, ra73. Hu, J. et al. (2013). Cell 154, 1036–1046. Garnett, M.J. et al. (2005). Mol. Cell *20*, 963–969. Wan, P.T.C., et al. (2004). Cell *116*, 855–867.
ActRIIa (ACtR2)	Both kinase-active and kinase-dead (K219R) variants of ActRIIa form a functional complex with constitutively active ALK2 Q207D mutant, which depends on dimerization with ActRIIa for signaling	K72	Bagarova, J. et al. (2013). Mol Cell Biol 33, 2413–2424.
Akt1, Akt2, Akt3	Studies where Akt was inactivated by inhibitors or by active site mutations (Akt1 K179M, Akt2 K181M, Akt2 G161V) demonstrated that Akt kinase domain conformation but not activity controls the ability of the adjacent PH domain to bind phosphoinositides	K72, G52	Okuzumi et al. (2009). Nat Chem Biol 5, 484–493. Vivanco, I. et al. (2014). eLife 3, e03571
AMPKα1, AMPKα2	Kinase-active and kinase-dead (AMPKα1 T172A, AMPKα2 D139A, AMPKα2 D157A) variants of AMPK interact with and co-activate PPARα	T179, D166, D184	Bronner, M. et al. Biochem. J. 384, 295–305 (2004).
Aurora A	The function of Aurora A kinase to prevent N-Myc degradation through interactions with N-Myc and SCF^{Fbxw7} is maintained even in the presence of type I Aurora A inhibitors	N/A	Otto, T. et al. (2009). Cancer Cell 15, 67–78.
Aurora C	Both kinase-active and kinase-dead (T202D) variants of Aurora C promote degradation of Aurora B and interfere with spindle assembly checkpoint	T172	Lin, B. W. et al. (2014) Cell Death Dis 5, e1106.
BMPR2	Both kinase-active and kinase-dead (K230R) variants of BMPR2 form a functional complex with constitutively active ALK2 Q207D mutant, which depends on dimerization with BMPR2 for signaling	K72	Bagarova, J. et al. (2013). Mol Cell Biol 33, 2413–2424.
Brk	Both kinase-active and kinase-dead (K219M) variants of Brk promote breast carcinoma cell proliferation	K72	Harvey, A.J. & Crompton, M.R. (2003). Oncogene 22, 5006–5010.
c-Kit	Inhibition of c-Kit kinase by small molecule inhibitor does not interfere with its signaling in a complex with the GM-CSF receptor	N/A	Lennartsson, J. et al. (2004). J Biol Chem 279, 44544–44553.
CDK2	Studies using an analog-sensitive CDK2 mutant defective in binding to cyclin A and activation loop phosphorylation revealed that kinase domain conformation and not activity regulates activation of CDK2. Binding of small molecule inhibitors can rescue both deficiencies	N/A	Merrick, K.A. et al. (2011). Mol Cell 42, 624–636.
CDK6	Both kinase-active and kinase-dead (K43M) variants of CDK6 promote transcription of p16INK4a and VEGF-A	K72	Kollmann, K. et al. (2013). Cancer Cell 24, 167–181.
CDK9	Both kinase-active and kinase-dead (D167N) variants of CDK9 inhibit glucocorticoid receptor-mediated gene induction and transactivation activity of B-Myb	D184	Zhu, R. et al. (2014). Biochemistry 53, 1753–1767. De Falco, G. et al (2000). Oncogene 19, 373–379.
CHK (CTK)	CHK binds to and inhibits Src family kinases in a phosphorylation-independent manner	N/A	Chong, Y.P. et al. (2004). J Biol Chem 279, 20752–20766.
DYRK2	Both kinase-active and kinase-dead variants of DYRK2 are capable of interacting with EDD ubiquitin ligase and substrate adaptors DDB1 and VPRBP to assemble the EDVP ubiquitin ligase complex	Mutation not specified in the original study	Maddika, S. & Chen, J. (2009). Nature 11, 409–419.

Kinase	Non-catalytic function(s)	Corresponding residue(s) in the active site of PKA[a]	References
EGFR HER2 HER4	Catalytically inactive (EGFR D813N, HER4 D843N) HER kinases can allosterically activate other HER kinases through asymmetric dimerization of kinase domains	D166	Zhang, X. et al. (2006). Cell 125, 1137–1149. Monsey, J. et al. (2010). J Biol Chem 285, 7035–7044. Qiu, C et al. (2008). Structure 16, 460–467.
EphA4	Expression of kinase-dead EphA4 (K653M) is sufficient for proper formation of anterior commissure (AC) in mice, whereas mice that are EphA4 null exhibit defects in AC formation	K72	Kullander, K. et al. (2001). Neuron 29, 73–84.
