Kinases in motion: impact of protein and small molecule interactions on kinase conformations

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Abstract Protein kinases act as central molecular switches in the control of cellular functions. Alterations in the regulation and function of protein kinases may provoke diseases including cancer. In this study we investigate the conformational states of such disease-associated kinases using the high sensitivity of the Kinase Conformation (KinCon)-reporter system. We first tracked BRAF-kinase activity conformation changes upon melanoma drug binding. Second, we also use the KinCon reporter technology to examine the impact of regulatory protein interactions on LKB1-kinase tumor suppressor functions. Third, we explore the conformational dynamics of RIP-kinases in response to TNF-pathway activation and small molecule interactions. Finally, we show that CDK4/6 interactions with regulatory proteins alter conformations which remain unaffected in the presence of clinically applied inhibitors. Apart from its predictive value, the KinCon technology helps identify cellular factors that impact drug efficacies. The understanding of the dynamics of full-length protein kinases when interacting with small molecule inhibitors or regulatory proteins is crucial for designing more effective therapeutic strategies.

Introduction

The human kinome is encoded by more than 500 genes which lead to the synthesis of functionally diverse kinases. Kinases are different in their domain composition but all contain a structurally conserved phosphotransferase domain (***Manning*** (***2009***), ***Kornev and Taylor*** (***2010***)). Conventionally, protein kinases function as enzymes that enable the transfer of a phosphate group from ATP to defined amino acids of a target protein. Protein kinases regulate various aspects of cellular functions including cell survival, apoptosis, cell division and metabolism via reversible and tightly regulated phosphorylations (Figure 1A) (***Bhullar et al.*** (***2018***), ***Blume-Jensen and Hunter*** (***2001***), ***Manning et al.*** (***2002***)). In addition to kinase phosphotransferase activities, kinase domains have scaffolding functions (***Shrestha et al.*** (***2020***), ***Reiterer et al.*** (***2014***)).

On the cellular level, kinases act as molecular switches, adopting conformational states that

align with an active (ON) or inactive (OFF) kinase state (***Huse and Kuriyan*** (***2002***), ***Yamaguchi and Hendrickson*** (***1996***), ***Lopez et al.*** (***2019***), ***Feichtner et al.*** (***2022***)). These ON and OFF states of protein kinases signify a switch-like behavior which is governed by a collection of molecular mechanisms (Figure 1A). Funneling the diverse signals underlines the center stage of kinases in signaling pathways, which frequently involve kinase regulation. Thus, one kinase directly modulates the activity level and role of the subsequent kinase in a cascade like structure (***Guo et al.*** (***2020***), ***Avruch et al.*** (***2001***)). This regulation is facilitated by the formation of multi-protein complexes to spatiotemporally control and amplify signal transmission (***Morrison*** (***2001***), ***Pouysségur et al.*** (***2002***)). Figure 1A highlights several factors affecting kinase functions. Selected modes of kinase regulation which are of relevance for the presented study are listed below:

First, the activity of a kinase can be altered by post-translational modifications (PTMs) of selected amino acids. PTMs alter kinase characteristics like its activity status, its location within the cell, rate of degradation, and its associations with other proteins (***Chou*** (***2020***), ***Cohen*** (***2000***), ***Deribe et al.*** (***2010***)).

Second, the scaffolding functions of kinase domains accompany the process of phosphotransferase reactions. These play central roles in the activation and deactivation process of interacting kinase protomers. This feature is key for pseudokinase activities, for relaying signaling inputs without catalytic functions (***Weinlich and Green*** (***2014***), ***Morrison and Davis*** (***2003***), ***Boudeau et al.*** (***2006***)).

Third, kinase functions depend on decisive regulatory protein interdependencies, for which several modes of regulation have been described. Intramolecular auto-inhibitory modules (AIM) alter kinase activity states by reducing the accessibility of the substrate protein for the subsequent kiss-and-run phosphotransferase reaction (***Mayrhofer et al.*** (***2020***), ***Pufall and Graves*** (***2002***), ***Xu et al.*** (***2002***)). In some cases, kinase domain activities are controlled by interacting with substrate-like sequences. Pseudo-substrate stretches bind to the catalytic cleft of the kinase and hinder the phosphorylation of the substrate. This binding to the catalytic site is altered in response to input signals and manifested in kinase conformation changes, which in turn coordinate protein kinase activation cycles (***Schmitt et al.*** (***2022***), ***Kemp et al.*** (***1994***)). Besides intramolecular inhibition, activating and inactivating regulatory protein interactions have been described for prototypical kinases such as PKA and CDKs (***Boudeau et al.*** (***2003***), ***Taylor et al.*** (***2005***)).

Fourth, kinase regulation is strongly dependent on the expression pattern and how the protein is stabilized in regard to the cell fate within the respective cell system or compartment (***Capra et al.*** (***2006***)).

Fifth, regulatory protein interactions of kinases depend on small molecule interactions. Besides different types of second messengers (e.a. Ca^{2+}, IP3, cAMP) (***Newton et al.*** (***2016***), ***Kasai and Petersen*** (***1994***)) a collection of metabolites and ions contribute in orchestrating cellular kinase functions (***Ramms et al.*** (***2021***)).

Alterations of the ON and OFF modes of regulating kinases are also pertinent to kinase-related disorders. Deregulation of kinase functions is associated with the development of numerous diseases, such as cancer, inflammatory, infectious and degenerative conditions (***Shchemelinin et al.*** (***2006***), ***Köstler and Zielinski*** (***2015***), ***Ferguson and Gray*** (***2018***)). Kinase malfunctions result from gene mutations, deletions, fusions or increased or aberrant expressions. These mechanisms result in gain or loss of function of the involved kinase pathways, thus driving disease etiology and progression (***Ochoa et al.*** (***2018***), ***Van et al.*** (***2021***), ***Cicenas et al.*** (***2018***)).

For this technical report, we have incorporated disease-relevant full-length phosphotransferases which exhibit different modes of regulation into the kinase conformation (KinCon) reporter system. The KinCon reporter platform is a *Renilla* luciferase (*R*Luc) based protein fragment complementation assay, which fuses two fragments of *R*Luc to the N and the C terminus of a full-length kinase. This reporter can be used to track conformational changes of kinases due to mutations, PTMs, PPIs, or inhibitor binding. We discuss the KinCon reporter principle at the beginning of the results section. The kinases listed below are characterized by distinctive modes of regulation, are viable

targets for inhibition, or are so far difficult to assess directly through traditional biochemical measurements: The liver kinase B1 (LKB1, STK11), the receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and the cyclin-dependent kinases 4 and 6 (CDK4/6).

LKB1 is active as trimeric cytoplasmic protein complex. It is the upstream regulator of the AMP-activated protein kinase (AMPK), which is the macromolecular key kinase complex in regulating the cellular energy metabolism (***Rodríguez et al.*** (***2021***)). LKB1 promotes AMPK signaling by forming an heterotrimeric complex with the pseudokinase STE20-related adaptor alpha (STRADα) and the scaffolding protein Mouse protein-25 (MO25) through phosphorylation and thus activation of cytoplasmic AMPK (***Boudeau et al.*** (***2004***), ***Narbonne et al.*** (***2010***)). Mutations in LKB1 can lead to the autosomal dominant disease Peutz-Jeghers syndrome (PJS) (***Mehenni et al.*** (***1998***), ***Beggs et al.*** (***2010***)). Inactivating mutations of LKB1 are frequently observed in non-small-cell lung cancer (NSCLC), cervical carcinoma and malignant melanoma (***Wingo et al.*** (***2009***), ***Ndembe et al.*** (***2022***)).

RIPK1 acts as a central stress sensor to control cell survival, inflammation and cell death signaling (***Clucas and Meier*** (***2023***)). Deregulation of RIPK1 and RIPK3-involved signaling cascades have been linked to inflammatory bowel diseases, rheumatoid arthritis, autoimmune conditions and neuroinflammatory diseases such as Alzheimer's and Parkinson's disease (***Martens et al.*** (***2020***), ***Li et al.*** (***2019***), ***Speir et al.*** (***2021***), ***Clucas and Meier*** (***2023***)). This is believed to be related to deregulations of both catalytic and scaffolding functions. Thus, a collection of small molecule blockers has been identified to interfere with RIPK1 signaling and function. It's intriguing to note that RIPK1 and RIPK3 may share a similar mechanism of action with BRAF, as their structural arrangements and the dimerization of their kinase domains bear resemblance (***Raju et al.*** (***2018***)). In addition to this, auto-phosphorylation of RIPK1 and RIPK3 represents a central regulatory element, with similar ties to BRAF (***Laurien et al.*** (***2020***)).

Unlike the auto-inhibitory mechanisms of kinase regulation, some kinases dynamically engage with activating and deactivating polypeptides (***Zhang et al.*** (***2021***)). One of the best studied examples is the interaction of regulatory proteins with the catalytic subunits of the cAMP-controlled serine/threonine protein kinase A (PKA) (***Taylor et al.*** (***2012***), ***Zhang et al.*** (***2020***)). In a similar manner CDKs form active and inactive protein complexes to directly promote the cell cycle (***Goel et al.*** (***2018***)). The central role of CDK4/6 lies in regulating cell cycle progression by phosphorylating and activating the key substrate retinoblastoma protein (Rb) that promotes G1 to S phase transition. On the molecular level this is controlled by complex formation of CDK4 and CDK6 with regulatory polypeptides (***James et al.*** (***2008***)). Binding to $p16^{INK4a}$, one of the most frequently mutated tumor suppressor proteins, blocks CDK4/6 functions (***Quelle et al.*** (***1997***)). Cancer mutations in $p16^{INK4a}$ counteract this. As a result, kinase activating cyclinD proteins bind to CDK4/6 to promote carcinogenesis (***VanArsdale et al.*** (***2015***)). Thus, CDK4/6 inhibitors (CDK4/6i) found the way into the clinic, in particular for treating breast cancer patients (***Nebenfuehr et al.*** (***2020***),***Yu et al.*** (***2006***). The development of drug resistance upon CDK4/6 inhibitors therapies underscores the need for personalized treatments that consider the patient's genetic profile and the underlying alterations of CDK4/6 complexes present in their cancer cells (***Álvarez-Fernández and Malumbres*** (***2020***), ***Knudsen and Witkiewicz*** (***2017***)).

Various cellular mechanisms are employed to control the molecular switch-like-behavior of protein kinases to temporally lock them into either an active or inactive conformation. These mechanisms include the PTMs of specific residues, the binding of regulatory proteins or co-factors, and allosteric changes induced by ligands (***Bhullar et al.*** (***2018***), ***Taylor et al.*** (***2021***), ***Newton*** (***2001***)). This precisely controlled mode of action can be hindered, amongst others by patient-specific mutations or modified by bio-active small molecules (Figure 1A). Conventional methods often fall short in capturing the dynamic functioning of kinases within their native cellular environments (***Klaeger et al.*** (***2017***), ***Croce et al.*** (***2019***)). This makes innovative biotech approaches essential for a comprehensive understanding of their roles in the cell. Here we show that genetically encoded KinCon reporters are extendable to many more kinases, to enable systematic monitoring of cellular kinase activity states in live cells. In addition to its predictive capabilities, the KinCon technology serves

as a valuable tool for uncovering cellular factors influencing drug efficacy (***Mayrhofer et al.*** (***2020***), ***Fleischmann et al.*** (***2023***), ***Röck et al.*** (***2019***)). Such insights into the molecular dynamics of kinases upon interactions with small molecule inhibitors or regulatory proteins is necessary for the design of more effective therapeutic strategies.

Results

Kinases act as molecular switches to integrate, amplify, restrict and/or relay signal propagation in spatiotemporal fashion and in cell-type specific manner. The precise coordination of usually oscillating kinase activities is a prerequisite for proper signal transmission (***Taylor et al.*** (***2012***), ***Pan and Heitman*** (***2002***)). When investigating pathological kinase functions for therapeutic purposes, it is crucial to take the cellular elements that influence kinase activities into account. In figure 1A we list a collection of factors which affect kinase activity states. It is thus essential to closely monitor the physiological and pathophysiological kinases functions of activation and deactivation in intact cell settings in the presence and absence of patient mutation and drug exposure.

One cell-based technology for studying cellular kinase activity states is the KinCon reporter technology (***Röck et al.*** (***2019***), ***Mayrhofer et al.*** (***2020***), ***Fleischmann et al.*** (***2023***)). It is a highly sensitive assay for tracking kinase activities which is mirrored by the alterations of full-length kinase structures in cells. With in-silico predictions it was projected that more than 200 protein kinases of the human kinome contain cis-regulatory elements. In many cases these sequence stretches may act as AIMs (***Mayrhofer et al.*** (***2020***), ***Yeon et al.*** (***2016***)). In figure 1B we depict exemplary how underlying kinase ON and OFF states can be tracked using KinCon reporter technology (***Enzler et al.*** (***2020***). The read out is based on the molecular motion of the full-length kinase containing diverse cis-regulatory elements such as AIMs. Cell-type specific KinCon measurement allow the testing of the influence of mutations, signaling pathway activation, binary protein interactions and drug binding on the respective reporter dynamics. We have previously applied the technology to gain insights into the functioning of two kinases that belong to the MAPK pathway. In these proof of concept studies, we showed that BRAF and MEK1 KinCon reporters are direct real-time read-outs for kinase activities in intact cell settings caused by mutation and drug treatments (***Röck et al.*** (***2019***), ***Mayrhofer et al.*** (***2020***), ***Fleischmann et al.*** (***2021***), ***Fleischmann et al.*** (***2023***)).

The overall construction principle of the KinCon reporter is modular. For the generation of the genetically encoded KinCon reporter the sequence of the kinase of choice is inserted into the multiple cloning site (MCS) of a mammalian expression construct. The MCS is flanked by the coding regions of a split luminescent protein for cellular over-expression experiments of the encoded hybrid reporter protein (see the KinCon reporter protein domain structure at the top of figure 1C). In many cases it is sufficient to fuse to the N and C terminus of the full-length kinase sequence the two fragments of the respective reporter protein (with intervening flexible linker stretches shown in red, Figure 1B, C).

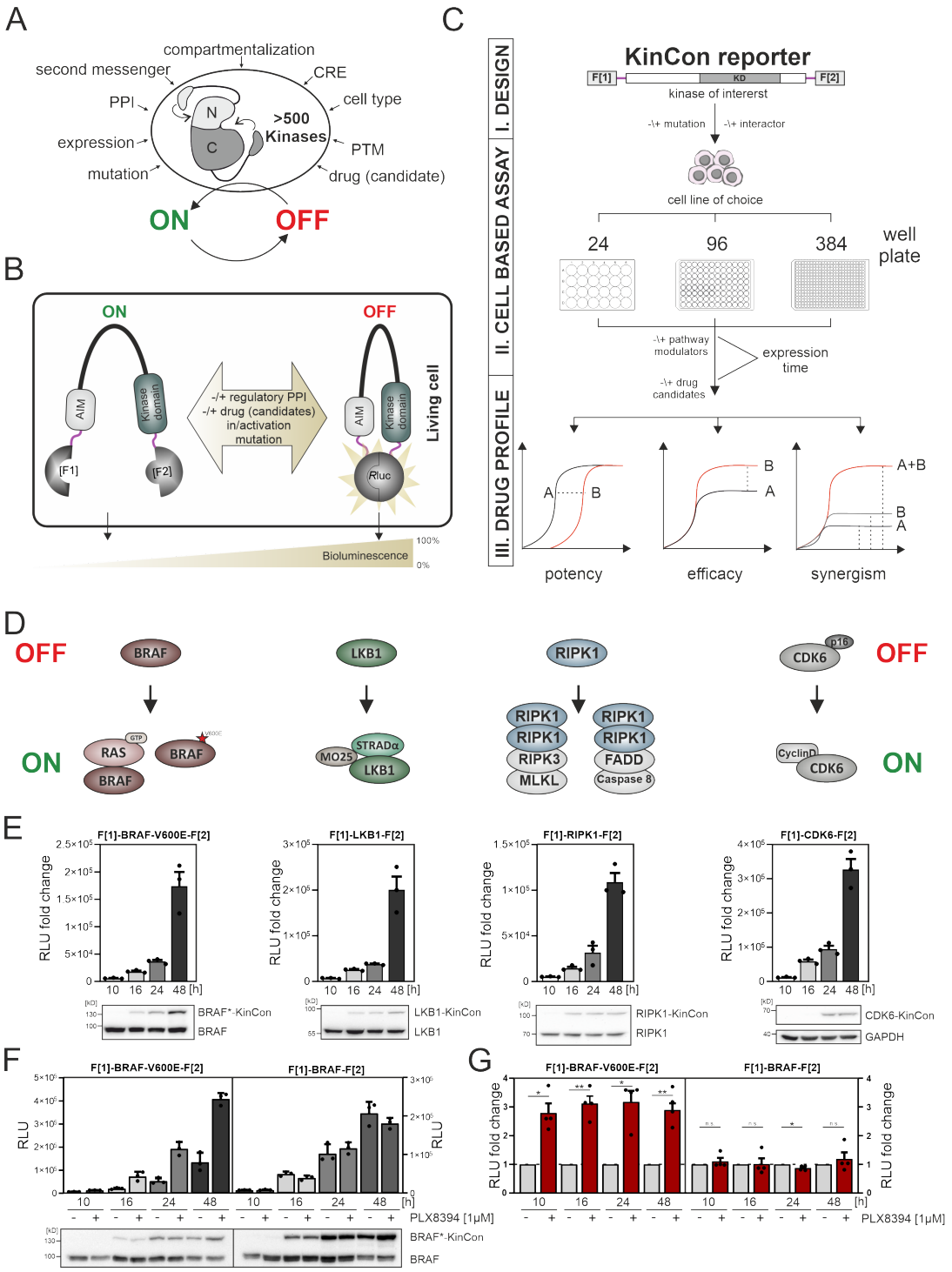
In this study we used the protein-fragment complementation fragments (PCA, -F[1] and -F[2]) of the *Renilla* luciferase (*R*Luc-PCA) (***Stefan et al.*** (***2007***)). The KinCon reporters are constructed to facilitate the intramolecular complementation of appended Rluc PCA fragments. Transient expressions offer the flexibility to analyze different time frames for KinCon reporter expressions and drug candidate exposures, in either low or high throughput format in intact cells (Figure 1C). Besides applying wild-type reporters it allows for the 'personalization' of the sensor set-up by integrating patient-specific mutations, co-expressing regulatory proteins, or making systematic changes to post translational modification sites.

