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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 track 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 to identify cellular factors that impact drug efficacies. The understanding of the structural dynamics of full-length protein kinases when interacting with small molecule inhibitors or regulatory proteins is crucial for designing more effective therapeutic strategies.

eLife assessment

This article reports an **important** bioluminescence-based reporter system to evaluate kinase conformations. This assay is applied to four different kinases that have unique, very special regulatory features, thereby indicating that the assay can be used to provide **convincing** evidence on the conformational state of a large number of kinases. This paper will be of interest to researchers working on kinases and their conformational states.

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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)).

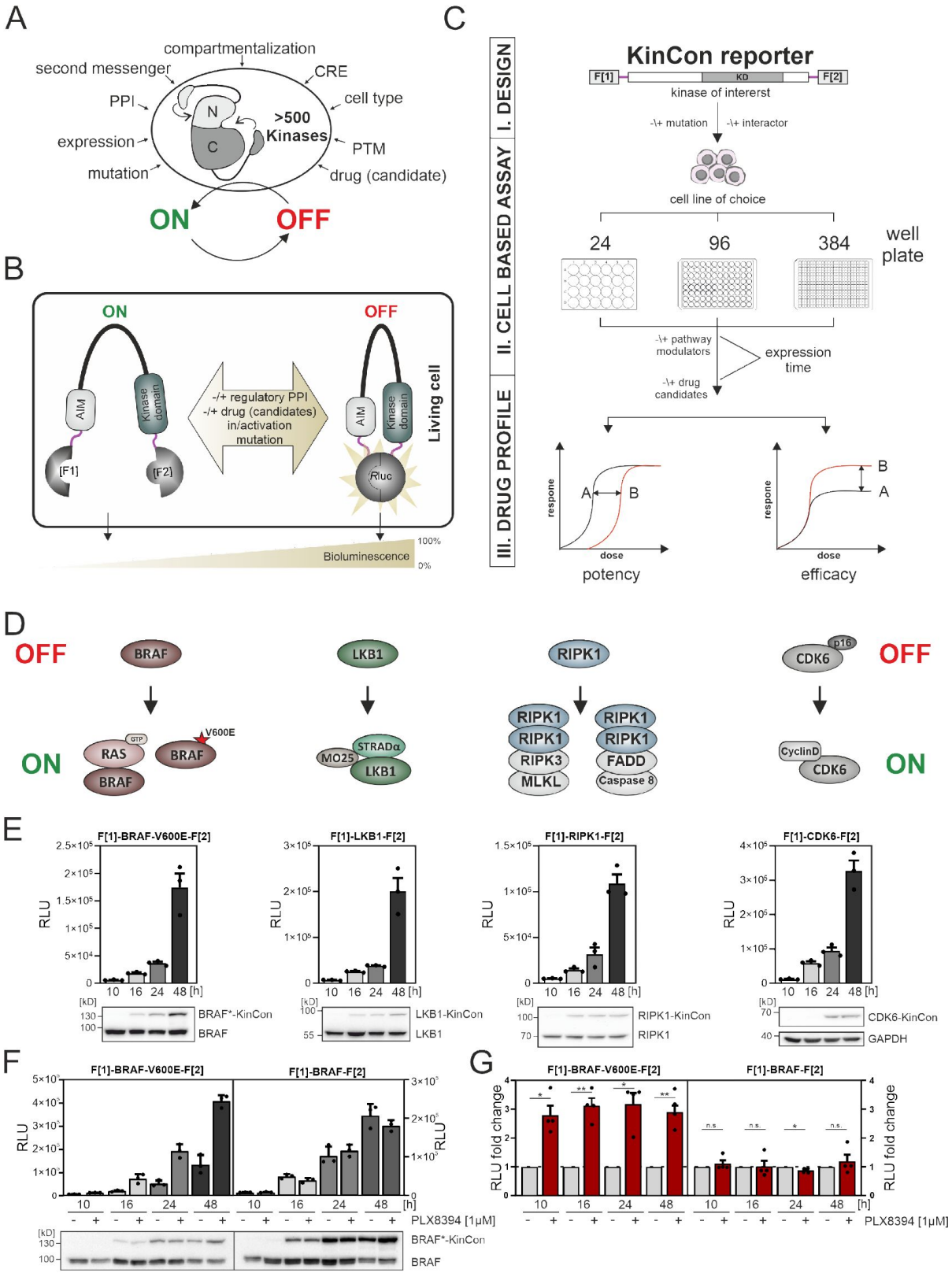
A
compartmentalization
second messenger
CRE
PPI
cell type
N
C
>500 Kinases
expression
PTM
mutation
drug (candidate)
ON
OFF
B
ON
OFF
AIM
Kinase domain
[F1]
[F2]
-/+ regulatory PPI
-/+ drug (candidates)
in/activation mutation
Rluc
Living cell
Bioluminescence
100%
0%
C
I. DESIGN
II. CELL BASED ASSAY
III. DRUG PROFILE
KinCon reporter
F[1]
KD
F[2]
kinase of interest
-\+ mutation
-\+ interactor
cell line of choice
24
96
384
well plate
-\+ pathway modulators
-\+ drug candidates
expression time
respone
dose
A
B
potency
efficacy
D
OFF
BRAF
LKB1
RIPK1
CDK6
p16
OFF
ON
RAS
GTP
BRAF
V600E
MO25
STRADα
LKB1
RIPK3
MLKL
FADD
Caspase 8
CyclinD
CDK6
ON
E
F[1]-BRAF-V600E-F[2]
F[1]-LKB1-F[2]
F[1]-RIPK1-F[2]
F[1]-CDK6-F[2]
RLU
10 16 24 48 [h]
[kD]
BRAF*-KinCon
BRAF
LKB1-KinCon
LKB1
RIPK1-KinCon
RIPK1
CDK6-KinCon
GAPDH
F
F[1]-BRAF-V600E-F[2]
F[1]-BRAF-F[2]
RLU
10 16 24 48 [h]
PLX8394 [1µM]
BRAF*-KinCon
BRAF
G
F[1]-BRAF-V600E-F[2]
F[1]-BRAF-F[2]
RLU fold change
n.s.
10 16 24 48 [h]
PLX8394 [1µM]