EphA8	Kinase-dead EphA8 (K666M, K666R) promotes cell attachment to fibronectin as well as the wild type EphA8	K72	Gu, C., & Park, S. (2001). Mol Cell Biol 21, 4579–4597.
EphB3	Both kinase-active and kinase-dead (K665R) EphB3 inhibit cell migration	K72	Miao, H.,et al. (2005). J. Biol. Chem. 280, 923–932.
ERK1	ERK1 is hypothesized to behave similarly to ERK2 in displacement of retinoblastoma (Rb) protein from lamin A	N/A	Rodriguez, J. et al. (2010). J Cell Biol 191, 967–979.
ERK2	Both kinase-active and kinase-dead (K54A, K54R) ERK2 are capable of: (i) displacing retinoblastoma (Rb) protein from lamin A; (ii) allosterically activating MKP3, PARP-1, and topoisomerase IIa; and (iii) repressing transcription of interferon-induced genes by binding directly to promoters	K72	Rodriguez, J. et al. (2010). J Cell Biol 191, 967–979. Camps, M. et al. (1998) Science 280, 1262–1265. Cohen-Armon, M. et al. (2007) Mol Cell 25, 297–308. Hu, S. et al. (2009) Cell 139, 610–622. Shapiro, P. S. et al. (1999) Mol. Cell. Biol. 19, 3551–3560.
FAK	Kinase-dead FAK (K454R) is sufficient to promote endothelial cell survival by regulating p21 expression; both kinase-active and kinase-dead FAK recruit paxillin to the plasma membrane to stimulate JNK2	K72	Zhao, X. et al. (2010) J. Cell Biol. 189, 955–965. Igishi, T. et al. (1999) J Biol Chem 274, 30738–30746.
FGFR3	Both kinase-active and kinase-dead (K508M) FGFR3 promote degradation of BMPR1a and BMPR1b by enhancing their association with Smurf1	K72	Qi, H.et al. (2014). Biochim Biophys Acta 1843, 1237–1247.
Fyn	Knockdown but not small molecule inhibition of Fyn kinase significantly decreases late stage T cell adhesion	N/A	Chapman, N.M. et al. (2012). PLoS ONE 7, e53011.
GRK2	Both kinase-active and kinase-dead (K220R) GRK2 suppress endothelin receptor signaling	K72	Freedman, N.J. et al. (1997). J. Biol. Chem. 272, 17734–17743.
IGF1R	IGF1R promotes phosphorylation of ERK1/2 and p38 even when its kinase is inactivated with a small molecule inhibitor or through a kinase-inactivating mutations (Y1131F/Y1135F/Y1136F)	T172	Perrault, R. et al. (2011). Cellular Signalling 23, 739–746.
IKKα	Both kinase-active and kinase-dead (K44M) IKKα are capable of inducing keratinocyte differentiation	K72	Hu, Y. et al. (2001). Nature 410, 710–714.
IKKβ	Both kinase-active and kinase-dead (K44A) IKKβ promote activation of Akt and localization of Akt to the membrane	K72	Ashida, N. et al. (2011). Nat Comms 2, 318.
INSR	Both kinase-active and kinase-dead (K1030R) Insulin Receptor (INSR) induce apoptosis in the absence of ligand and activate the phosphatase PHLPP1 in response to insulin	K72	Boucher, J.(2010). Science Signaling 3, ra87–ra87. Zhang, M. & Riedel, H. (2009). J. Cell. Biochem. 107, 65–75.
Ire1	Binding of ADP or type I inhibitors to the Ire1 kinase domain is sufficient to promote oligomerization and activation of the RNase domain	N/A	Papa, F.R. et al. (2003). Science 302, 1533–1537.
Lck	Studies using Lck mutants known to adopt different conformational states were used to demonstrate that the	K72	Rossy, J. et al. (2012). Nat Immunol 14, 82–89.

Kinase	Non-catalytic function(s)	Corresponding residue(s) in the active site of PKA[a]	References
	conformation of Lck and not its activity regulates Lck clustering in early T cell signaling		
LRRK2	Mice deficient in LRRK2, but not mice expressing kinase dead LRRK2 (D1994S), develop early onset pathophysiological changes in the lung	D166	Herzig, M.C. et al. (2011). Hum. Mol. Genet. 20, 4209–4223.
Lyn	Both kinase-active and kinase-dead (K275L) Lyn inhibit BCR-mediated activation of PKC	K72	Katsuta, H. et al. (1998). J Immunol 160, 1547–1551.
MARK2	Expression of kinase-dead (T208A/S212A) MARK2 is sufficient to rescue the multipolar to bipolar transition in neurons in which MARK2 has been knocked out	T172	Sapir, T. et al. (2008). J. Neurosci. 28, 5710–5720.