Following KinCon reporter expression along with co-expression of interacting molecules in the appropriate cell plate format, systematic perturbations can be applied. Following addition of the luciferase substrate to cells grown in a mono-layer or in suspension cellular bioluminescence signals are emanating from complemented *R*Luc PCA fragments (Figure 1B, C) (***Röck et al.*** (***2019***), ***Mayrhofer et al.*** (***2020***)). Light recordings and subsequent calculations of time-dependent dosage

variations of bioluminescence signatures of parallel implemented KinCon configurations aid in establishing dose-response curves. These curves are used for discerning pharmacological characteristics such as drug potency, effectiveness of drug candidates, and potential drug synergies (Figure 1C). In order to enhance our understanding of kinase dynamics we selected a group of kinases which activities are altered in different pathological settings. These examples emphasize how mutations, PTMs, PPIs, or kinase drugs induce context-dependent effects on the conformation states of kinases. In figure 1D we present a schematic and simplified depiction of the kinase's ON and OFF conditions for complexes emanating from the kinases BRAF, LKB1, RIPK1 and CDK6. Exemplary, we show how protein complex formation and patient mutations contribute or perturb kinase activation cycles. As starting point, we illustrated the high sensitivity of the reporter system for tracking basal activity conformations of the kinases BRAF-V600E, LKB1, RIPK1, and CDK6 respectively. We showed that transient over-expression of these KinCon reporters for 10h, 16h, 24h or 48h in HEK293T cells delivers consistently of increasing signals for all KinCon reporters (Figure 1E, Figure Supplement 1A). Immunoblotting cell lysates after luminescence measurements showed expression levels of the reporters in the range and below the endogenous expressed kinases (Figure 1E).

Next, we analyzed the BRAF kinase activity conformations using wild-type and mutated KinCon BRAF reporters. The V600E mutation, found primarily in melanoma patients, effectively immobilized BRAF and the respective KinCon reporter in its and opened and active conformation (***Davies et al.*** (***2002***), ***Lavoie et al.*** (***2020***), ***Karoulia et al.*** (***2017***), ***Lito et al.*** (***2013***), ***Röck et al.*** (***2019***)). Previously we have shown that FDA-approved melanoma drugs (Vemurafenib, Encorafenib, Dabrafenib) and one drug candidate from clinical studies (PLX8394) converted the opened BRAF-V600E reporter back to the more closed and thus inactive conformation (***Röck et al.*** (***2019***), ***Mayrhofer et al.*** (***2020***), ***Yao et al.*** (***2019***)). Using this readout, we showed that at expression levels of the BRAF KinCon reporter below the immunoblotting detection limit, one hour of drug exposure exclusively converted BRAF-V600E to the more closed conformation (Figure 1F, G, Figure Supplement 1B). These data underline that at expression levels far below the endogenous kinase, protein activity conformations can be tracked in intact cells. This may represent the more authentic (patho)physiological context and takes the molecular interactions with endogenous factors into consideration.

Next, we adapted the KinCon biosensor technology to investigate the correlation between conformation and activity-regulation of key kinase pathways. We analyzed three different kinase pathways displaying different modes of kinase ON-OFF regulation.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Rluc
Living cell
Bioluminescence
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I. DESIGN
II. CELL BASED ASSAY
III. DRUG PROFILE
KinCon reporter
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kinase of interest
-\+ mutation
-\+ interactor
cell line of choice
24
96
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well plate
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A
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BRAF
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MO25
STRADα
LKB1
RIPK1
RIPK3
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Caspase 8
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CDK6
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F[1]-BRAF-V600E-F[2]
F[1]-LKB1-F[2]
F[1]-RIPK1-F[2]
F[1]-CDK6-F[2]
RLU fold change
10 16 24 48 [h]
BRAF*-KinCon
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LKB1-KinCon
LKB1
RIPK1-KinCon
RIPK1
CDK6-KinCon
GAPDH
F
F[1]-BRAF-V600E-F[2]
F[1]-BRAF-F[2]
RLU
PLX8394 [1μM]
BRAF*-KinCon
BRAF
G
F[1]-BRAF-V600E-F[2]
F[1]-BRAF-F[2]
RLU fold change
PLX8394 [1μM]

Figure 1. Kinase regulation and KinCon reporter technology features. A) Impact of indicated factors/features (e.g. Protein-protein interactions (PPI), post-translational modifications (PTM) cis-regulatory elements (CRE)) on the switch-like behavior of kinases. **B)** Schematic representation of the KinCon reporter technology using the *Renilla* Luciferase (*R*luc) protein-fragment complementation assay (PCA) as it works for kinases such as BRAF which contain autoinhibitory modules (AIM); *R*Luc fragments are N and C terminally fused to the kinase of interest (with interjacent linker in red) and are labeled with F[1] and F[2]. PPIs, drug (candidate) or small molecule binding, mutations and/or PTMs may convert the KinCon reporter into different conformation states. Protein movements are quantified through measuring alterations of bioluminescence signals using either intact cell populations or lysed cells upon *R*Luc substrate addition. **C)** Shown is the workflow for the KinCon reporter construct engineering and analyses using KinCon technology. The kinase gene of interest is inserted into the multiple cloning site of a mammalian expression vector which is flanked by respective PCA fragments (-F[1]-, -F[2]) and separated with interjacent flexible linkers. Transient expression of the genetically encoded reporter in different multi-well formats allows to vary expression levels and define a coherent drug treatment plan. Moreover, it is possible to alter the kinase sequence (mutations) or to co-express or knock-down the kinase, interlinked kinases or proteinogenic regulators of the pathway. After systematic administration of pathway modulating drugs or drug candidates, analyses of KinCon dynamics reveal changes in potency, efficacy, and potential synergistic effects of the tested bioactive small molecules. **D)** Shown is a simplified schematic representation of the activating mechanisms of BRAF, LKB1, RIPK1 and CDK6 complexes (with indication of selected regulators or complex components) engaged in altering OFF (top) or ON (bottom) kinase states. **E)** Representative KinCon experiments of time-dependent expressions of indicated KinCon reporter constructs in HEK293T cells are shown (mean ±SEM). BRAF-V600E, LKB1, RIPK1 and CDK6 KinCon reporters were transiently over-expressed in 24-well format in HEK293T cells for 10h, 16h, 24h and 48h each. Immunoblotting with respective antibodies show expression levels of endogenous and over-expressed kinases. **F)** Impact of 1µM PLX8394 exposure (for 1h) on BRAF and BRAF-V600E KinCon reporters transiently over-expressed for indicated time frames in HEK293T cells is shown. One representative experiment with the respective immunoblots of in total n=4 independent performed experiments is presented. **G)** *R*Luc PCA values have been normalized on the untreated conditions for every time point. The mean ±SEM of PLX8394 exposure on BRAF dynamics of n=4 experiments is shown. RLU, relative light units. Statistical significance for G: One-sample t-test (*p<0.05, **p<0.01, ***p<0.001)

Trimeric LKB1 complexes

Upon heterotrimeric complex formation with the pseudokinase STRADα and the scaffolding protein MO25, the kinase LKB1 contributes to the activation of AMPK by phosphorylation at the position Thr172 (Figure 2A). This activation mechanism acts as a key step in regulating glucose and lipid metabolism in response to cellular energy fluctuations in the cell. Additionally, AMPK has been shown to have a conserved role in cell polarity. (***Shackelford and Shaw*** (***2009***), ***Boudeau et al.*** (***2004***),***Baas et al.*** (***2003***)). It is the pseudokinase domain of STRADα that directly interacts with the kinase domain of LKB1, thus triggering the activation of LKB1's tumor-suppressing phosphotransferase functions (Figure 2B, C). It is assumed that upon ATP binding STRADα occupies an active conformation. In this scenario LKB1 affinities for binding rise and it binds as a pseudosubstrate. MO25 acts as scaffold for the kinase dimer to promote the activated LKB1 conformation state (***Zeqiraj et al.*** (***2009a***), ***Zeqiraj et al.*** (***2009b***)). Further, MO25 binding stabilizes this trimeric cytoplasmic complex (Figure 2A, B) (***Boudeau et al.*** (***2003***), ***Baas et al.*** (***2003***)).

The capacity of STRADα for allosterically modulating LKB1 functions through direct interaction underlines that pseudokinases are more than inert scaffolds. They are involved in modulating interacting enzyme entities (***Rajakulendran and Sicheri*** (***2010***), ***Reiterer et al.*** (***2014***)). This is in line with the common belief that pseudokinases can employ switch-like transitions to regulate signaling networks (***Kung and Jura*** (***2019***), ***Shrestha et al.*** (***2020***)). Given the discovery of LKB1 inactivating mutations in diseases like PJS, NSCLC, and colorectal cancer, there is growing interest in exploring strategies to therapeutically restore the function of mutated LKB1 (***Sanchez-Cespedes*** (***2007***), ***Launonen*** (***2005***), ***Kitajima et al.*** (***2019***)).

In contrast to blockers of kinase functions it is more challenging to identify activator molecules for promoting reactivation of the LKB1-AMPK axis. Thus, we investigated the impact of trimeric complex formation of LKB1:STRADα:MO25 on downstream activity of LKB1. We adopted the KinCon technology for measuring involved kinase conformation states. First, we transiently over-

expressed the three flag-tagged polypeptides LKB1, STRADα and MO25 in HeLa cells. Next, we employed the pT172-AMPK/AMPK ratio as a measure of cellular LKB1 activity, observing the highest increase of LKB1 mediated downstream phosphorylation of AMPKα-T172 following co-expression of both, MO25 and STRADα respectively. Using the chosen cellular setting and transfection protocol with a 1:1:1 ratio of transfected expression constructs, a similar effect was observed when solely over-expressing STRADα. No impact on AMPK phosphorylation was observed when MO25 was over-expressed alone (Figure 2D).

First, we started to investigate the impact of LKB1 and MO25 co-expression on STRADα KinCon dynamics. Notable is the fact that only faint STRADα KinCon signals were detected in the absence of MO25 and LKB1 co-expression. To our surprise we have observed a more than 10-fold elevation of the reporter signals following co-expression of both interacting partners in HEK293T cells (48h of expression, Figure 2E). This data supports the notion that STRADα engages an opened conformation, where C- and N- termini are separated and thus almost no bioluminescence signal can be detected under basal conditions. Upon interaction with LKB1 this conformation shifts to a partially closed intermediate conformation state. The trimeric complex further promotes structure closing. We have observed the same tendency - but to a lower extent - using LKB1 KinCon readouts in the presence and absence of both interacting proteins (Figure 2F).

To validate these findings, we then tested the impact of LKB1-binding deficient STRADα-H231A/F233A (HF) mutant proteins (***Boudeau et al.*** (***2004***)) in co-expression experiments with the LKB1 KinCon reporter. It has previously been reported that this mutation prevents STRADα-LKB1 dimer and greatly hinders STRADα-MO25-LKB1 trimer formation and thereby AMPK activation (***Zeqiraj et al.*** (***2009a***)). We observed that the so-called HF double mutation of STRADα is sufficient to abolish the elevating effect of trimer complex formation (Figure 2G). Therefore, we assume that the active, opened LKB1 conformation is not present upon coexpression of STRADα-HF. This might me due to a lack of onteraction between LKB1 and STRADα-HF. Since both mutations are located at the LKB1/STRADα binding interface, we hypothesize that allosteric communication between LKB1 and STRADα is essential for its function (***Qing et al.*** (***2022***), ***Zeqiraj et al.*** (***2009a***)) (Figure 2H). These findings underline that indeed the trimeric complex formation alters KinCon dynamics. With this reporter technology we monitored the interaction based activities of of LKB1 directly in a living cell setting.

LKB1-loss of function mutations have been identified in a plethora of pathological conditions including autosomal diseases and many different forms of cancers (***Sanchez-Cespedes*** (***2007***), ***Launonen*** (***2005***), ***Molaei et al.*** (***2022***)). Thus, we set out to analyze the impact of patient mutations on alterations of involved kinase conformations (Figure 2C).

In figure 2C we have listed the assumed functions of the tested mutations. Three 'tool' mutations (K48R, R74A and K78I) (***Zubiete-Franco et al.*** (***2019***), ***Qing et al.*** (***2022***), ***Yang et al.*** (***2019***)) and three patient mutations (D176N, D194N and W308C, in red) (***Ui et al.*** (***2014***), ***Al Bakir et al.*** (***2023***), ***Islam et al.*** (***2019***)) were analyzed.

Figure 2H highlights the location of these mutations within LKB1. K78, D176, and D194 are highly conserved residues within the ATP binding pocket and are critical for kinase activity: K78 forms a salt bridge with E98 on the αC-helix which stabilizes a functional active conformation, the catalytic D176 is crucial for phosphoryl transfer, and D194 is part of the DFG motif involved in binding of the Mg^{2+} ion (***Fabbro et al.*** (***2015***), ***Meharena et al.*** (***2016***)). The residue W308 is part of a hydrophobic cluster and thus surrounded by lipophilic residues (***Zeqiraj et al.*** (***2009a***)). R74 is located within the STRADα binding interface, and has been reported to interact with STRADα Q251 (***Zeqiraj et al.*** (***2009a***)), and K48 is a solvent-exposed acetylation site (***Zubiete-Franco et al.*** (***2019***), ***Zeqiraj et al.*** (***2009a***)) located on the back of the N-lobe. Among these mutations, only the W308C mutation prevented the closing of the LKB1 conformation when co-expressed with STRADα and MO25 (Figure 2I). This indicates, that the catalytic site residues are critical for enzymatic activity, but play a less important role in maintaining the LKB1 active conformation in the presence of both STRADα and MO25. The W308C mutation has previously been identified to cause a reduction in

LKB1 stability when compared to the wild-type variant (***Islam et al.*** (***2019***)), suggesting the polar side chain of cysteine likely disrupts the interaction network within the hydrophobic cluster. Additionally, the LKB1 mutant W308C diminishes its catalytic activity (***Islam et al.*** (***2019***), ***Boudeau et al.*** (***2004***), ***Mehenni et al.*** (***1998***)), consistent with the observed disruption of the LKB1 active conformation (Figure 2I). These findings suggest that LKB1-W308C lost its ability to form the heterotrimeric complex, with implications on hindering downstream activation and thus affecting its tumor suppressor functions. Overall these findings underline that KinCon technology can be extended to track the impact of binary protein complexes and related cancer mutations on kinase activity dynamics. Moreover, this data demonstrated that in contrast to the previously published MEK1 and BRAF KinCons (***Fleischmann et al.*** (***2021***), ***Fleischmann et al.*** (***2023***), ***Röck et al.*** (***2019***), ***Mayrhofer et al.*** (***2020***)) the more closed STRADα and LKB1 KinCon conformations represent the more active kinase states.

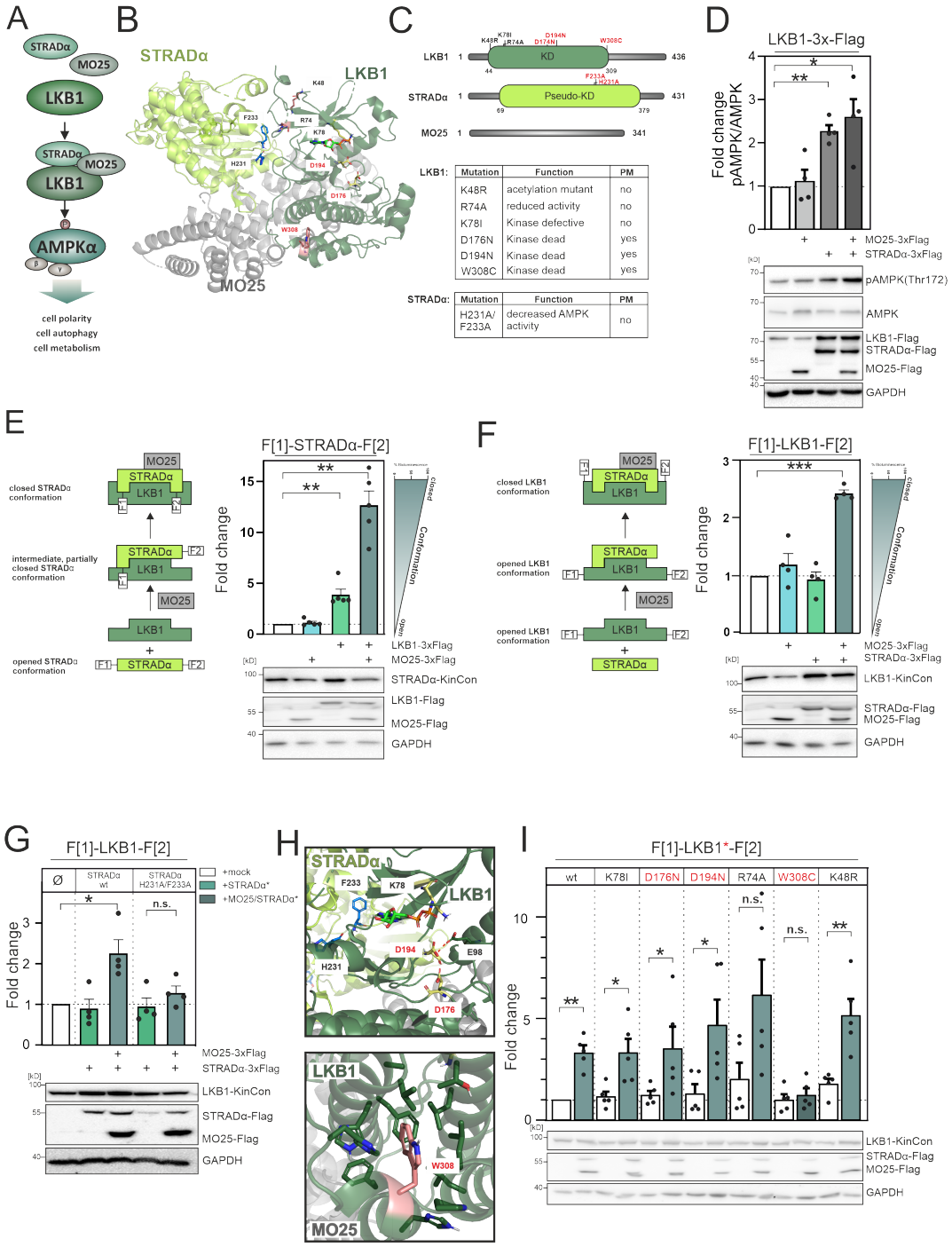
A
STRADα
MO25
LKB1
STRADα
MO25
LKB1
AMPKα
cell polarity
cell autophagy
cell metabolism
B
STRADα
LKB1
MO25
C
LKB1
KD
STRADα
Pseudo-KD
MO25
LKB1:
Mutation | Function | PM
K48R | acetylation mutant | no
R74A | reduced activity | no
K78I | Kinase defective | no
D176N | Kinase dead | yes
D194N | Kinase dead | yes
W308C | Kinase dead | yes
STRADα:
Mutation | Function | PM
H231A/ F233A | decreased AMPK activity | no
D
LKB1-3x-Flag
Fold change pAMPK/AMPK
MO25-3xFlag
STRADα-3xFlag
pAMPK(Thr172)
AMPK
LKB1-Flag
STRADα-Flag
MO25-Flag
GAPDH
E
F[1]-STRADα-F[2]
closed STRADα conformation
intermediate, partially closed STRADα conformation
opened STRADα conformation
Fold change
LKB1-3xFlag
MO25-3xFlag
STRADα-KinCon
LKB1-Flag
MO25-Flag
GAPDH
F
F[1]-LKB1-F[2]
closed LKB1 conformation
opened LKB1 conformation
opened LKB1 conformation
Fold change
MO25-3xFlag
STRADα-3xFlag
LKB1-KinCon
STRADα-Flag
MO25-Flag
GAPDH
G
F[1]-LKB1-F[2]
STRADα wt
STRADα H231A/F233A
+mock
+STRADα*
+MO25/STRADα*
Fold change
MO25-3xFlag
STRADα-3xFlag
LKB1-KinCon
STRADα-Flag
MO25-Flag
GAPDH
H
STRADα
LKB1
MO25
I
F[1]-LKB1*-F[2]
wt
K78I
D176N
D194N
R74A
W308C
K48R
Fold change
LKB1-KinCon
STRADα-Flag
MO25-Flag
GAPDH