Figure 1.

Kinase regulation and KinCon reporter technology features.

A) Impact of indicated factors/features (e.g. Protein-protein interactions (PPIs), 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 1 and 2 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 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. Expression of the genetically encoded reporter in indicated 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 respective endogenous kinase, interlinked kinases or proteinogenic regulators of the respective pathway. After systematic administration of pathway modulating drugs or drug candidates, analyses of KinCon structure dynamics may reveal alterations in potency, efficacy, and potential synergistic effects of the tested bioactive small molecules (schematic dose response curves are depicted). **D**) Simplified schematic representation of the activation 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). Indicated KinCon reporters were transiently over-expressed in 24-well format in HEK293T cells for 10h, 16h, 24h and 48h each. Immunoblotting show expression levels of endogenous kinases and over-expressed KinCon reporters. **F**) Impact of 1µM PLX8394 exposure (for 1h) on BRAF and BRAF-V600E KinCon reporters (HEK293T cells) is shown. Representative of n=4 independent experiments is presented. **G**) *R*Luc PCA values have been normalized on the untreated conditions. The mean ±SEM of PLX8394 exposure on BRAF conformation opening and closing 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)

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 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 substratelike 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 both 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.g. 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 dynamic changes of kinases conformations due to mutations, PTMs, protein:protein interactions (PPIs), or binding of bioselective small molecules. 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 cyclindependent kinases 4 and 6 (CDK4/6).