MSK2	Both kinase-active and kinase-dead (D179A/D551A) MSK2 interact with and stimulate autophosphorylation of PKR; it remains unclear whether one or both kinase domains of MSK2 are involved in this interaction	D184	Kang, J.-I., and Ahn, B.-Y. (2011). Biochem Biophys Res Comm 407, 248–253.
MST1	Both kinase-active and kinase-dead (K59R, T183A) MST1 bind to and inhibit androgen receptor	K72, T172	Cinar, B. et al. (2011). Cancer Research 71, 4303–4313.
mTOR	Both kinase-active and kinase-dead (D2357E) rapamycin-resistant mTOR variants rescue myogenesis in C2C12 cells treated with rapamycin	D184	Erbay, E. & Chen, J. (2001). J. Biol. Chem. 276, 36079–36082.
p38α	Knockdown of p38α blocks cell proliferation, whereas inhibition with small molecules has no effect on the role of p38α in cell cycle progression	N/A	Fan, L. et al. (2005). Cell Cycle 4, 1616–1624.
PAK1	Both kinase-active and kinase-dead (K298R) PAK1 bind to Akt and PDK1 to promote recruitment of Akt to the membrane and phosphorylation of Akt by PDK1	K72	Higuchi, M. et al. (2008). Nat Cell Biol 10, 1356–1364.
PAK2	Both kinase-active and kinase-dead (K278R) PAK2 interact with βpix to regulate spindle orientation	K72	Mitsushima, M. et al. (2009). Mol. Cell. Biol 29, 2816–2827.
PAK4	Both kinase-active and kinase-dead (K350M) PAK4 inhibit TNFα-induced apoptosis	K72	Gnesutta, N., and Minden, A. (2003). Mol Cell Biol 23, 7838–7848.
PERK	Catalytically inactivated PERK (through binding of an inhibitor) can allosterically activate another PERK kinase through dimerization	N/A	Dey, M. et al. (2014). J Biol Chem 289, 5747–5757. Mendez, A.S. et al. (2015) eLife 4, e05434
PKR	Dimerization of the PKR kinase domain seems sufficient to allosterically active PKR kinase activity as observed for RAF and PERK kinases	N/A	Dey, M. et al. (2014). J Biol Chem 289, 5747–5757.
Pyk2	Knockdown but not inhibition of Fyn kinase with a small molecule significantly decreases late stage T cell adhesion	N/A	Chapman, N.M. et al. (2012). PLoS ONE 7, e53011.
RIPK1	RIPK1-deficient mice die shortly after birth due to extensive apoptosis, whereas mice expressing a kinase-dead RIPK1 K45A or D138N mutant are viable	K72, D166	Berger, S.B. et al. (2014). J. Immunol. 192, 5476–5480. Newton, K. et al. (2014). Science 343, 1357–1360.
RIPK3	Mice lacking RIPK3 or expressing a kinase-dead RIPK3 K51A mutant are healthy, but inactivation of RIPK3 through a kinase-dead D161N mutation is lethal. The D161N mutation or inhibition with small molecules enhances the ability of RIPK3 to assemble an apoptosis-inducing complex	K72, D166	Mandal, P. et al. (2014). Mol. Cell.56, 481–495. Newton, K. et al. (2014). Science 343, 1357–1360.
TAK1	Both kinase-active and kinase-dead (K63W) TAK1 are capable of acting as adaptors for ubiquitin ligases to promote degradation of Bcl10	K72	Moreno-García, M.E. et al. (2013). Mol. Cell. Biol. 33, 1149–1163.
TrkA	Both kinase-active and kinase-dead (K538A) TrkA promote neuronal cell death	K72	Nikoletopoulou, V. et al. (2010). Nature 467, 59–63.

Kinase	Non-catalytic function(s)	Corresponding residue(s) in the active site of PKA[a]	References
TGFβRI	A kinase-dead (G217E, K232R) mutant of TGFβRI can complement an activation-defective mutant (G261E, G322D) to form a functional signaling complex	K72	Weis-Garcia, F. & Massagué, J. (1996) EMBO J. 15, 276–289.
WNK2	Both kinase-active and kinase-dead (K207A, S352A/ S356A) WNK2 inhibit growth of glioma cells	K72, T172	Hong, C. et al. (2007). Proc. Natl. Acad. Sci. U.S.a. 104, 10974–10979.
ZAP70	Inactivation of ZAP70 with a small molecule inhibitor has no effect on its ability to activate Rap1 GTPase	N/A	Au-Yeung, B.B. et al. (2010) Nat Immunol. 11, 1085–1092.

[a]Indicates the residues in the active site of Protein Kinase A (PKA) that correspond to those mutated to generate kinase-dead alleles for cases where kinase-inactivating mutations were used to identify non-catalytic functions.