Figure 2. LKB1 emanating complexes and mutation-related kinase activity conformations in intact cells. A) Simplified view of the LKB1-complex composition which promotes AMPKα signaling via phosphorylation at position Thr172.**B)** The crystal structure of the LKB1-STRADα-MO25 complex (PDB code 2WTK (***Zeqiraj et al.*** (***2009a***))) represents a snapshot of trimeric complex assembly. The missense mutations we have analyzed are indicated in blue (STRADα) and pale yellow and rose (LKB1). The uncleavable ATP analogue AMP-PNP bound to the catalytic pocket is depicted in light green sticks. **C)** Domain organization of human LKB1, STRADα and MO25 (Accession numbers: Q15831, Q7RTN6, Q9Y376) with indication of the kinase and pseudokinase domains (KD). Shown in red are tested missense mutations. These are summarized in the table together with their origin and assumed functions (PM stands for patient mutation) (***Zubiete-Franco et al.*** (***2019***), ***Qing et al.*** (***2022***), ***Yang et al.*** (***2019***), ***Ui et al.*** (***2014***),***Al Bakir et al.*** (***2023***), ***Islam et al.*** (***2019***), ***Boudeau et al.*** (***2004***)). **D)** Effect of LKB1 co-expression in the presence and absence of the complex partners MO25 and STRADα on the pAMPK/AMPK ratio was determined (HeLa cells, 48h post transfection) (mean ±SEM, n=4 ind. experiments). Representative western blots showing the presence/absence of interaction partners are shown below (3x-Flag expressed constructs indicated as flag). **E)** Illustration of the KinCon reporter setup for STRADα KinCon measurements: Effect of LKB1-STRADα-MO25 complex formation on the STRADα KinCon reporter (HEK293T cells, 48h post transfection). Bioluminescence signals were normalized on reporter expression levels and compared to the STRADα-KinCon signal (mean ±SEM, n=4 ind. experiments). Representative western blots are shown below (3x-Flag expressed constructs indicated as flag). **F)** Illustration of the KinCon reporter setup for LKB1 KinCon measurements: Effect of LKB1-STRADα-MO25 complex formation on the LKB1 KinCon reporter conformation. Bioluminescence signals were measured 48h post transfection, were normalized on protein expression levels and compared to the LKB1-KinCon signal (HEK293T cells, 48h post transfection) (mean ±SEM, n=5 ind. experiments). **G)** LKB1-KinCon measurements upon co-expression of wt and mutated STRADα proteins displaying the binding deficient STRADα mutations H231A/F233A (HF; see binding interface in Figure 2B/H). Bioluminescence signals were normalized on protein expression levels and are displayed relative to the LKB1-KinCon signal (HEK293T cells, 48h post transfection) (mean ±SEM, n=4 ind. experiments). One representative immunoblot is shown below (3x-Flag expressed constructs indicated as flag). **H)** A more detailed depiction of the trimeric complex from figure 2B highlights the localization of mutations conferring altered LKB1 functions. LKB1 residues K78, D176, and D194 (pale yellow sticks) are located within the catalytic cleft and in close proximity to AMP-PNP (light green sticks). Please note that K79 forms a salt bridge with E98 (dark green sticks) on the αC-helix. STRADα residues H231 and F233 (blue sticks) are located within the LKB1 interaction interface. LKB1 residue W308 (rose sticks) is part of a hydrophobic cluster of residues and is surrounded by the lipophilic side chains of neighboring residues (dark green sticks). **I)** Impact of LKB1 missense mutations (three patient mutations D176N, D194N and W308C and three 'non-patient' mutations K48R, R74A, K78I) on KinCon conformation changes upon co-expression of flag-tagged STRADα and MO25. Bioluminescence signals were normalized on protein expression levels and are displayed relative to the LKB1-wt KinCon reporter (HEK293T cells, 48h post transfection) (mean ±SEM, n=4 ind. experiments). Representative immunoblots are shown below (3x-Flag expressed constructs indicated as flag). Statistical significance for D, E, F, G, and I: One-sample t-test (*p<0.05, **p<0.01, ***p<0.001)

Mutations and inhibitors induce RIPK1 conformation changes

RIPK1 is a multi-functional protein and central regulator of cell death, inflammatory processes, and immune responses (Figure 3A). Generally, RIPK1 can promote both, cell survival as well as cell death, highly depending on the regulatory inputs received from the cellular environment (***Degterev et al.*** (***2019***), ***Clucas and Meier*** (***2023***), ***Silke and Meier*** (***2013***)). Genetic alterations of RIPK1 are linked to immune and autoinflammatory diseases. Examples are missense mutations of aspartate 324, which act as gain-of-function mutations of the protein kinase. Patients harboring these mutations suffer from chronic auto-inflammation caused by hypersensitive RIPK1 (***Lalaoui et al.*** (***2020***), ***Tao et al.*** (***2020***)). Small molecule mediated inhibition of RIPK1 kinase activity were shown to counteract the necroptotic phenotype in disease models (***Tao et al.*** (***2020***)). This exemplifies the potential of RIPK1 targeting drugs for the treatment of certain inflammatory diseases.

We established a KinCon reporter platform for monitoring the conformational changes of kinases and pseudo-kinases involved in the necroptosis signaling pathway. Additionally, we examined the effects of binding of allosteric kinase blockers to both wild-type and mutant RIPK1 reporters. We depict the domain organization of RIPK1, RIPK3 and MLKL. We also included analyses of RIPK2 - which is not part of the necroptosis pathway (Figure 3B). We cloned the human versions of these kinase genes into KinCon reporter expression constructs. After transient over-expression

of these kinases in HEK293T cells, we quantified the basal *R*Luc PCA signals and compared these to the basal signal of the BRAF KinCon reporter (Figure 3C).

As starting point, we evaluated whether RIP kinases 1 and 3 activation would manifest in dynamic conformational changes. We promoted necroptosis introducing stimuli by activating the TNFα pathway with TNFα (***Christofferson et al.*** (***2012***)), while preventing repressive RIPK1 ubiquitylation with the SMAC mimetic BV-6 (***Li et al.*** (***2011***)). To prevent the onset of apoptosis and instead induce activation of RIPK3, we blocked caspase 8 activation with the caspase inhibitor zVAD-FMK (***Festjens et al.*** (***2007***)) (as illustrated in figure 3A).

We used HEK293T cells and transiently expressed the RIPK1 and RIPK3 KinCon reporters for 48 hours. Bioluminescence readouts of the KinCon reporters were quantified after indicated timings of TNFα, BV-6, and zVAD-FMK (TBZ) treatments. We observed successive accumulation of the respective KinCon reporter protein levels over the treatment time course (Figure Supplement 2A). We assume that this is caused by blocking of caspase 8, thus preventing the cleavage of both RIPK KinCon sensors. As consequence we normalized the obtained *R*Luc PCA signals on reporter expression levels. In doing so we found that both, the RIPK1 as well as the RIPK3 KinCon reporter showed markedly reduced bioluminescence in the necroptotic cellular environment (Figure 3D). These results support the notion that both kinases shift upon pathway activation to a more opened (ON state) full-length kinase conformation.

Conformational modulation of the RIPK1 KinCon following TNFα pathway activation suggests that the reporter is incorporated into complex I. We were interested to test the degree to which the RIPK1 KinCon represents a functional RIPK1 entity, despite the substantial modifications introduced to the polypeptide sequence. For this purpose, we conducted co-expression experiments of various RIPK1 KinCon and flag-tagged RIPK1 variants in RIPK1-deficient HEK293T cells. We used auto-phosphorylation at S166 as the primary readout for RIPK1 catalytic activity. Our results showed that the RIPK1 KinCon reporter was not capable of auto-phosphorylation (Figure Supplement 3B). In the presence of active flag-tagged RIPK1 however, trans-phosphorylation of the KinCon reporter hybrid proteins was evident (Figure Supplement 3B).

To further deepen our understanding of modes of RIPK1 activation we integrated a collection of missense mutations into the KinCon reporter. The assumed functions have been listed in Figure 3E. At the kinase N terminus a stretch of phosphorylation sites are attributed to kinase activation. Auto-phosphorylations at the positions S14, S15 and S166 have been shown to trigger downstream activation (***Laurien et al.*** (***2020***), ***Wang et al.*** (***2018***)). The localizations are highlighted in the domain organization and the structure depiction of RIPK1 dimers (Figure 3E, F). Additional kinases like IKKα, IKKβ, TBK1, and MK2 have been linked to control the phosphorylation and activity state of RIPK1 (***Dondelinger et al.*** (***2019***), ***Xu et al.*** (***2018***), ***Jaco et al.*** (***2017***)).

Auto-phosphorylation events have been described for the positions S14/15/166 and are critical for RIPK1 activation (***Wang et al.*** (***2021***)) thus, we integrated mutations that mimic protein phosphorylation (S to E amino acid substitutions) and those that are deficient of phosphorylation (S to A amino acid substitutions), aiming to track their impact on molecular motions of RIPK1 to simulate the more activate kinase status. In addition, the kinase deficient mutant K45A was investigated (***Shutinoski et al.*** (***2016***)). Interestingly, K45 in RIPK1 corresponds to K78 in LKB1, highlighting the importance of this lysine residue in the functionality of various kinases. Our KinCon findings unexpectedly demonstrated for RIPK1 that in addition to the kinase-deficient mutant (K45A) all the phosphorylation mimetic (S14/15/166E) and phosphorylation preventing (S14/15/166A) mutations tested led to an opening (ON state) of RIPK1 conformation (Figure 3G).

We also incorporated prevalent patient mutations of RIPK1, observed in distinct inflammatory diseases, into the KinCon biosensor. Besides the patient mutations of the regulatory caspase 8 cleavage site in RIPK1 (D324A/E/H) we integrated the C601Y missense mutation, which causes immunodeficiency and inflammatory bowel disease, into the reporter (***Li et al.*** (***2019***)). Again, we observed an opening of RIPK1 conformations with all mutations tested (Figure 3H). This data supports the idea that in addition to preventing caspase 8 cleavage the mutations at position 324 affect

the activity conformation of RIPK1. The C601Y substitution promotes RIPK1 opening as well.

We next tested RIPK1 KinCon settings with the allosteric RIPK1 modulators GSK547 and Necrostatin (***Puylaert et al.*** (***2022***), ***Cho et al.*** (***2011***)). When bound to RIPK1, both of these compounds occupy a pocket located behind the ATP binding site (Figure 3I, Figure Supplement 2B). The kinase-deficient mutation (K45A) of the KinCon biosensor displayed the strongest opening of the kinase conformation. For this reason we used it to benchmark the effects of Necrostation and GSK547. Application of both compounds for various time points showed that these inhibitors engaged with all the tested RIPK1 KinCon reporters. We observed an increase of bioluminescence for all RIPK1 inhibitors tested, which reflects the closing of the kinase conformation. The inhibitors also affected the phosphorylation mimetic (S14/15/166E) and phosphorylation preventing (S14/15/166A) KinCon reporters to the more closed configuration when compared to the unrelated control, the MEK1 kinase inhibitor Cobimetinib (Figure 3J, Figure Supplement 3D). This data underlines that application of these two kinase blockers alter the conformations and we assume scaffolding functions of active and inactive RIPK1 protomers.

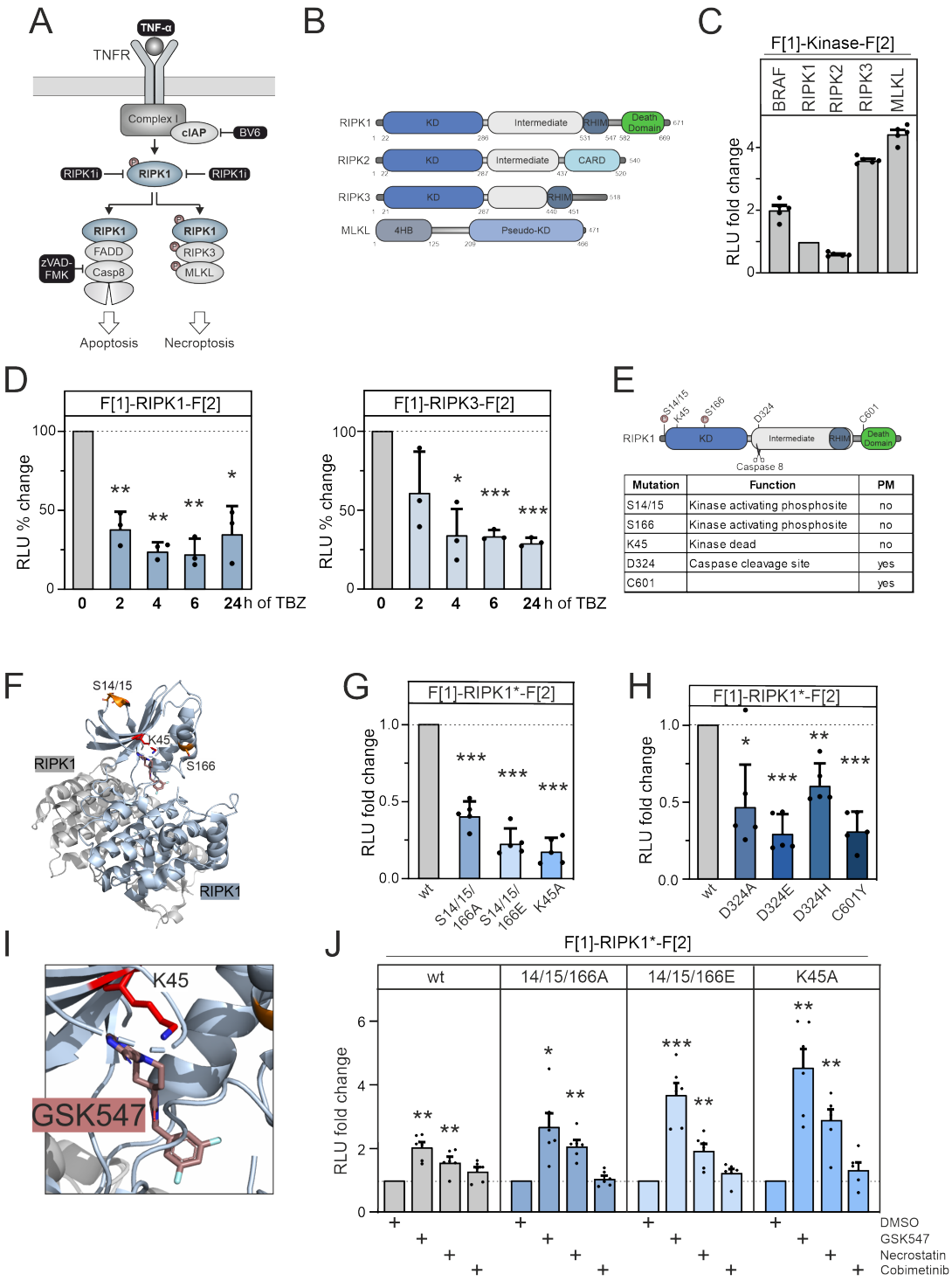
A
TNF-α
TNFR
Complex I
cIAP
BV6
RIPK1i
RIPK1
RIPK1i
RIPK1
FADD
zVAD-FMK
Casp8
RIPK1
RIPK3
MLKL
Apoptosis
Necroptosis
B
RIPK1
KD
Intermediate
RHIM
Death Domain
RIPK2
KD
Intermediate
CARD
RIPK3
KD
RHIM
MLKL
4HB
Pseudo-KD
C
F[1]-Kinase-F[2]
BRAF
RIPK1
RIPK2
RIPK3
MLKL
RLU fold change
D
F[1]-RIPK1-F[2]
RLU % change
0 2 4 6 24 h of TBZ
F[1]-RIPK3-F[2]
RLU % change
0 2 4 6 24 h of TBZ
E
S14/15
K45
S166
D324
C601
RIPK1
KD
Intermediate
RHIM
Death Domain
Caspase 8
Mutation | Function | PM
S14/15 | Kinase activating phosphosite | no
S166 | Kinase activating phosphosite | no
K45 | Kinase dead | no
D324 | Caspase cleavage site | yes
C601 | | yes
F
S14/15
K45
S166
RIPK1
RIPK1
G
F[1]-RIPK1*-F[2]
RLU fold change
wt
S14/15/166A
S14/15/166E
K45A
H
F[1]-RIPK1*-F[2]
RLU fold change
wt
D324A
D324E
D324H
C601Y
I
K45
GSK547
J
F[1]-RIPK1*-F[2]
wt
14/15/166A
14/15/166E
K45A
RLU fold change
DMSO
GSK547
Necrostatin
Cobimetinib