LKB1 is active as trimeric cytoplasmic protein complex. It is the upstream regulator of the AMPactivated protein kinase (AMPK) (Rodríguez et al. (2021)). LKB1 promotes AMPK signaling by forming an heterotrimeric complex with the pseudokinase STE20-related adaptor alpha (STRAD*α*) 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 bioactive small molecules (**Figure 1A**). Conventional methods often fall short in capturing the dynamics of kinase phosphotransferase and scaffolding activities 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 structure dynamics of kinases in intact cells 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 auto-inhibitory modules, so called 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 reflecting conformation changes of full-length kinases. 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 expression offer the flexibility to analyze different time frames for KinCon reporter expression 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 structure 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 a time frame of 10h, 16h, 24h or 48h in HEK293T cells delivers consistently increasing signals for all KinCon reporters (**Figure 1E**, Figure Supplement 1A). Immunoblotting of cell lysates following 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.

Trimeric LKB1 complexes

Upon heterotrimeric complex formation with the pseudokinase STRAD*α* and the scaffolding protein MO25, the kinase LKB1 contributes to the activation of AMPK by phosphorylation at the position Thr172 (**Figure 2A)** (Shackelford and Shaw (2009), Boudeau et al. (2004),Baas et al. (2003)). It is the pseudokinase domain of STRAD*α* 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 STRAD*α* 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)).

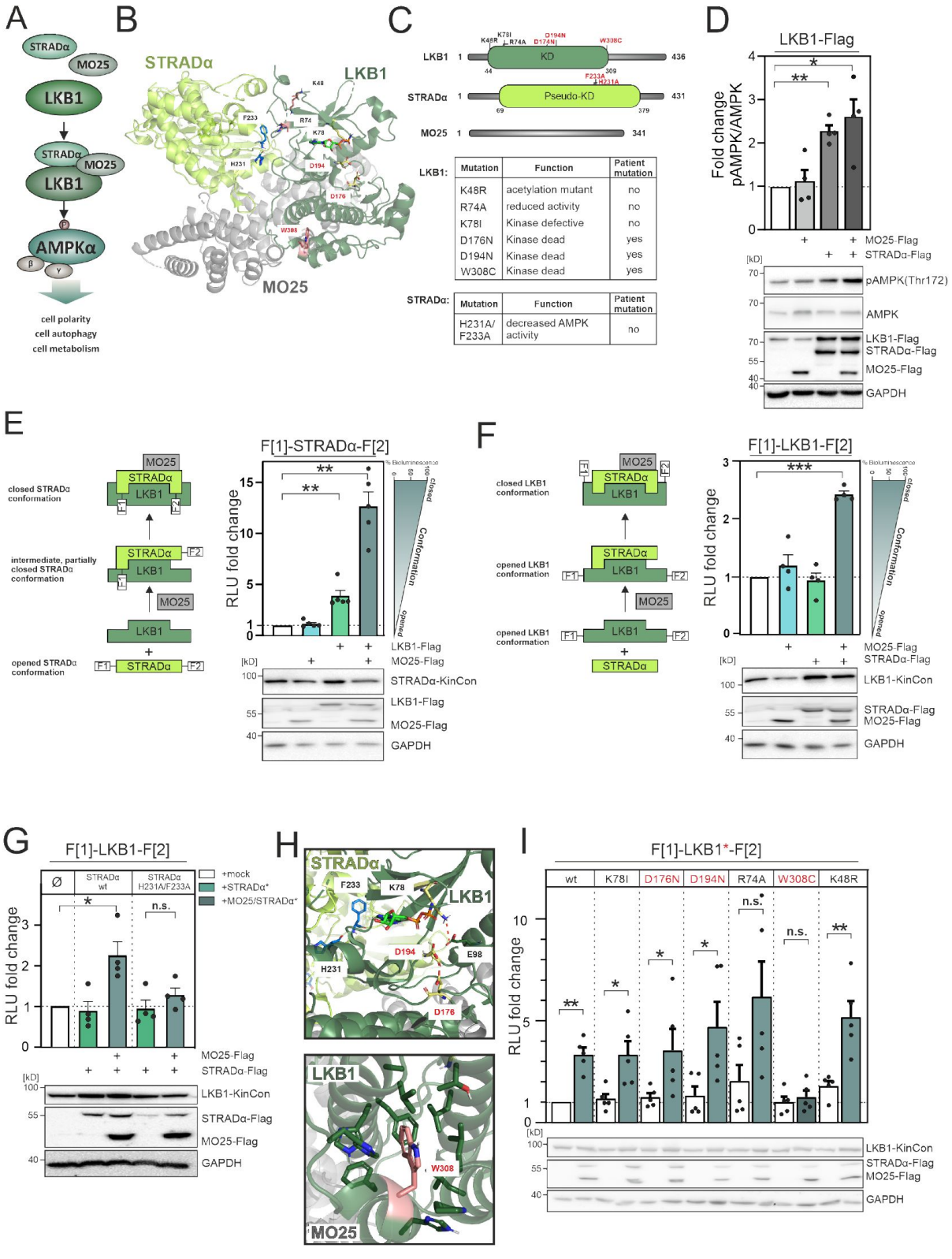
A
STRADα
MO25
LKB1
AMPKα
cell polarity
cell autophagy
cell metabolism
B
STRADα
LKB1
MO25
C
LKB1
KD
STRADα
Pseudo-KD
MO25
LKB1:
Mutation | Function | Patient mutation
K48R | acetylation mutant | no
R74A | reduced activity | no
K78I | Kinase defective | no
D176N | Kinase dead | yes
D194N | Kinase dead | yes
W308C | Kinase dead | yes
STRADα:
Mutation | Function | Patient mutation
H231A/ F233A | decreased AMPK activity | no
D
LKB1-Flag
Fold change pAMPK/AMPK
MO25-Flag
STRADα-Flag
pAMPK(Thr172)
AMPK
LKB1-Flag
STRADα-Flag
MO25-Flag
GAPDH
E
closed STRADα conformation
intermediate, partially closed STRADα conformation
opened STRADα conformation
F[1]-STRADα-F[2]
RLU fold change
Conformation
LKB1-Flag
MO25-Flag
STRADα-KinCon
LKB1-Flag
MO25-Flag
GAPDH
F
closed LKB1 conformation
opened LKB1 conformation
opened LKB1 conformation
F[1]-LKB1-F[2]
RLU fold change
Conformation
MO25-Flag
STRADα-Flag
LKB1-KinCon
STRADα-Flag
MO25-Flag
GAPDH
G
F[1]-LKB1-F[2]
RLU fold change
+mock
+STRADα*
+MO25/STRADα*
MO25-Flag
STRADα-Flag
LKB1-KinCon
STRADα-Flag
MO25-Flag
GAPDH
H
STRADα
LKB1
F233
K78
D194
E98
H231
D176
LKB1
W308
MO25
I
F[1]-LKB1*-F[2]
wt
K78I
D176N
D194N
R74A
W308C
K48R
RLU 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 AMPK*α* signaling via phosphorylation at position Thr172.B) Crystal structure of the LKB1-STRAD*α*-MO25 complex (PDB code 2WTK *(Zeqiraj et al. (2009a))*) representing a snapshot of trimeric complex assembly. The missense mutations we have analyzed are indicated in blue (STRAD*α*) and pale yellow and rose (LKB1). The ATP analogue AMP-PNP is depicted in light green sticks. **C**) Domain organization of human LKB1, STRAD*α* and MO25 (Accession numbers: Q15831, Q7RTN6, Q9Y376) with indication of the kinase and pseudo-kinase domains (KD). Shown in red are tested missense mutations. These are summarized in the table together with their origin and assumed functions (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 co-expressions of indicated kinase complex components on AMPK phosphorylation (HeLa cells, 48h post transfection) (mean ±SEM, n=4 ind. experiments; 3x-Flag is indicated as flag). **E**) Illustration of the KinCon reporter setup for STRAD*α* KinCon measurements: Effect of LKB1-STRAD*α*-MO25 complex formation on the STRAD*α* KinCon reporter opening and closing (HEK293T cells, 48h post transfection). Expression corrected signals (STRAD*α*-KinCon) are shown (mean ±SEM, n=4 ind. experiments). **F**) KinCon reporter setup for LKB1 KinCon measurements: Effect of LKB1-STRAD*α*-MO25 complex formation on the LKB1 KinCon reporter conformation. Expression corrected signals are shown (LKB1-KinCon; (HEK293T cells, 48h post transfection) (mean ±SEM, n=5 ind. experiments). **G**) LKB1-KinCon measurements upon co-expression of indicated proteins displaying the binding deficient STRAD*α* mutations H231A/F233A (HF; see binding interface in **Figure 2B/H)**. Expression corrected signals are displayed (HEK293T cells, 48h post transfection) (mean ±SEM, n=4 ind. experiments). **H**) Structure depiction 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). **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 interactors. Expression corrected signals are displayed (HEK293T cells, 48h post transfection) (mean ±SEM, n=4 ind. experiments). Statistical significance for D, E, F, G, and I: One-sample t-test (*p<0.05, **p<0.01, ***p<0.001)