Figure 3. RIPK1 conformation dynamics. A) Simplified chematic representations of the activation pathways for apoptosis and necroptosis are depicted. Highlighted are the compounds for the TBZ (10pg/ml TNFα, 10nM BV-6, and 20nM zVAD-FMK) treatment that induce necroptosis in black. **B)** Domain organization of human RIPK1 (accession number: Q13546), RIPK2 (accession number: O43353), RIPK3 (accession number: Q9Y572) and MLKL (accession number: Q8NB16). **C)** Basal signals of indicated KinCon reporters following transient over-expression in HEK293T cells. Bars represent the RLU fold change relative to RIPK1, which have been normalized on the KinCon protein expression levels (mean ±SEM;, n=5 ind. experiments). **D)** Time-dependent treatments with TBZ of HEK293T cells transiently expressing wt RIPK1 (left) and wt RIPK3 (right) KinCon reporters. Bioluminescence signals were normalized on protein expression levels and are displayed relative to the untreated reporter signals (mean ±SEM, n=3 ind. experiments). **E)** Domain organization of RIPK1 with indication of missense mutation sites in RIPK1 and their suggested mode of regulation. **F)** 3D structure of RIPK1 dimers with functional mutations highlighted (PDB code: 6HHO (***Wang et al.*** (***2018***))). The kinase-bound inhibitor GSK547 is depicted as brown sticks **G)** KinCon reporter signals of functional RIPK1 mutations (S14/15/166A, S14/15/166E, K45A) measured in a HEK293T RIPK1 knock-out cell line. Bioluminescence signals were normalized on KinCon protein expression levels and are displayed relative to wt-RIPK1 (mean ±SEM, n=5 ind. experiments). **H)** KinCon reporter signals of RIPK1 mutations originating from patient loci (D324A, D324E, D324H, C601Y) were measured in HEK293T RIPK1 KO cells. Bioluminescence signals were normalized on KinCon protein expression levels and are displayed relative to wt-RIPK1 (mean ±SEM, n=5 ind. experiments). **I)** 3D structure of RIPK1 with the inhibitor GSK547, which binds to an allosteric site in close proximity to the ATP binding site. **J)** Dynamics of RIPK1 reporters displaying indicated mutations (described in G) upon exposure to the two RIPK1i (GSK547 and Necrostatin 1μM), and the MEKi Cobimetinib (1μM, control experiment) or DMSO for 1h. Bars represent the RLU fold-change relative to the DMSO control of the respective reporter (mean ±SEM, n=6 ind. experiments, HEK293T RIPK1 KO). Statistical significance for C, D, G, H and J: One-sample t-test (*p<0.05, **p<0.01, ***p<0.001)

CDK4 and CDK6 interactions and conformations

CDK4 and CDK6 exhibit proto-oncogenic properties that rely on the presence and interaction with regulatory proteins (***Scheiblecker et al.*** (***2020***), ***Eferl and Wagner*** (***2003***)). Further, CDK6 functions as a chromatin-bound factor and transcriptional regulator, thereby promoting the initiation of tumorigenesis (***Kollmann et al.*** (***2013***), ***Semczuk and Jakowicki*** (***2004***), ***Kollmann and Sexl*** (***2013***)). These activities are modulated by its molecular interaction partners, particularly cyclins and proteinogenic inhibitors. CDKs are protein kinases that regulate a plethora of processes, most importantly cell cycle progression (***Goel et al.*** (***2018***)). On the molecular level CDK4/6 control phosphorylation of the retinoblastoma protein (Rb) which acts as a transcriptional corepressor. This phosphorylation event leads to the dissociation of the Rb-E2F complex, enabling the transcription of target genes that facilitate cell cycle progression (***Giacinti and Giordano*** (***2006***)). To activate CDK4/6 they form a complex with D-type cyclins (***Gao et al.*** (***2020***)). The phosphotransferase function of CDK4/6 is inhibited by a number of different proteins, including the INK4 family of proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) and members from the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$) (Figure 4A) (***Lin et al.*** (***2001***)). Over 80% of all human cancers (e.g. breast, melanoma and pancreatic cancer) exhibit alterations of different kind in the CDK4/6-cyclinD-INK4-Rb cascade (***Ortega et al.*** (***2002***)). Deregulation is caused by alterations of protein expression levels and/or by mutations within the crucial regulatory proteins. Prime example is the tumor suppressor protein $p16^{INK4a}$ which is one of the most frequently mutated genes in human cancers (***Romagosa et al.*** (***2011***)), ***Liggett Jr and Sidransky*** (***1998***)).

The CDK4/6-cyclinD protein complex has been found to be hyperactivated in many cancers and therefore promote tumor growth (***Choi et al.*** (***2012***)). This implies that CDK4/6 serves as a significant therapeutic target, and chemical inhibitors have been developed to target CDK4/6 phosphotransferase activities. Efficient CDK4/6 inhibitors such as Palbocilicb, Ribociclib and Abemacilib are currently in clinical use against breast cancer (***Otto and Sicinski*** (***2017***)). A major drawback of these therapies is the emergence of drug resistance (***Fassl et al.*** (***2022***)). The development of precision medicine oriented polypharmacology therapies is one strategy to avoid the advent of such underlying resistance mechanisms (***Álvarez-Fernández and Malumbres*** (***2020***), ***Yang et al.*** (***2017***)). In this context it needs to be noted that CDK6 possesses a kinase independent function as nuclear tran-

scriptional regulator (***Kollmann et al.*** (***2013***), ***Kollmann and Sexl*** (***2013***), ***Scheicher et al.*** (***2015***)). This involves feedback loops involving STAT3 and cyclinD regulations of p16^{INK4a} expression (***Kollmann et al.*** (***2013***)).

On the molecular level CDK4/6 activity states depend on the formation of PPIs with cyclinD or p16^{INK4a}, wherein cyclinD activates them, while p16^{INK4a} inhibits their activity of promoting Rb phosphorylation (Figure 4A). Distinct to the other kinases tested here, the CDK protein coding sequences are composed almost exclusively of the kinase domain with short additional stretches at the N and C termini (Figure 4B). In contrast to BRAF and RIPK1, the regulatory protein motifs are encoded by separate polypeptides. In the following, we tested the binary interaction of p16 with CDK4 and CDK6. Structures of these complexes have been described. It is evident that substitution of R at the position 31 of CDK6 with C (R31C) alters the binding affinities. CDK6-R31 is a crucial amino acid in the binding interface with p16^{INK4a}. It forms two ionic interactions with p16^{INK4a} residues D74 and D84, which are lost in the R31C mutant (Figure 4C). Experimental data further supports this notion (***Rodríguez-Díez et al.*** (***2014***)). We included the respective mutations by generating CDK6-R31C and also the corresponding R24C expression construct in CDK4 (CDK4-R24C). We conceived a work program with PPI and kinase conformation reporters to elucidate the mechanism of dimerization in relation to drug binding.

We started analyzing binary protein interaction of p16 with CDK4 and CDK6. We fused the two *R*Luc fragments that are used in the KinCon reporter to two different proteins, in this case CDK4 or CDK6 and p16^{ink4a}. When two proteins interact, the two Rluc PCA fragments form a complemented luciferase and an increased light signal can be detected (Figure 4D, left). Originally the underlying technology had been developed for the dynamic measurements of regulatory kinase interactions of PKA (***Stefan et al.*** (***2007***)). Besides testing the kinase mutants CDK6-R31C and CDK4-R24C we generated the murine homolog of the p16^{INK4a} displaying the cancer mutation P40L. P40L showed reduced affinities for CDKs and thus no longer inhibits phosphotransferase functions of CDK4/6 (***Yarbrough et al.*** (***1999***)). Upon complex formation of CDKs with p16^{INK4a} the C terminal fused *R*Luc PCA reporter fragments complement and bioluminescence signals can be detected using intact cells transiently expressing the hybrid proteins. After co-expression of indicated constructs for 48 hours the cells were subjected to bioluminescence measurements (Figure 4D, Figure Supplement 1C). Using this cell based PPI reporter we observed a significant reduction of p16^{INK4a}:CDK4/6 complex formation when the respective R residues are mutated to C. Integration of the P40L mutation in p16^{INK4a} prevented complex formation as expected and further validates the reporter system applied (Figure 4D).

Next, we set out to test the impact of these CDK mutations on kinase conformations. We have previously shown that basal CDK4/6 KinCon conformation states can be tracked using the KinCon reporter technology (***Mayrhofer et al.*** (***2020***)). Thus, we evaluated if a reduction of interaction with inhibitory proteins such as p16^{INK4a} alter CDK full length conformations. We transiently overexpressed the wt and mutant CDK4/6 KinCon reporter in HEK293T cells. After 48 hours of expression the cells were subjected to bioluminescence measurement. The results showed that the reduced affinity for p16^{INK4a} binding alters the conformations of both CDKs. CDK4- and CDK6-p16^{INK4a} binding deficient mutants exhibited a more opened conformation, as indicated by a decrease in bioluminescence (Figure 4E). In the following, all KinCon reporters have been subjected to analyzes upon CDK4/6 inhibitor binding (***Yam et al.*** (***2018***)). Again we used HEK293T cells and transiently expressed the wt and mutated KinCon reporters for 48 hours. We then exposed the cells to three clinically applied CDK4/6i at a concentration of 1µM for 3h. Overall we could not detect any major effects of drug exposure on the dynamics of the four CDK KinCon reporters which showed different affinities for p16^{INK4a} binding (Figure 4F). This data underlines that these breast cancer drugs which should bind to the active protein kinase conformation did not induce full length CDK4 and CDK6 conformation changes in the tested cell culture settings.

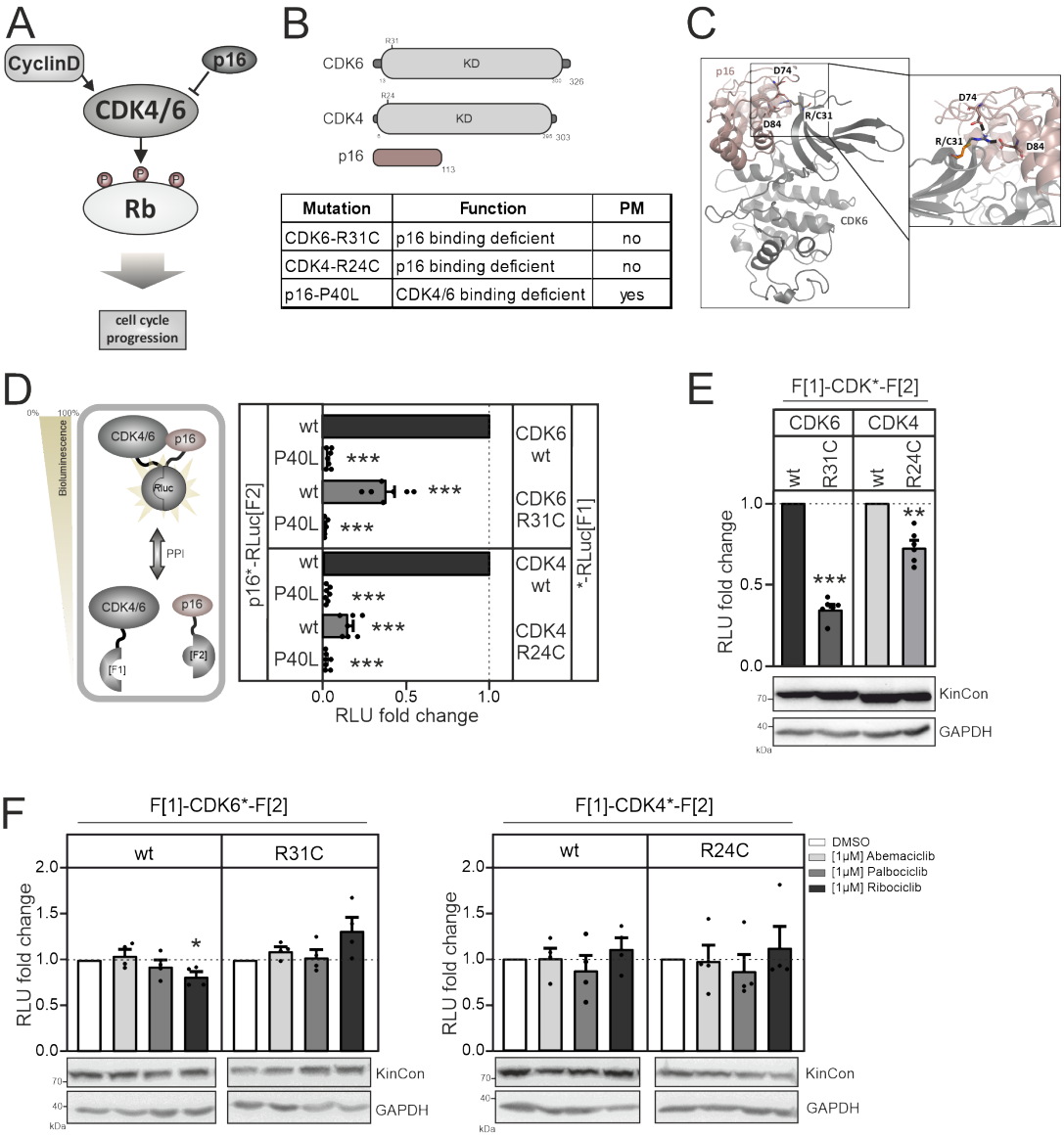

Figure 4. CDK4/6 interactions and conformations. A) Schematic illustration of regulatory CDK4/6 interactions and Rb activation. **B)** Domain organization of CDK4, CDK6 and p16^{INK4a}; tested point mutations are listed below. **C)** 3D structure of CDK6 in complex with p16^{INK4a}. Crucial amino acids involved in the interaction of the two proteins are highlighted. The R31C mutant is depicted in orange. (PDB code 1BI7 (***Russo et al.*** (***1998***))). **D)** PPI analyses of the kinases CDK4 and CDK6 with p16^{INK4a}. Scheme illustrates CDK4/6 hetero-dimer formation with p16^{INK4a} analyzed using a PCA *R*Luc PPI reporter system. PPI induces the complementation of *R*Luc PCA fragments promoting an increase in bioluminescence. HEK293T cells were exposed to measurements after 48h of transient reporter expression. Bars represent the RLU fold change of PPI in relation to wt CDK4/6-p16^{INK4a} (mean ±SEM, n=7 ind. experiments). **E)** Basal signal of CDK4/6 KinCon reporters with indicated mutations are shown. Constructs were transiently expressed for 48h in HEK293T cells. Bars represent the RLU fold change relative to the wt CDK4/6 reporters (mean ±SEM, n=6 ind. experiments). One representative western blot is shown below. **F)** Dynamics of CDK4/6 reporters expressed in HEK293T cells for 48h upon exposure to the three indicated CDK4/6i (1µM) or DMSO for 3h. Bars represent the RLU fold change relative to the DMSO control of the respective reporter (mean ±SEM, n=4 ind. experiments). Statistical significance for D-F: One-sample t-test (*p<0.05, **p<0.01, ***p<0.001)

The inhibitors used in this study, fall into different inhibitor categories (Type I, Type I 1/2, Type II and Type III). The classification of inhibitors is determined by the activation state of the protein

kinase, particularly the positioning of the DFG-Asp (active in, inactive out) and the αC-helix (active in, inactive out) (***Zhang et al.*** (***2009***)). However, type III (allosteric) inhibitors deviate from this pattern. They bind adjacent to the ATP-binding pocket, enabling simultaneous binding of ATP and the inhibitor (***Wu et al.*** (***2015***)). Type I inhibitors selectively bind to the active conformation of the kinase, characterized by DFG in and αC in. Type I 1/2 inhibitors, on the other hand, bind to the inactive state of the kinase, represented by DFG in and αC out. Type II inhibitors bind to the inactive kinase, where DFG is out and αC can be either in or out (***Arter et al.*** (***2022***)) (Figure 5A and B). In this study we have observed alterations of KinCon activity conformations upon changes of protein interactions and through type I 1/2 and type III inhibitor binding (Figure 5A-C), but not type I inhibitors. We provide a summary of the changes in KinCon activity conformations. We have previously shown that for both kinases of the MAPK pathway, MEK1 and BRAF the respective inhibitors affect primarily the active kinase conformation (***Röck et al.*** (***2019***), ***Mayrhofer et al.*** (***2020***), ***Fleischmann et al.*** (***2021***), ***Fleischmann et al.*** (***2023***)). Exemplarily we illustrate that both kinases which are activated through cancer patient mutations (BRAF-V600E and MEK1-K57E) can bind the respective kinase inhibitor, resulting in a change in the activity conformations (Figure 5A, B). The same observation was made with the MEK1 KinCon upon activation through BRAF phosphorylation (***Fleischmann et al.*** (***2023***)). In contrast it was of interest that for all tested RIPK1 activity conformations we observed that the binding of allosteric RIPK1i promoted a closing of the RIPK1 conformation. In Figure 5B we exemplarily illustrate the impact of inhibitor binding on the kinase-dead version RIPK1-K45A. This observation underlines that also inactive RIPK1 complexes are target of drug binding with feasible consequences on kinase scaffolding functions. Kinase activities of CDK4/6 are regulated via defined PPIs (***Nebenfuehr et al.*** (***2020***)). We showed that reducing binding affinities for $p16^{INK4a}$ and related inhibitory proteins triggers the opening of the kinase conformation (Figure 5C). However, using the applied standard protocols we have chosen for all KinCon we have not observed that any of the three clinically applied CDK4/6i tested (such as Abemaciclib) altered the kinase conformation significantly.

In this context we would like to introduce another kinase example displaying regulatory PPI controlling the catalytic kinase protomer. When the regulatory (R) and the catalytic (C) subunit interact a tetrameric PKA holoenzyme complex (R2:C2) is inactive. When second messenger cAMP molecules bind to a R dimer the complex dissociates and catalytic PKA subunits are activated (***Taylor et al.*** (***2013***)). Mutations such as L206R lock the catalytic subunit in its active state. Thus, interactions of regulatory subunits are significantly reduced (***Mayrhofer et al.*** (***2020***), ***Bolger*** (***2022***)). Reduction of binding affinities through the use of the general cAMP elevating agent Forskolin, triggers conformation changes to the more opened state; mechanistically similar to CDK6-R31C. The PKAc-L206R KinCon mutant already engages this opened activity conformation state (Figure 5D). Based on these two examples we assume that binary protein interactions are the prime factors for altering CDK4/6 and PKA-C activity conformations.

In this study we focused on a collection of well-studied kinase pathways. In this regard it is important to note that only a limited number of approved drugs target human kinases. We have depicted this visually by employing an evolutionary-based representation of kinase relationships in the human kinome tree (Figure 5E). The blue squares indicate the kinase branches displaying approved kinase blockers. It is evident that numerous protein kinases lack drug candidates and a more suitable tool for basic research analyses of pathological kinase functions (these kinases are referred to as the 'dark kinome') (***Berginski et al.*** (***2021***), ***Southekal et al.*** (***2021***)). In the kinome tree illustration we have integrated our growing catalog of KinCon reporters (red dots, Figure 5E). This represents an extendable toolbox for gaining first but also deeper insights into the dynamic alterations of kinase activity structures induced by different means of inhibitors, activators or mutations. Systematic implementation of this technology in kinase research programs would assist in advancing our understanding of kinase and pseudokinase regulations and functions.

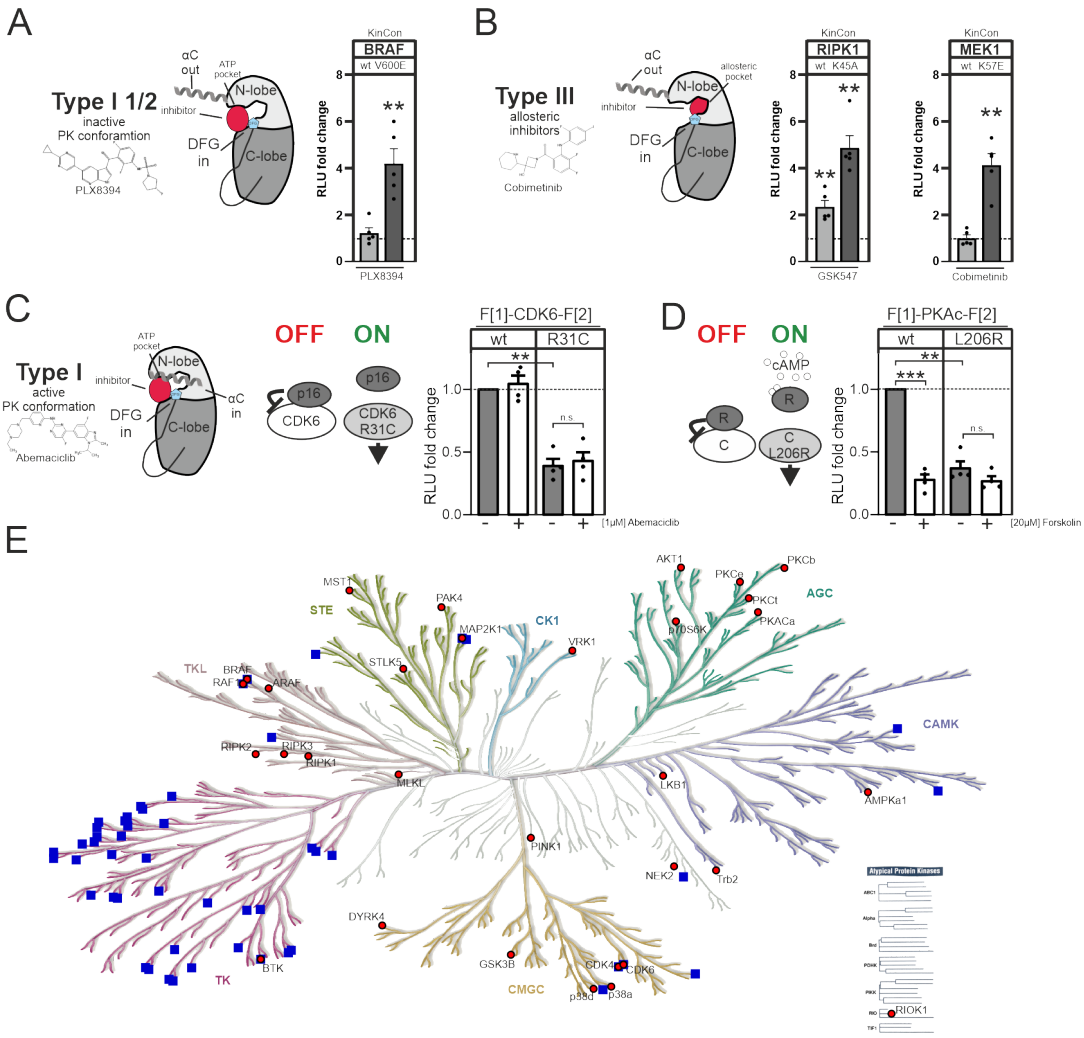

Figure 5. Impact of small bioactive or second messenger molecules and protein interactions on kinase activity conformations. A+B) Depiction of molecular interactions of a type I 1/2 and type III kinase inhibitors with a kinase domains (N and C lobe). Impact of PLX8394, Cobimetinib GSK547 and on wt and mutated versions of BRAF, RIPK1 and MEK1 KinCon reporters. 48h after transfection of HEK293T cells with respective reporter constructs, the cells were treated with the indicated inhibitors for 1h (1μM) followed by Rluc PCA analyses. Bars represent the RLU fold change relative to the DMSO control of the respective reporter (mean ±SEM, n=4 ind. experiments). **C)** Depiction of molecular interactions of a type I kinase inhibitor with a kinase domain (N and C lobe). Impact of p16-deficient binding (R31C mutation) and abemaciclib on indicated CDK6 kinase conformations. 48h after transfection of HEK293T cells with respective reporter constructs, the cells were treated with the indicated inhibitors for 3h (1μM) followed by Rluc PCA analyses. Bars represent the RLU fold change in relation to untreated wt CDK6 (mean ±SEM, n=4 ind. experiments). **D)** Bioluminescence measurement of PKAc wt and L206R KinCon reporters. After 48h of transient expressions in HEK293T cells, the cells were treated with 20μM of Forskolin for 15 min followed by Rluc PCA analyses. Bars represent the RLU fold change in relation to untreated wt PKAc (mean ±SEM, n=4 ind. experiments). **E)** Kinase tree displays kinases for which KinCon reporter have been generated (red dots). The blue squares highlight the kinase for which approved drugs are available. Generated with kinhub.org/kinmap/. Statistical significance for A-D: One-sample t-test (*p<0.05, **p<0.01, ***p<0.001)

Discussion

In the field of kinase-related diseases, mutations of different kind and at different stages of kinase pathways have prompted the development of kinase-specific and small molecule-based inhibitors as primary therapeutic approach (***Ferguson and Gray*** (***2018***)). Despite significant efforts in this

direction, only a small fraction of the entire kinome has been targeted by FDA-approved kinase blockers so far (***Lahiry et al.*** (***2010***), ***Politi et al.*** (***2015***)). Once a kinase inhibitor is introduced into clinical use, it often encounters the development of drug resistance mechanisms over time (***Longley and Johnston*** (***2005***)). Especially in cancer therapy, these facts pose a significant challenge for identifying and applying efficient small molecule based therapies. Hence, it is crucial to gain a more extensive insight into the molecular consequences of kinase patient mutations and the evolving patterns of drug resistance mechanisms. For both endeavors new technologies are indispensable for identifying, accurately monitoring and targeting the dysregulated functions of kinases on the cellular level.

Anticipating and unveiling the cellular mechanism of drug actions may help to develop new treatment concepts by reducing the diminishing of drug efficacies (***Chan and Ginsburg*** (***2011***), ***Goetz and Schork*** (***2018***)). The KinCon technology, introduced here, seeks to address the previously mentioned challenges. It has the potential to become a valuable asset for tracking kinase functions in living cells which are hard to measure solely via phosphotransferase activities. Overall, it offers an innovative solution for understanding kinase activity conformations, which could pave the way for more novel intervention strategies for kinase entities with limited pharmaceutical targeting potential. This relates to the tracking of kinase-scaffold and pseudo-kinase functions.

At the beginning of our studies, we underlined the exceptional sensitivity of the KinCon-reporter system for tracking kinase activity conformations. We demonstrated that at expression levels far below the endogenous kinase of interest we tracked kinase conformations and their alterations upon drug exposure (Figure 1E-G). Tracking enzyme activities at low expression levels is paramount for understanding the details of cellular functions, as many regulatory and spatiotemporal controlled kinase interactions occur under such settings. Such precise measurements are essential for unraveling and targeting pathological kinases functions.

So far, KinCon reporters have been used to assess different conformational states of kinases which are altered by phosphotransferase-activating patient mutations and/or kinase drug binding (***Röck et al.*** (***2019***), ***Mayrhofer et al.*** (***2020***), ***Fleischmann et al.*** (***2021***), ***Fleischmann et al.*** (***2023***)). Here we set out to evaluate consequences of inactivating patient mutations on the complex formation emanating from the kinase LKB1. We aimed to broaden the application spectrum of this reporter technology for investigating how regulatory protein interactions influence the tumor suppressor functions of the kinase LKB1 (***Partanen et al.*** (***2012***)). This holds particular implication for inactivated LKB1, as it suggests that activator compounds could potentially counteract its loss-of-function mutations in cancer. In order to screen or test such re-activator molecules, bioluminescence measurements in living cells would be advantageous, since the complex cellular environment needs to be taken into account. In in-vitro assays, which mostly focus on kinase domain activities, regulatory protein interactions are neglected. We have confirmed the notion that the formation of the trimeric complex between STRADα-LKB1-MO25 was necessary for signal propagation to AMPK. In line with this, the LKB1 and STRADα KinCon bioluminescence signals increased upon complex formation and vice-versa were reduced back to the baseline signal when a complex breaking mutation in the pseudokinase STRADα was introduced (Figure 2G). Further, we showed that tracking structural rearrangements induced by patient or regulatory mutations can be quantified using KinCon technology (Figure 2I). In this setting KinCon measurements of either the kinase (LKB1) or pseudokinase (STRADα) report trimeric complex formation which is pivotal for cytoplasmic kinase pathway activation (Figure 2E, F). In contrast to MEK1 and BRAF KinCons, the closed STRADα and LKB1 KinCon conformations seem to correspond to the more active kinase units.

In the next step, we expanded the application of the KinCon reporter to RIPK. Examining the molecular mechanisms that regulate RIPK1 activity poses a challenge because of the intricate nature of the pathway. Functioning as a molecular scaffold, RIPK1 orchestrates signal transduction by coordinating multiple kinases participating in the NFκB pathway (***Moynagh*** (***2005***), ***Meylan et al.*** (***2004***)). Moreover, as a regulator of cell death, RIPK1 forms a substantial amyloid-like signalosome in conjunction with RIPK3 and various other factors (***Degterev et al.*** (***2019***)). We have shown that

indeed pathway activation at different levels of the cascade can be tracked. Further, all tested mutations converted RIPK1 to the more opened conformation (Figure 3G, H). Both tested and allosterically acting RIPK1i converted the KinCon reporter back to a more closed conformation (Figure 3J, 5B). This consistent impact on the open kinase conformation, indicates a uniform response in altering the enzymes structural state. We assume that this unexpected drug driven transition of inactive and active RIPK1 complexes to more closed kinase conformation may have relevance for scaffold functions of RIPK1. Further, we speculate that it may affect drug efficacies.

The proto-oncogenic characteristics of CDK4 and CDK6 depend on expression levels and their interactions with a collection of regulatory proteins (***Lin et al.*** (***2001***). We applied the KinCon technology for tracking activity states of both kinases. We demonstrated that regulatory CDK4/6 interactions induce conformational changes that remain unaltered when clinically used type I inhibitors bind to them (Figure 4F). However, we would like to state that the involvement of cyclinD interactions will need to be taken into account for follow up studies. In general these observations will be relevant for targeting other kinases which are disease relevant and are thus current or future targets for drug discovery efforts. Overall the details of drug binding and action have been elaborated in last section of the results (Figure 5).

In summary, we have extended the KinCon reporter application spectrum. Besides monitoring conformational changes induced by drugs and drug candidates, it can be applied to accurately track the formation of activated and kinase centered protein complexes as well. Apart from its predictive value, the KinCon technology helps consider or identify cellular factors that impact drug candidate efficacies. This understanding of the molecular kinase dynamics as they interact with small molecule inhibitors or regulatory proteins is crucial for designing more effective therapeutic strategies, especially considering that many kinase pathways have so far remained untargeted. The KinCon technology offers a potential avenue to change this.

Materials & Methods

Expression Constructs

All constructs were cloned into the pcDNA3.1 expression vector containing F[1] and F[2] of the KinCon reporter. Linear DNA fragments were produced by PCR using Q5 DNA Polymerase. After removing the PCR overhangs with AgeI and HpaI (NEB) respectively and subsequent DNA fragment gel extraction, the PCR insert fragments were isolated using the innuPREP DOUBLEpure Kit (Analytik Jena). The DNA Fragments were the ligated using T4 DNA ligase (NEB) and amplified using XL10-gold ultracompetent cells. Plasmids were purified by mini- or midiprep (Quiagen) and verified by Sanger Sequencing (Microsynth/Eurofins).

Cell culture

HEK293T cells were obtained from ATCC (CRL-11268). HEK293T cells were grown in DMEM supplemented with 10% FBS. Transient transfections were performed with Transfectin reagent (Biorad, 1703352). HeLa cells were obtained from ATCC (CCL-2). HeLa cells were grown in DMEM supplemented with 10% FBS. HEK293T RIPK1 knock-out cell lines were provided by Pascal Meier. Transient transfections were performed with JetPRIME DNA and siRNA transfection reagent (Polyplus supplied by VWR, 101000046).

All cells are tested regularly for mycoplasma by PCR using suitable primers and/or Universal Mycoplasma Detection Kit (ATCC, 30-1012K).

Immunoblotting

Cells were lysed in ice-cold RIPA buffer (50mM Tris-HCl pH 7.4, 1% NP-40, 0,25% Na-Deoxycholate, 120mM NaCl, 1mM EDTA, 1mM PMSF, 1µg/mL Leupeptine/ Aprotinin/ Pepstatin , 1mM Na_3VO_4/ $Na_4P_2O_7$/ NAF) and mixed with 5xLaemmli Buffer. After heating to 95°C for 10 minutes, the samples were loaded on 10% Acylamide SDS gels for subsequent electrophoresis. Gels were transferred to

a PDVF membrane (Roth) using either the Trans-Blot SD Semi-Dry Transfer Cell (Bio-rad) or the Mini Trans-Blot Cell (Biorad), blocked in TBS-T with 2,5% BSA for 30 minutes at room temperature and incubated in the primary antibody over night at 4°C. Blots were incubated with secondary antibodies for 1h at room temperature and washed with TBS-T before imaging. Imaging was performed with a FUSION FX (Vilber). Immunoblot images were analyzed using ImageJ (NHI). The signal of the target protein was then normalized on the indicated loading control (e.g GAPDH or Vinculin). To normalize AMPK phosphorylation (pAMPK) levels to those of total AMPK, GAPDH-normalized pAMPK levels were divided by their respective GAPDH-normalized total AMPK levels from the same experiment. Primary antibodies used were the rabbit anti-GAPDH (14C10) (Cell Signaling, 2118), rabbit anti-AMPKα (Thr172) (40H9) (Cell Signaling, 2532), rabbit-anti-Phospho-AMPKα (Thr172) (40H9) (Cell Signaling 2535), rabbit-anti-LKB1 (D60C5) (Cell Signaling 3047), rabbit-anti-Vinculin (Cell Signaling 4650), rabbit-anti-RIP (D94C12) XP (Cell Signaling 3493), rabbit-anti RIP3 (E1Z1D) (Cell Signaling 13526), anti-CDK6 (DCS83) mouse (Cell Signaling 3136S), mouse-anti-FLAG M2 (Sigma Aldrich F3165-1MG), rabbit-anti- Renilla Luciferase (EPR17792) (Abcam ab185926), mouse-anti- Renilla Luciferase clone 1D5.2 (Millipore MAB4410).

Luciferase PPI Assay

PCA was performed by growing HEK293T cells in DMEM supplemented with 10% FBS in a 24-well plate format. PCA analyses was performed similarly as previously described in (***Röck et al.*** (***2019***)). HEK293T cells were grown in DMEM supplemented with 10% FBS. The indicated *R*Luc-tagged constructs, one with *R*Luc[F1] and one with *R*Luc[F2], were transiently over-expressed with TransFectin reagent (Bio-Rad, 1703352) in a 24-well plate format. Fourty-eight hours after transfection the medium was carefully aspirated, the cells were washed once with PBS (1mM Sodium phosphate ph 7,2; 15mM NaCl) and after addition of 150µL of PBS to each well, transferred to a 96-well plate (Grainer 96 F-Bottom) . Bioluminescence was measured after addition of 20µL h-Coelentracine (Nanolight Technology) using the PHERAstar FSX (BMG Labtech) (Measurement start time [s]: 0,2 ; Measurement interval time [s]: 10,00; Optic module LUM plus; Gain: 3600; Focal height [mm] 12,5). Data was evaluated using the MARS Data evaluation Software (BMG Labtech).

Luciferase PCA Assay (KinCon Assay)

HEK293T cells, cultured in StableCell DMEM (Sigma Aldrich), were split into 24-well plates at 90.000c/well and after 24h the indicated plasmids were transfected using TransFectin Lipid reagent (Bio-Rad 1703352) at a total of 50-66ng/well. After 48 hours of Protein expression cells were washed once with PBS (1mM Sodium phosphate ph 7,2; 15mM NaCl) and after addition of 150µL of PBS to each well, transferred to a 96-well plate (Grainer 96 F-Bottom) . Bioluminescence was measured after addition of 20µL h-Coelentracine (Nanolight Technology) using the PHERAstar FSX (BMG Labtech) (Measurement start time [s]: 0,2 ; Measurement interval time [s]: 10,00; Optic module LUM plus; Gain: 3600; Focal height [mm] 12,5). Data was evaluated using the MARS Data evaluation Software (BMG Labtech).

Inhibitors

Inhibitors used were Palbociclib (PD 0332991) (MCE Med Chem Express, HY-50767), Abemaciclib (LY2835219) (MCE Med Chem Express, HY-16297A), Ribociclib (LEE011) (MCE Med Chem Express, HY-15777), PLX8394 (MCE Med Chem Express, HY-18972), GSK-547 (MCE Med Chem Express, HY-114492), Necrostatin 2 racemate (1S; Nec-1S; 7-Cl-O-Nec1) (MCE Med Chem Express, HY-14622A), Cobimetinib (GDC-0973; XL518) (MCE Med Chem Express HY13064), TNFα (MCE Med Chem Express, HY-P7090A), z-VAD(OMe)-FMK (MCE Med Chem Express, HY-16658), BV6 (MCE Med Chem Express, HY-16701).

Statistical analyses

The data were analyzed using GraphPad Prism 8.0. If not indicated otherwise, one-sample t-tests were used to evaluate statistical significance. Values are expressed as the mean ±SEM as indicated. Significance was set at the 95% confidence level and ranked as *p<0.05, **p<0.01, ***p<0.001.

Preparation of structures

The LKB1-STRADα-MO25α (PDB code 2WTK (***Zeqiraj et al.*** (***2009a***))) and CDK6-p16^{INK4a} (PDB code 1BI7 (***Russo et al.*** (***1998***))) complex structures were prepared using the default setting of the Protein Preparation Wizard (***Madhavi Sastry et al.*** (***2013***), ***Schrödinger*** (***2019***)) in Maestro Schrödinger release 2019-4 (Schrödinger Release 2019-4: Maestro, Schrödinger, LLC, New York, NY, 2019). The construct used to generate the LKB1 structure contained the inactivating mutation D194A, intended to prevent Mg^{2+} binding (***Zeqiraj et al.*** (***2009a***)). This mutation was converted back to the wildtype Asp residue using MOE version v2022.02 (Molecular Operating Environment (MOE), 2022.02; Chemical Computing Group ULC, 1010 Sherbrooke St. West, Suite 910, Montreal, QC, Canada, H3A 2R7, 2022). For the calculation of the CDK6 R31C mutant, only the CDK6 residues within 12Å of p16^{INK4a} were retained. The mutation was then generated using Osprey v2.2beta (***Chen et al.*** (***2009***), ***Gainza et al.*** (***2013***)) as described before (***Kaserer and Blagg*** (***2018***)). Figures were generated using PyMOL version 2.5.0 (The PyMOL Molecular Graphics System, Version 2.5.0 Schrödinger, LLC).

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Other Chemistry Niceties

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Conflicts of Interest: Aspects of the present study are the subject of patents and pending patent applications. The founders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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References

Al Bakir M, Huebner A, Martínez-Ruiz C, Grigoriadis K, Watkins TB, Pich O, Moore DA, Veeriah S, Ward S, Laycock J, et al. The evolution of non-small cell lung cancer metastases in TRACERx. Nature. 2023; p. 1–10.

Álvarez-Fernández M, Malumbres M. Mechanisms of sensitivity and resistance to CDK4/6 inhibition. Cancer cell. 2020; 37(4):514–529.

Arter C, Trask L, Ward S, Yeoh S, Bayliss R. Structural features of the protein kinase domain and targeted binding by small-molecule inhibitors. Journal of Biological Chemistry. 2022; 298(8).

Avruch J, Khokhlatchev A, Kyriakis JM, Luo Z, Tzivion G, Vavvas D, Zhang X. Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. Recent progress in hormone research. 2001; 56:127–155.

Baas A, Boudeau J, Sapkota G, Smit L, Medema R, Morrice N, Alessi D, Clevers H. Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD. The EMBO journal. 2003; 22(12):3062–3072.

Beggs A, Latchford AR, Vasen HF, Moslein G, Alonso A, Aretz S, Bertario L, Blanco I, Bülow S, Burn J, et al. Peutz–Jeghers syndrome: a systematic review and recommendations for management. Gut. 2010; 59(7):975–986.

Berginski ME, Moret N, Liu C, Goldfarb D, Sorger PK, Gomez SM. The Dark Kinase Knowledgebase: an online compendium of knowledge and experimental results of understudied kinases. Nucleic acids research. 2021; 49(D1):D529–D535.

Bhullar KS, Lagarón NO, McGowan EM, Parmar I, Jha A, Hubbard BP, Rupasinghe HV. Kinase-targeted cancer therapies: progress, challenges and future directions. Molecular cancer. 2018; 17:1–20.

Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature. 2001; 411(6835):355–365.

Bolger GB. The cAMP-signaling cancers: Clinically-divergent disorders with a common central pathway. Frontiers in Endocrinology. 2022; 13:1024423.

Boudeau J, Baas AF, Deak M, Morrice NA, Kieloch A, Schutkowski M, Prescott AR, Clevers HC, Alessi DR. MO25α/β interact with STRADα/β enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. The EMBO journal. 2003; 22(19):5102–5114.

Boudeau J, Miranda-Saavedra D, Barton GJ, Alessi DR. Emerging roles of pseudokinases. Trends in cell biology. 2006; 16(9):443–452.

Boudeau J, Scott JW, Resta N, Deak M, Kieloch A, Komander D, Hardie DG, Prescott AR, van Aalten DM, Alessi DR. Analysis of the LKB1-STRAD-MO25 complex. Journal of cell science. 2004; 117(26):6365–6375.

Capra M, Nuciforo PG, Confalonieri S, Quarto M, Bianchi M, Nebuloni M, Boldorini R, Pallotti F, Viale G, Gishizky ML, et al. Frequent alterations in the expression of serine/threonine kinases in human cancers. Cancer research. 2006; 66(16):8147–8154.

Chan IS, Ginsburg GS. Personalized medicine: progress and promise. Annual review of genomics and human genetics. 2011; 12:217–244.

Chen CY, Georgiev I, Anderson AC, Donald BR. Computational structure-based redesign of enzyme activity. Proceedings of the National Academy of Sciences. 2009; 106(10):3764–3769.

Cho Y, McQuade T, Zhang H, Zhang J, Chan FKM. RIP1-dependent and independent effects of necrostatin-1 in necrosis and T cell activation. PloS one. 2011; 6(8):e23209.

Choi YJ, Li X, Hydbring P, Sanda T, Stefano J, Christie AL, Signoretti S, Look AT, Kung AL, von Boehmer H, et al. The requirement for cyclin D function in tumor maintenance. Cancer cell. 2012; 22(4):438–451.

Chou KC. Progresses in predicting post-translational modification. International Journal of Peptide Research and Therapeutics. 2020; 26(2):873–888.

Christofferson D, Li Y, Hitomi J, Zhou W, Upperman C, Zhu H, Gerber S, Gygi S, Yuan J. A novel role for RIP1 kinase in mediating TNFα production. Cell death & disease. 2012; 3(6):e320–e320.

Cicenas J, Zalyte E, Bairoch A, Gaudet P, Kinases and cancer. MDPI; 2018.

Clucas J, Meier P. Roles of RIPK1 as a stress sentinel coordinating cell survival and immunogenic cell death. Nature Reviews Molecular Cell Biology. 2023; p. 1–18.

Cohen P. The regulation of protein function by multisite phosphorylation–a 25 year update. Trends in biochemical sciences. 2000; 25(12):596–601.

Croce L, Coperchini F, Magri F, Chiovato L, Rotondi M. The multifaceted anti-cancer effects of BRAF-inhibitors. Oncotarget. 2019; 10(61):6623.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, et al. Mutations of the BRAF gene in human cancer. Nature. 2002; 417(6892):949–954.

Degterev A, Ofengeim D, Yuan J. Targeting RIPK1 for the treatment of human diseases. Proceedings of the National Academy of Sciences. 2019; 116(20):9714–9722.

Deribe YL, Pawson T, Dikic I. Post-translational modifications in signal integration. Nature structural & molecular biology. 2010; 17(6):666–672.

759 **Dondelinger Y**, Delanghe T, Priem D, Wynosky-Dolfi MA, Sorobetea D, Rojas-Rivera D, Giansanti P, Roelandt R,
760 Gropengiesser J, Ruckdeschel K, et al. Serine 25 phosphorylation inhibits RIPK1 kinase-dependent cell death
761 in models of infection and inflammation. Nature communications. 2019; 10(1):1729.

762 **Eferl R**, Wagner EF. AP-1: a double-edged sword in tumorigenesis. Nature Reviews Cancer. 2003; 3(11):859–868.

763 **Enzler F**, Tschaikner P, Schneider R, Stefan E. KinCon: cell-based recording of full-length kinase conformations.
764 IUBMB life. 2020; 72(6):1168–1174.

765 **Fabbro D**, Cowan-Jacob SW, Moebitz H. Ten things you should know about protein kinases: IUPHAR R eview
766 14. British journal of pharmacology. 2015; 172(11):2675–2700.

767 **Fassl A**, Geng Y, Sicinski P. CDK4 and CDK6 kinases: From basic science to cancer therapy. Science. 2022;
768 375(6577):eabc1495.

769 **Feichtner A**, Kugler V, Schwaighofer S, Nuener T, Fleischmann J, Stefan E. Tracking mutation and drug-driven
770 alterations of oncokinase conformations. memo-Magazine of European Medical Oncology. 2022; 15(2):137–
771 142.

772 **Ferguson FM**, Gray NS. Kinase inhibitors: the road ahead. Nature reviews Drug discovery. 2018; 17(5):353–377.

773 **Festjens N**, Vanden Berghe T, Cornelis S, Vandenabeele P. RIP1, a kinase on the crossroads of a cell's decision
774 to live or die. Cell Death & Differentiation. 2007; 14(3):400–410.

775 **Fleischmann J**, Feichtner A, DeFalco L, Kugler V, Schwaighofer S, Huber RG, Stefan E. Allosteric kinase inhibitors
776 reshape MEK1 kinase activity conformations in cells and in silico. Biomolecules. 2021; 11(4):518.

777 **Fleischmann J**, Schwaighofer S, De Falco L, Enzler F, Feichtner A, Kugler V, Tschaikner P, Huber RG, Stefan E.
778 Tracking and blocking interdependencies of cellular BRAF-MEK oncokinase activities. PNAS Nexus. 2023; p.
779 pgad185.

780 **Gainza P**, Roberts KE, Georgiev I, Lilien RH, Keedy DA, Chen CY, Reza F, Anderson AC, Richardson DC, Richard-
781 son JS, et al. OSPREY: protein design with ensembles, flexibility, and provable algorithms. In: *Methods in*
782 *enzymology*, vol. 523 Elsevier; 2013.p. 87–107.

783 **Gao X**, Leone GW, Wang H. Cyclin D-CDK4/6 functions in cancer. Advances in cancer research. 2020; 148:147–
784 169.

785 **Giacinti C**, Giordano A. RB and cell cycle progression. Oncogene. 2006; 25(38):5220–5227.

786 **Goel S**, DeCristo MJ, McAllister SS, Zhao JJ. CDK4/6 inhibition in cancer: beyond cell cycle arrest. Trends in cell
787 biology. 2018; 28(11):911–925.

788 **Goetz LH**, Schork NJ. Personalized medicine: motivation, challenges, and progress. Fertility and sterility. 2018;
789 109(6):952–963.

790 **Guo YJ**, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. Experimental
791 and therapeutic medicine. 2020; 19(3):1997–2007.

792 **Huse M**, Kuriyan J. The conformational plasticity of protein kinases. Cell. 2002; 109(3):275–282.

793 **Islam MJ**, Khan AM, Parves MR, Hossain MN, Halim MA. Prediction of deleterious non-synonymous SNPs of hu-
794 man STK11 gene by combining algorithms, molecular docking, and molecular dynamics simulation. Scientific
795 Reports. 2019; 9(1):1–16.

796 **Jaco I**, Annibaldi A, Lalaoui N, Wilson R, Tenev T, Laurien L, Kim C, Jamal K, John SW, Liccardi G, et al. MK2
797 phosphorylates RIPK1 to prevent TNF-induced cell death. Molecular cell. 2017; 66(5):698–710.

798 **James MK**, Ray A, Leznova D, Blain SW. Differential modification of p27Kip1 controls its cyclin D-cdk4 inhibitory
799 activity. Molecular and cellular biology. 2008; 28(1):498–510.

800 **Karoulia Z**, Gavathiotis E, Poulikakos PI. New perspectives for targeting RAF kinase in human cancer. Nature
801 Reviews Cancer. 2017; 17(11):676–691.

802 **Kasai H**, Petersen OH. Spatial dynamics of second messengers: IP3 and cAMP as long-range and associative
803 messengers. Trends in neurosciences. 1994; 17(3):95–101.

Kaserer T, Blagg J. Combining mutational signatures, clonal fitness, and drug affinity to define drug-specific resistance mutations in cancer. Cell chemical biology. 2018; 25(11):1359–1371.

Kemp BE, Parker MW, Hu S, Tiganis T, House C. Substrate and pseudosubstrate interactions with protein kinases: determinants of specificity. Trends in biochemical sciences. 1994; 19(11):440–444.

Kitajima S, Ivanova E, Guo S, Yoshida R, Campisi M, Sundararaman SK, Tange S, Mitsuishi Y, Thai TC, Masuda S, et al. Suppression of STING associated with LKB1 loss in KRAS-driven lung cancer. Cancer discovery. 2019; 9(1):34–45.

Klaeger S, Heinzlmeir S, Wilhelm M, Polzer H, Vick B, Koenig PA, Reinecke M, Ruprecht B, Petzoldt S, Meng C, et al. The target landscape of clinical kinase drugs. Science. 2017; 358(6367):eaan4368.

Knudsen ES, Witkiewicz AK. The strange case of CDK4/6 inhibitors: mechanisms, resistance, and combination strategies. Trends in cancer. 2017; 3(1):39–55.

Kollmann K, Heller G, Schneckenleithner C, Warsch W, Scheicher R, Ott RG, Schäfer M, Fajmann S, Schlederer M, Schiefer AI, et al. A kinase-independent function of CDK6 links the cell cycle to tumor angiogenesis. Cancer cell. 2013; 24(2):167–181.

Kollmann K, Sexl V. CDK6 and p16INK4A in lymphoid malignancies. Oncotarget. 2013; 4(11):1858.

Kornev AP, Taylor SS. Defining the conserved internal architecture of a protein kinase. Biochimica Et Biophysica Acta (BBA)-Proteins and Proteomics. 2010; 1804(3):440–444.

Köstler WJ, Zielinski CC. Targeting receptor tyrosine kinases in cancer. Receptor tyrosine kinases: Structure, functions and role in human disease. 2015; p. 225–278.

Kung JE, Jura N. Prospects for pharmacological targeting of pseudokinases. Nature Reviews Drug Discovery. 2019; 18(7):501–526.

Lahiry P, Torkamani A, Schork NJ, Hegele RA. Kinase mutations in human disease: interpreting genotype–phenotype relationships. Nature Reviews Genetics. 2010; 11(1):60–74.

Lalaoui N, Boyden SE, Oda H, Wood GM, Stone DL, Chau D, Liu L, Stoffels M, Kratina T, Lawlor KE, et al. Mutations that prevent caspase cleavage of RIPK1 cause autoinflammatory disease. Nature. 2020; 577(7788):103–108.

Launonen V. Mutations in the human LKB1/STK11 gene. Human mutation. 2005; 26(4):291–297.

Laurien L, Nagata M, Schünke H, Delanghe T, Wiederstein JL, Kumari S, Schwarzer R, Corona T, Krüger M, Bertrand MJ, et al. Autophosphorylation at serine 166 regulates RIP kinase 1-mediated cell death and inflammation. Nature communications. 2020; 11(1):1747.

Lavoie H, Gagnon J, Therrien M. ERK signalling: a master regulator of cell behaviour, life and fate. Nature reviews Molecular cell biology. 2020; 21(10):607–632.

Li W, Li B, Giacalone NJ, Torossian A, Sun Y, Niu K, Lin-Tsai O, Lu B. BV6, an IAP antagonist, activates apoptosis and enhances radiosensitization of non-small cell lung carcinoma in vitro. Journal of Thoracic Oncology. 2011; 6(11):1801–1809.

Li Y, Führer M, Bahrami E, Socha P, Klaudel-Dreszler M, Bouzidi A, Liu Y, Lehle AS, Magg T, Hollizeck S, et al. Human RIPK1 deficiency causes combined immunodeficiency and inflammatory bowel diseases. Proceedings of the National Academy of Sciences. 2019; 116(3):970–975.

Liggett Jr WH, Sidransky D. Role of the p16 tumor suppressor gene in cancer. Journal of clinical oncology. 1998; 16(3):1197–1206.

Lin J, Jinno S, Okayama H. Cdk6-cyclin D3 complex evades inhibition by inhibitor proteins and uniquely controls cell's proliferation competence. Oncogene. 2001; 20(16):2000–2009.

Lito P, Rosen N, Solit DB. Tumor adaptation and resistance to RAF inhibitors. Nature medicine. 2013; 19(11):1401–1409.

Longley D, Johnston P. Molecular mechanisms of drug resistance. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland. 2005; 205(2):275–292.

Lopez ED, Burastero O, Arcon JP, Defelipe LA, Ahn NG, Marti MA, Turjanski AG. Kinase activation by small conformational changes. Journal of chemical information and modeling. 2019; 60(2):821–832.

Madhavi Sastry G, Adzhigirey M, Day T, Annabhimoju R, Sherman W. Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. Journal of computer-aided molecular design. 2013; 27:221–234.

Manning BD. Challenges and opportunities in defining the essential cancer kinome. Science Signaling. 2009; 2(63):pe15–pe15.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002; 298(5600):1912–1934.

Martens S, Hofmans S, Declercq W, Augustyns K, Vandenabeele P. Inhibitors targeting RIPK1/RIPK3: old and new drugs. Trends in pharmacological sciences. 2020; 41(3):209–224.

Mayrhofer JE, Enzler F, Feichtner A, Röck R, Fleischmann J, Raffeiner A, Tschaikner P, Ogris E, Huber RG, Hartl M, et al. Mutation-oriented profiling of autoinhibitory kinase conformations predicts RAF inhibitor efficacies. Proceedings of the National Academy of Sciences. 2020; 117(49):31105–31113.

Meharena HS, Fan X, Ahuja LG, Keshwani MM, McClendon CL, Chen AM, Adams JA, Taylor SS. Decoding the interactions regulating the active state mechanics of eukaryotic protein kinases. PLoS Biology. 2016; 14(11):e2000127.

Mehenni H, Gehrig C, Nezu Ji, Oku A, Shimane M, Rossier C, Guex N, Blouin JL, Scott HS, Antonarakis SE. Loss of LKB1 kinase activity in Peutz-Jeghers syndrome, and evidence for allelic and locus heterogeneity. The American Journal of Human Genetics. 1998; 63(6):1641–1650.

Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, Tschopp J. RIP1 is an essential mediator of Toll-like receptor 3–induced NF-κB activation. Nature immunology. 2004; 5(5):503–507.

Molaei A, Molaei E, Sadeghnia H, Hayes AW, Karimi G. LKB1: An emerging therapeutic target for cardiovascular diseases. Life Sciences. 2022; p. 120844.

Morrison DK. KSR: a MAPK scaffold of the Ras pathway? Journal of cell science. 2001; 114(9):1609–1612.

Morrison DK, Davis RJ. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annual review of cell and developmental biology. 2003; 19(1):91–118.

Moynagh PN. The NF-κB pathway. Journal of cell science. 2005; 118(20):4589–4592.

Narbonne P, Hyenne V, Li S, Labbé JC, Roy R. Differential requirements for STRAD in LKB1-dependent functions in C. elegans. Development. 2010; 137(4):661–670.

Ndembe G, Intini I, Perin E, Marabese M, Caiola E, Mendogni P, Rosso L, Broggini M, Colombo M. LKB1: Can we target an hidden target? focus on NSCLC. Frontiers in Oncology. 2022; 12:889826.

Nebenfuehr S, Kollmann K, Sexl V. The role of CDK6 in cancer. International journal of cancer. 2020; 147(11):2988–2995.

Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chemical reviews. 2001; 101(8):2353–2364.

Newton AC, Bootman MD, Scott JD. Second messengers. Cold Spring Harbor perspectives in biology. 2016; 8(8):a005926.

Ochoa D, Bradley D, Beltrao P. Evolution, dynamics and dysregulation of kinase signalling. Current opinion in structural biology. 2018; 48:133–140.

Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2002; 1602(1):73–87.

Otto T, Sicinski P. Cell cycle proteins as promising targets in cancer therapy. Nature Reviews Cancer. 2017; 17(2):93–115.

Pan X, Heitman J. Protein kinase A operates a molecular switch that governs yeast pseudohyphal differentiation. Molecular and cellular biology. 2002; .

Partanen JI, Tervonen TA, Myllynen M, Lind E, Imai M, Katajisto P, Dijkgraaf GJ, Kovanen PE, Mäkelä TP, Werb Z, et al. Tumor suppressor function of Liver kinase B1 (Lkb1) is linked to regulation of epithelial integrity. Proceedings of the National Academy of Sciences. 2012; 109(7):E388–E397.

Politi K, Ayeni D, Lynch T. The next wave of EGFR tyrosine kinase inhibitors enter the clinic. Cancer cell. 2015; 27(6):751–753.

Pouysségur J, Volmat V, Lenormand P. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. Biochemical pharmacology. 2002; 64(5-6):755–763.

Pufall MA, Graves BJ. Autoinhibitory domains: modular effectors of cellular regulation. Annual review of cell and developmental biology. 2002; 18(1):421–462.

Puylaert P, Coornaert I, Neutel CH, Dondelinger Y, Delanghe T, Bertrand MJ, Guns PJ, De Meyer GR, Martinet W. The impact of RIPK1 kinase inhibition on atherogenesis: a genetic and a pharmacological approach. Biomedicines. 2022; 10(5):1016.

Qing T, Liu J, Liu F, Mitchell DC, Beresis RT, Gordan JD. Methods to assess small molecule allosteric modulators of the STRAD pseudokinase. In: *Methods in Enzymology*, vol. 667 Elsevier; 2022.p. 427–453.

Quelle DE, Cheng M, Ashmun RA, Sherr CJ. Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. Proceedings of the National Academy of Sciences. 1997; 94(2):669–673.

Rajakulendran T, Sicheri F. Allosteric protein kinase regulation by pseudokinases: insights from STRAD. Science Signaling. 2010; 3(111):pe8–pe8.

Raju S, Whalen DM, Mengistu M, Swanson C, Quinn JG, Taylor SS, Webster JD, Newton K, Shaw AS. Kinase domain dimerization drives RIPK3-dependent necroptosis. Science signaling. 2018; 11(544):eaar2188.

Ramms DJ, Raimondi F, Arang N, Herberg FW, Taylor SS, Gutkind JS. Gαs–Protein kinase a (PKA) pathway signalopathies: the emerging genetic landscape and therapeutic potential of human diseases driven by aberrant Gαs-PKA signaling. Pharmacological Reviews. 2021; 73(4):1326–1368.

Reiterer V, Eyers PA, Farhan H. Day of the dead: pseudokinases and pseudophosphatases in physiology and disease. Trends in cell biology. 2014; 24(9):489–505.

Röck R, Mayrhofer JE, Torres-Quesada O, Enzler F, Raffeiner A, Raffeiner P, Feichtner A, Huber RG, Koide S, Taylor SS, et al. BRAF inhibitors promote intermediate BRAF (V600E) conformations and binary interactions with activated RAS. Science advances. 2019; 5(8):eaav8463.

Rodríguez C, Muñoz M, Contreras C, Prieto D. AMPK, metabolism, and vascular function. The FEBS Journal. 2021; 288(12):3746–3771.

Rodríguez-Díez E, Quereda V, Bellutti F, Prchal-Murphy M, Partida D, Eguren M, Kollmann K, Gomez de Cedron M, Dubus P, Canamero M, et al. Cdk4 and Cdk6 cooperate in counteracting the INK4 family of inhibitors during murine leukemogenesis. Blood, The Journal of the American Society of Hematology. 2014; 124(15):2380–2390.

Romagosa C, Simonetti S, Lopez-Vicente L, Mazo A, Lleonart M, Castellvi J, Ramon y Cajal S. p16Ink4a overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene. 2011; 30(18):2087–2097.

Russo AA, Tong L, Lee JO, Jeffrey PD, Pavletich NP. Structural basis for inhibition of the cyclin-dependent kinase Cdk6 by the tumour suppressor p16INK4a. Nature. 1998; 395(6699):237–243.

Sanchez-Cespedes M. A role for LKB1 gene in human cancer beyond the Peutz–Jeghers syndrome. Oncogene. 2007; 26(57):7825–7832.

Scheiblecker L, Kollmann K, Sexl V. CDK4/6 and MAPK—Crosstalk as Opportunity for Cancer Treatment. Pharmaceuticals. 2020; 13(12):418.

Scheicher R, Hoelbl-Kovacic A, Bellutti F, Tigan AS, Prchal-Murphy M, Heller G, Schneckenleithner C, Salazar-Roa M, Zöchbauer-Müller S, Zuber J, et al. CDK6 as a key regulator of hematopoietic and leukemic stem cell activation. Blood, The Journal of the American Society of Hematology. 2015; 125(1):90–101.

Schmitt DL, Mehta S, Zhang J. Study of spatiotemporal regulation of kinase signaling using genetically encodable molecular tools. Current opinion in chemical biology. 2022; 71:102224.

Schrödinger L. Schrödinger release 2019-4: protein preparation wizard; Epik; impact; prime; glide; LigPrep; induced fit docking protocol. New York, NY. 2019; .

Semczuk A, Jakowicki JA. Alterations of pRb1-cyclin D1-cdk4/6-p16INK4A pathway in endometrial carcinogenesis. Cancer letters. 2004; 203(1):1–12.

Shackelford DB, Shaw RJ. The LKB1–AMPK pathway: metabolism and growth control in tumour suppression. Nature Reviews Cancer. 2009; 9(8):563–575.

Shchemelinin I, Sefc L, Necas E. Protein kinases, their function and implication in cancer and other diseases. Folia biologica. 2006; 52(3):81.

Shrestha S, Byrne DP, Harris JA, Kannan N, Eyers PA. Cataloguing the dead: breathing new life into pseudokinase research. The FEBS Journal. 2020; 287(19):4150–4169.

Shutinoski B, Alturki N, Rijal D, Bertin J, Gough P, Schlossmacher M, Sad S. K45A mutation of RIPK1 results in poor necroptosis and cytokine signaling in macrophages, which impacts inflammatory responses in vivo. Cell Death & Differentiation. 2016; 23(10):1628–1637.

Silke J, Meier P. Inhibitor of apoptosis (IAP) proteins–modulators of cell death and inflammation. Cold Spring Harbor perspectives in biology. 2013; 5(2):a008730.

Southekal S, Mishra NK, Guda C. Pan-cancer analysis of human kinome gene expression and promoter DNA methylation identifies dark kinase biomarkers in multiple cancers. Cancers. 2021; 13(6):1189.

Speir M, Djajawi TM, Conos SA, Tye H, Lawlor KE. Targeting RIP kinases in chronic inflammatory disease. Biomolecules. 2021; 11(5):646.

Stefan E, Aquin S, Berger N, Landry C, Nyfeler B, Bouvier M, Michnick S. Quantification of dynamic protein complexes using Renilla luciferase fragment complementation applied to protein kinase A activities in vivo. Proceedings of the National Academy of Sciences. 2007; 104(43):16916–16921.

Tao P, Sun J, Wu Z, Wang S, Wang J, Li W, Pan H, Bai R, Zhang J, Wang Y, et al. A dominant autoinflammatory disease caused by non-cleavable variants of RIPK1. Nature. 2020; 577(7788):109–114.

Taylor SS, Ilouz R, Zhang P, Kornev AP. Assembly of allosteric macromolecular switches: lessons from PKA. Nature reviews Molecular cell biology. 2012; 13(10):646–658.

Taylor SS, Kim C, Vigil D, Haste NM, Yang J, Wu J, Anand GS. Dynamics of signaling by PKA. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2005; 1754(1-2):25–37.

Taylor SS, Wu J, Bruystens JG, Del Rio JC, Lu TW, Kornev AP, Ten Eyck LF. From structure to the dynamic regulation of a molecular switch: A journey over 3 decades. Journal of Biological Chemistry. 2021; 296.

Taylor SS, Zhang P, Steichen JM, Keshwani MM, Kornev AP. PKA: lessons learned after twenty years. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2013; 1834(7):1271–1278.

Ui A, Ogiwara H, Nakajima S, Kanno S, Watanabe R, Harata M, Okayama H, Harris C, Yokota J, Yasui A, et al. Possible involvement of LKB1-AMPK signaling in non-homologous end joining. Oncogene. 2014; 33(13):1640–1648.

Van AAN, Kunkel MT, Baffi TR, Lordén G, Antal CE, Banerjee S, Newton AC. Protein kinase C fusion proteins are paradoxically loss of function in cancer. Journal of Biological Chemistry. 2021; 296.

VanArsdale T, Boshoff C, Arndt KT, Abraham RT. Molecular pathways: targeting the cyclin D–CDK4/6 axis for cancer treatment. Clinical cancer research. 2015; 21(13):2905–2910.

Wang Q, Fan D, Xia Y, Ye Q, Xi X, Zhang G, Xiao C. The latest information on the RIPK1 post-translational modifications and functions. Biomedicine & Pharmacotherapy. 2021; 142:112082.

Wang W, Marinis JM, Beal AM, Savadkar S, Wu Y, Khan M, Taunk PS, Wu N, Su W, Wu J, et al. RIP1 kinase drives macrophage-mediated adaptive immune tolerance in pancreatic cancer. Cancer cell. 2018; 34(5):757–774.

Weinlich R, Green DR. The two faces of receptor interacting protein kinase-1. Molecular cell. 2014; 56(4):469–480.

Wingo SN, Gallardo TD, Akbay EA, Liang MC, Contreras CM, Boren T, Shimamura T, Miller DS, Sharpless NE, Bardeesy N, et al. Somatic LKB1 mutations promote cervical cancer progression. PloS one. 2009; 4(4):e5137.

Wu P, Clausen MH, Nielsen TE. Allosteric small-molecule kinase inhibitors. Pharmacology & therapeutics. 2015; 156:59–68.

Xu Be, Min X, Stippec S, Lee BH, Goldsmith EJ, Cobb MH. Regulation of WNK1 by an autoinhibitory domain and autophosphorylation. Journal of Biological Chemistry. 2002; 277(50):48456–48462.

Xu D, Jin T, Zhu H, Chen H, Ofengeim D, Zou C, Mifflin L, Pan L, Amin P, Li W, et al. TBK1 suppresses RIPK1-driven apoptosis and inflammation during development and in aging. Cell. 2018; 174(6):1477–1491.

Yam C, Hung MC, Hortobagyi GN. CDK4/6 inhibitors in hormone receptor-positive, human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer: Are we at the finish line? Oncotarget. 2018; 9(76):34193.

Yamaguchi H, Hendrickson WA. Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. Nature. 1996; 384(6608):484–489.

Yang C, Li Z, Bhatt T, Dickler M, Giri D, Scaltriti M, Baselga J, Rosen N, Chandarlapaty S. Acquired CDK6 amplification promotes breast cancer resistance to CDK4/6 inhibitors and loss of ER signaling and dependence. Oncogene. 2017; 36(16):2255–2264.

Yang R, Li SW, Chen Z, Zhou X, Ni W, Fu DA, Lu J, Kaye FJ, Wu L. Role of INSL4 signaling in sustaining the growth and viability of LKB1-inactivated lung cancer. JNCI: Journal of the National Cancer Institute. 2019; 111(7):664–674.

Yao Z, Gao Y, Su W, Yaeger R, Tao J, Na N, Zhang Y, Zhang C, Rymar A, Tao A, et al. RAF inhibitor PLX8394 selectively disrupts BRAF dimers and RAS-independent BRAF-mutant-driven signaling. Nature medicine. 2019; 25(2):284–291.

Yarbrough WG, Buckmire RA, Bessho M, Liu ET. Biologic and biochemical analyses of p16INK4a mutations from primary tumors. Journal of the National Cancer Institute. 1999; 91(18):1569–1574.

Yeon JH, Heinkel F, Sung M, Na D, Gsponer J. Systems-wide identification of cis-regulatory elements in proteins. Cell Systems. 2016; 2(2):89–100.

Yu Q, Sicinska E, Geng Y, Ahnström M, Zagozdzon A, Kong Y, Gardner H, Kiyokawa H, Harris LN, Stål O, et al. Requirement for CDK4 kinase function in breast cancer. Cancer cell. 2006; 9(1):23–32.

Zeqiraj E, Filippi BM, Deak M, Alessi DR, Van Aalten DM. Structure of the LKB1-STRAD-MO25 complex reveals an allosteric mechanism of kinase activation. Science. 2009; 326(5960):1707–1711.

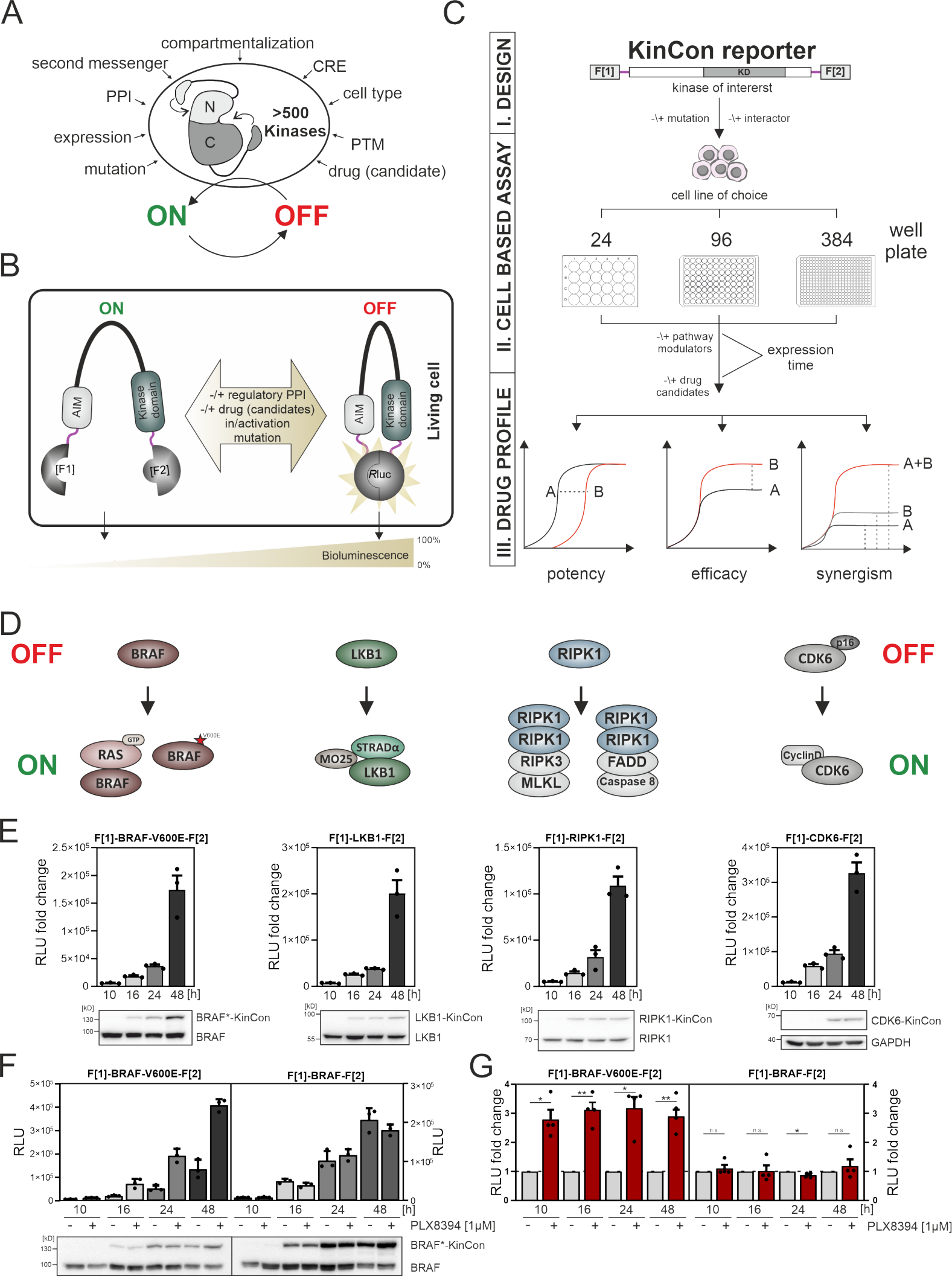
Zeqiraj E, Filippi BM, Goldie S, Navratilova I, Boudeau J, Deak M, Alessi DR, van Aalten DM. ATP and MO25α regulate the conformational state of the STRADα pseudokinase and activation of the LKB1 tumour suppressor. PLoS biology. 2009; 7(6):e1000126.

Zhang JZ, Lu TW, Stolerman LM, Tenner B, Yang JR, Zhang JF, Falcke M, Rangamani P, Taylor SS, Mehta S, et al. Phase separation of a PKA regulatory subunit controls cAMP compartmentation and oncogenic signaling. Cell. 2020; 182(6):1531–1544.

Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. Nature reviews cancer. 2009; 9(1):28–39.

Zhang M, Maloney R, Jang H, Nussinov R. The mechanism of Raf activation through dimerization. Chemical Science. 2021; 12(47):15609–15619.

Zubiete-Franco I, García-Rodríguez JL, Lopitz-Otsoa F, Serrano-Macia M, Simon J, Fernández-Tussy P, Barbier-Torres L, Fernández-Ramos D, Gutiérrez-de Juan V, de Davalillo SL, et al. SUMOylation regulates LKB1 localization and its oncogenic activity in liver cancer. EBioMedicine. 2019; 40:406–421.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Rluc
Living cell
100%
Bioluminescence
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ON
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F[1]-BRAF-V600E-F[2]
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RLU fold change
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LKB1
RIPK1-KinCon
RIPK1
CDK6-KinCon
GAPDH
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RLU
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PLX8394 [1μM]
BRAF*-KinCon
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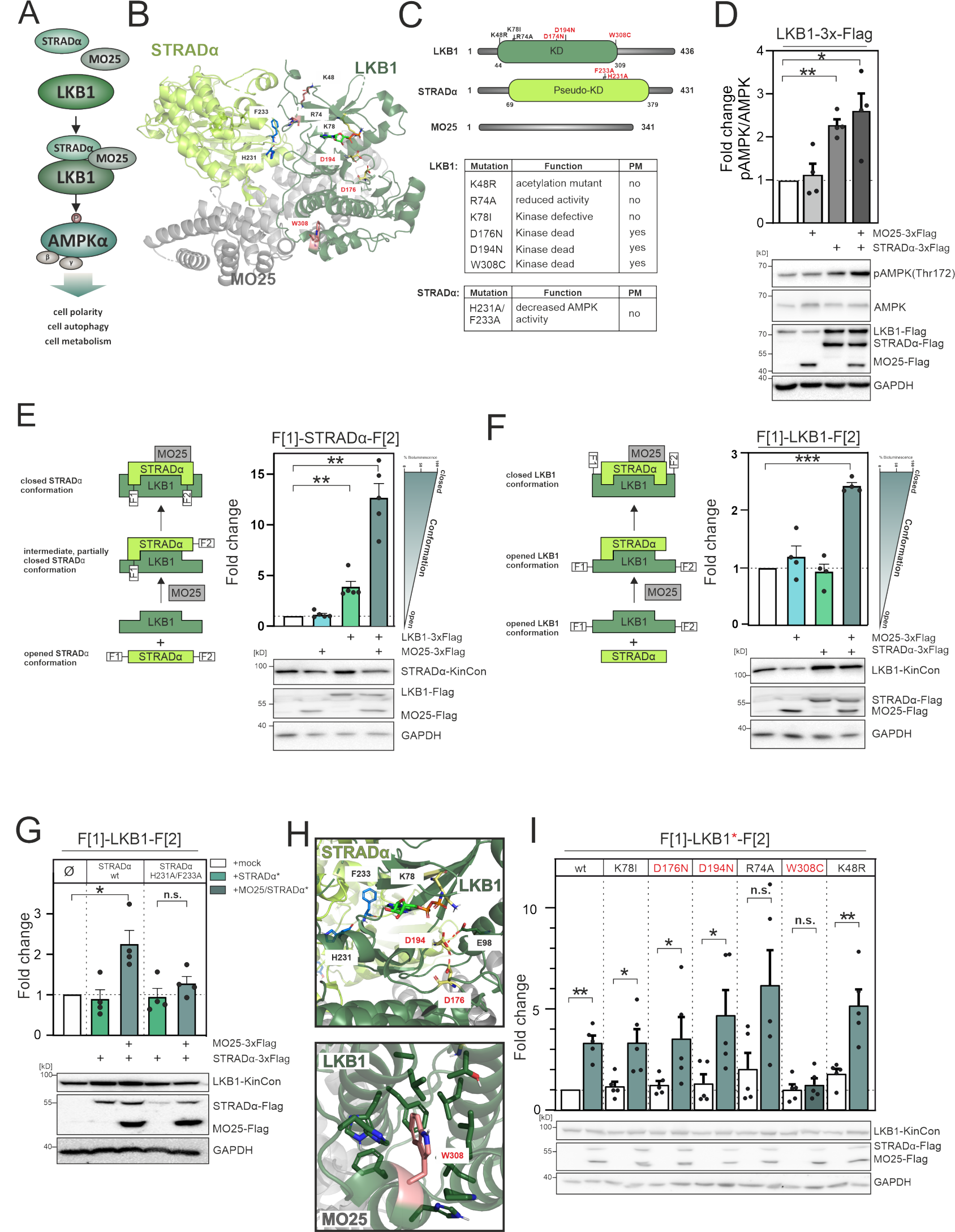
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LKB1:
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LKB1-3x-Flag
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AMPK
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MO25-Flag
GAPDH
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closed STRADα conformation
intermediate, partially closed STRADα conformation
opened STRADα conformation
MO25
STRADα
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F1
F2
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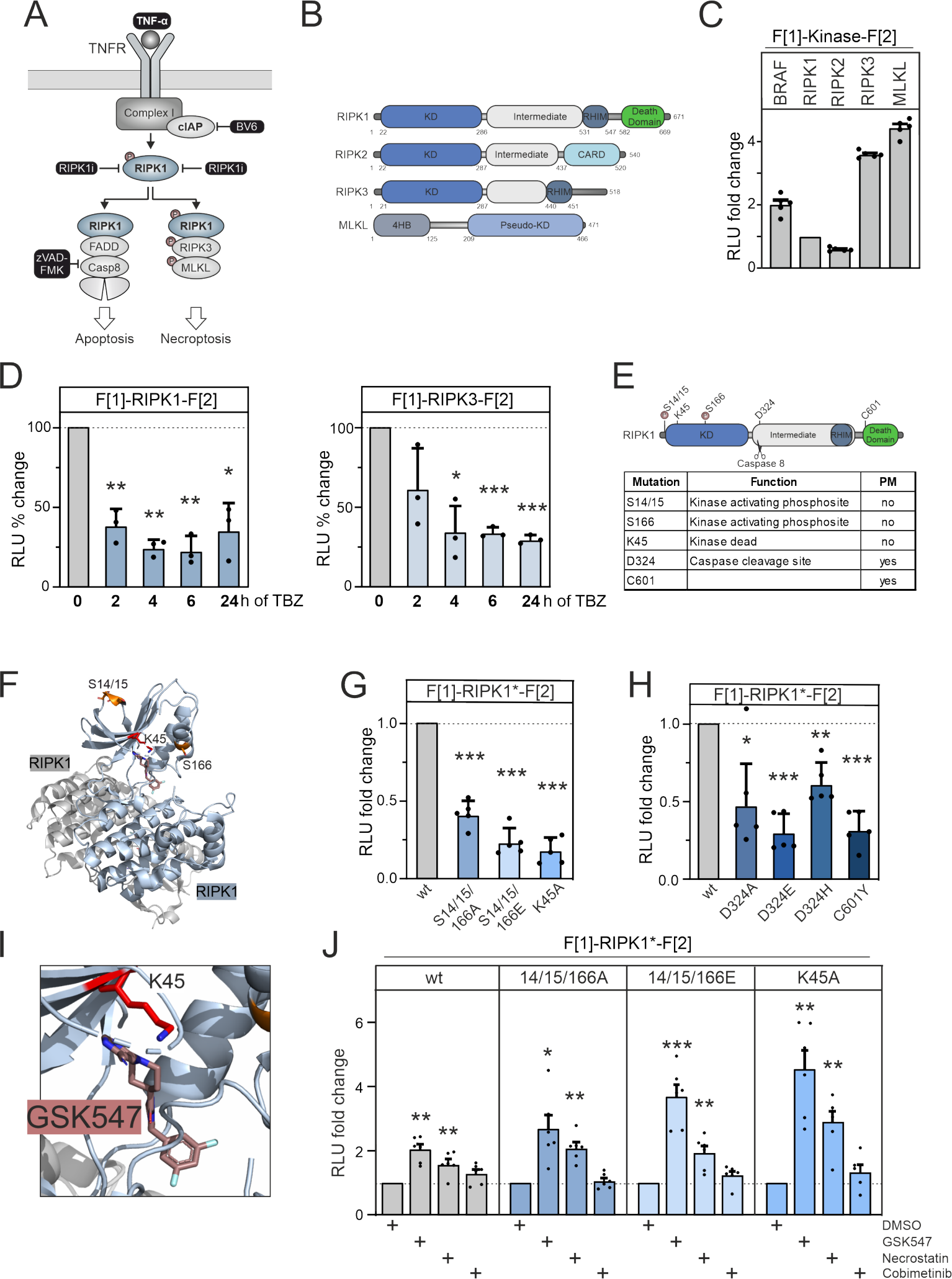
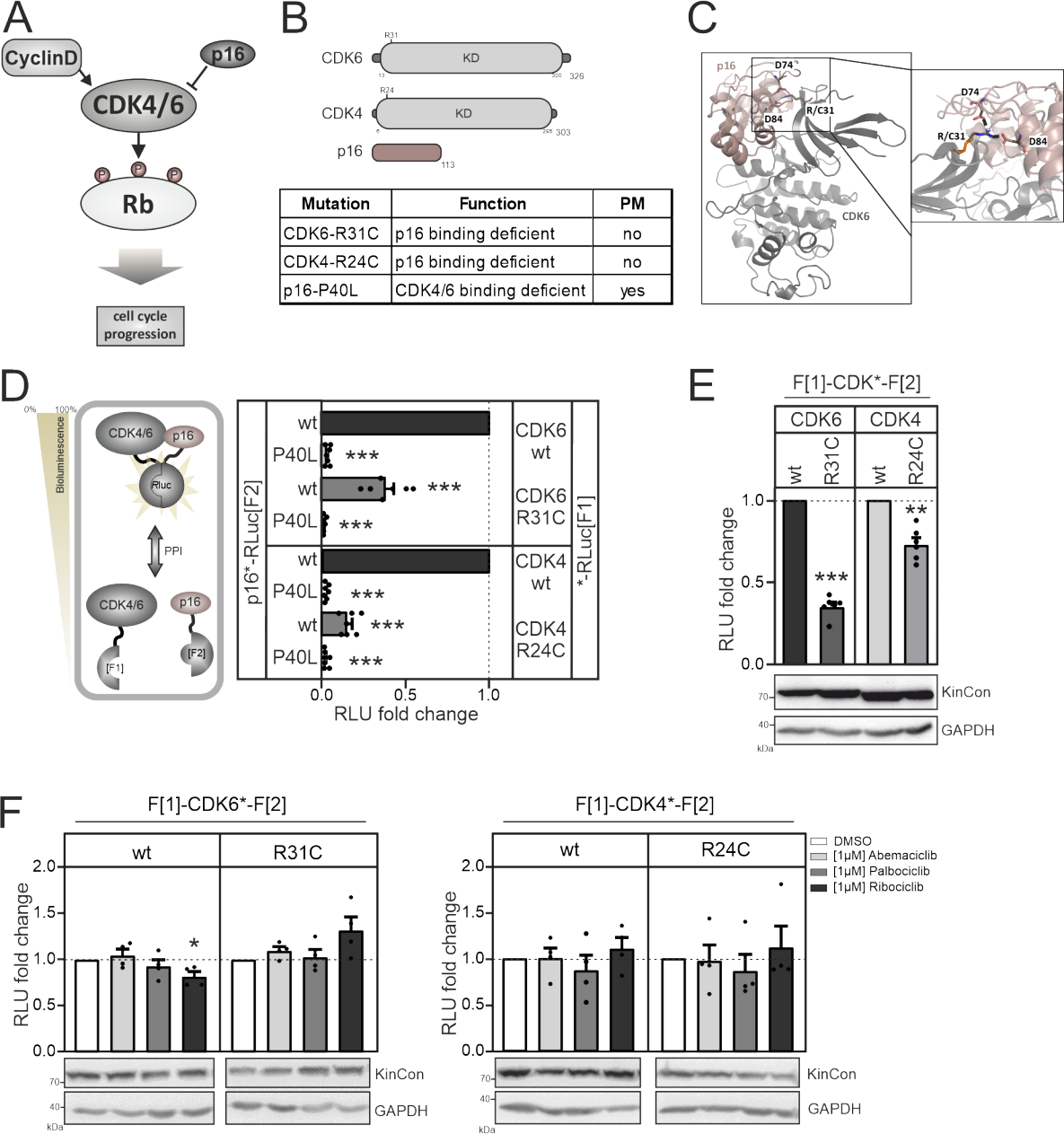
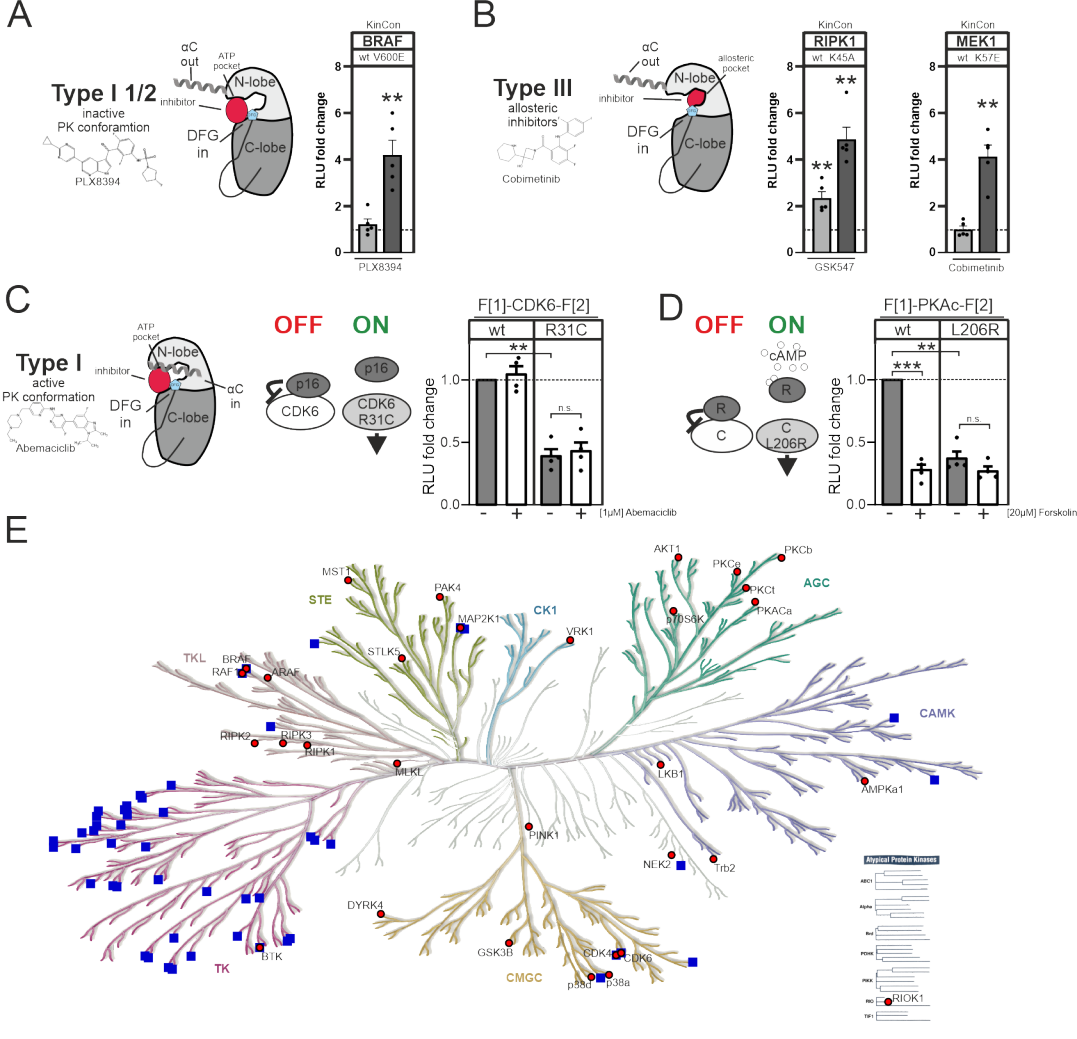
F[1]-LKB1-F[2]
MO25-3xFlag
STRADα-3xFlag
LKB1-KinCon
STRADα-Flag
MO25-Flag
GAPDH
G
F[1]-LKB1-F[2]
Ø
STRADα wt
STRADα H231A/F233A
+mock
+STRADα*
+MO25/STRADα*
n.s.
MO25-3xFlag
STRADα-3xFlag
LKB1-KinCon
STRADα-Flag
MO25-Flag
GAPDH
H
STRADα
F233
K78
LKB1
D194
E98
H231
D176
LKB1
W308
MO25
I
F[1]-LKB1*-F[2]
wt
K78I
D176N
D194N
R74A
W308C
K48R
n.s.
LKB1-KinCon
STRADα-Flag
MO25-Flag
GAPDH


A
TNF-α
TNFR
Complex I
cIAP
BV6
RIPK1i
RIPK1
RIPK1i
RIPK1
FADD
zVAD-FMK
Casp8
RIPK1
RIPK3
MLKL
Apoptosis
Necroptosis
B
RIPK1
KD
Intermediate
RHIM
Death Domain
RIPK2
CARD
RIPK3
MLKL
4HB
Pseudo-KD
C
F[1]-Kinase-F[2]
BRAF
RIPK1
RIPK2
RIPK3
MLKL
RLU fold change
D
F[1]-RIPK1-F[2]
RLU % change
0 2 4 6 24 h of TBZ
F[1]-RIPK3-F[2]
E
S14/15
K45
S166
D324
C601
RIPK1
KD
Intermediate
RHIM
Death Domain
Caspase 8
Mutation | Function | PM
S14/15 | Kinase activating phosphosite | no
S166 | Kinase activating phosphosite | no
K45 | Kinase dead | no
D324 | Caspase cleavage site | yes
C601 | | yes
F
S14/15
K45
S166
RIPK1
RIPK1
G
F[1]-RIPK1*-F[2]
RLU fold change
wt
S14/15/166A
S14/15/166E
K45A
H
F[1]-RIPK1*-F[2]
RLU fold change
wt
D324A
D324E
D324H
C601Y
I
K45
GSK547
J
F[1]-RIPK1*-F[2]
wt
14/15/166A
14/15/166E
K45A
RLU fold change
DMSO
GSK547
Necrostatin
Cobimetinib



Mutation	Function	PM
CDK6-R31C	p16 binding deficient	no
CDK4-R24C	p16 binding deficient	no
p16-P40L	CDK4/6 binding deficient	yes


A
Type I 1/2
inactive
PK conforamtion
PLX8394
αC out
ATP pocket
N-lobe
inhibitor
DFG in
C-lobe
KinCon
BRAF
wt V600E
RLU fold change
PLX8394
B
Type III
allosteric inhibitors
Cobimetinib
αC out
allosteric pocket
N-lobe
inhibitor
DFG in
C-lobe
KinCon
RIPK1
wt K45A
GSK547
KinCon
MEK1
wt K57E
Cobimetinib
C
Type I
active
PK conformation
Abemaciclib
ATP pocket
N-lobe
inhibitor
αC in
DFG in
C-lobe
OFF
ON
p16
CDK6
CDK6 R31C
F[1]-CDK6-F[2]
wt
R31C
n.s
[1μM] Abemaciclib
D
OFF
ON
cAMP
R
C
L206R
F[1]-PKAc-F[2]
wt
L206R
n.s
[20μM] Forskolin
E
STE
CK1
AGC
TKL
CAMK
TK
CMGC
MST1
PAK4
MAP2K1
VRK1
STLK5
BRAF
RAF1
ARAF
RIPK2
RIPK3
RIPK1
MEKK
AKT1
PKCb
PKCa
PKCt
PKACa
p70S6K
LKB1
AMPKa1
PINK1
NEK2
Trb2
DYRK4
GSK3B
CDK4
CDK6
p38d
p38a
BTK
RIOK1
Atypical Protein Kinases