The capacity of STRAD*α* 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 a 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:STRAD*α*:MO25 on downstream activity of LKB1. We adopted the KinCon technology for measuring involved kinase conformation states. First, we transiently overexpressed the three flag-tagged polypeptides LKB1, STRAD*α* 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 AMPK*α*-T172 following co-expression of both, MO25 and STRAD*α* 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 STRAD*α*. No impact on AMPK phosphorylation was observed when MO25 was over-expressed alone (**Figure 2D**).

Next, we started to investigate the impact of LKB1 and MO25 co-expression on STRAD*α* KinCon conformation dynamics. Notable is the fact that only faint STRAD*α* 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**, Figure Supplement 5A). This data supports the notion that STRAD*α* engages an opened conformation, where C- and N-termini are separated and thus almost no bioluminescence signals can be detected under basal conditions. Upon interaction with LKB1 this conformation shifts to a partially closed intermediate 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**, Figure Supplement 5B).

To validate these findings, we then tested the impact of LKB1-binding deficient STRAD*α*-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 STRAD*α*-LKB1 dimer and greatly hinders STRAD*α*-MO25-LKB1 trimer formation and thereby AMPK activation *(Zeqiraj et al. (2009a))*. We observed that the so-called HF double mutation of STRAD*α* is sufficient to abolish the elevating effect of trimer complex formation (**Figure 2G)**. Indeed both mutations are located in the LKB1/STRAD*α* binding interface, thereby preventing complex formation (Qing et al. (2022), Zeqiraj et al. (2009a)) (**Figure 2H)**. These findings underline that indeed the trimeric complex formation alters the opening and closing of the tested full-length kinase structures using the applied KinCon reporter read out. With this reporter technology we monitored the interaction controlled 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.

In **Figure 2C** we have listed the proposed 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 *α*C-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 STRAD*α* binding interface, and has been reported to interact with STRAD*α* 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 and R74A mutation prevented significant closing of the LKB1 conformation when co-expressed with STRAD*α* 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 STRAD*α* 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, in this case closed kinase 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 the 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 STRAD*α* and LKB1 KinCon conformations represent the more active kinase states.

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)** (Degterev et al. (2019), Clucas and Meier (2023), Silke and Meier (2013)). Genetic alterations of RIPK1 are linked to immune and autoinflammatory diseases(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 central 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 display conformational changes. We promoted necroptosis introducing stimuli by activating the TNF*α* pathway with TNF*α* (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 TNF*α*, 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 biosensors. As consequence we normalized the obtained *R*Luc PCA signals on reporter expression levels. We showed that both KinCon reporters showed markedly reduced bioluminescence in the necroptotic cellular environment (**Figure 3D)**. These results support the notion that both kinases shift to a more opened (ON state) full-length kinase conformation upon pathway activation.

Conformational modulation of the RIPK1 KinCon following TNF*α* 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. 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).

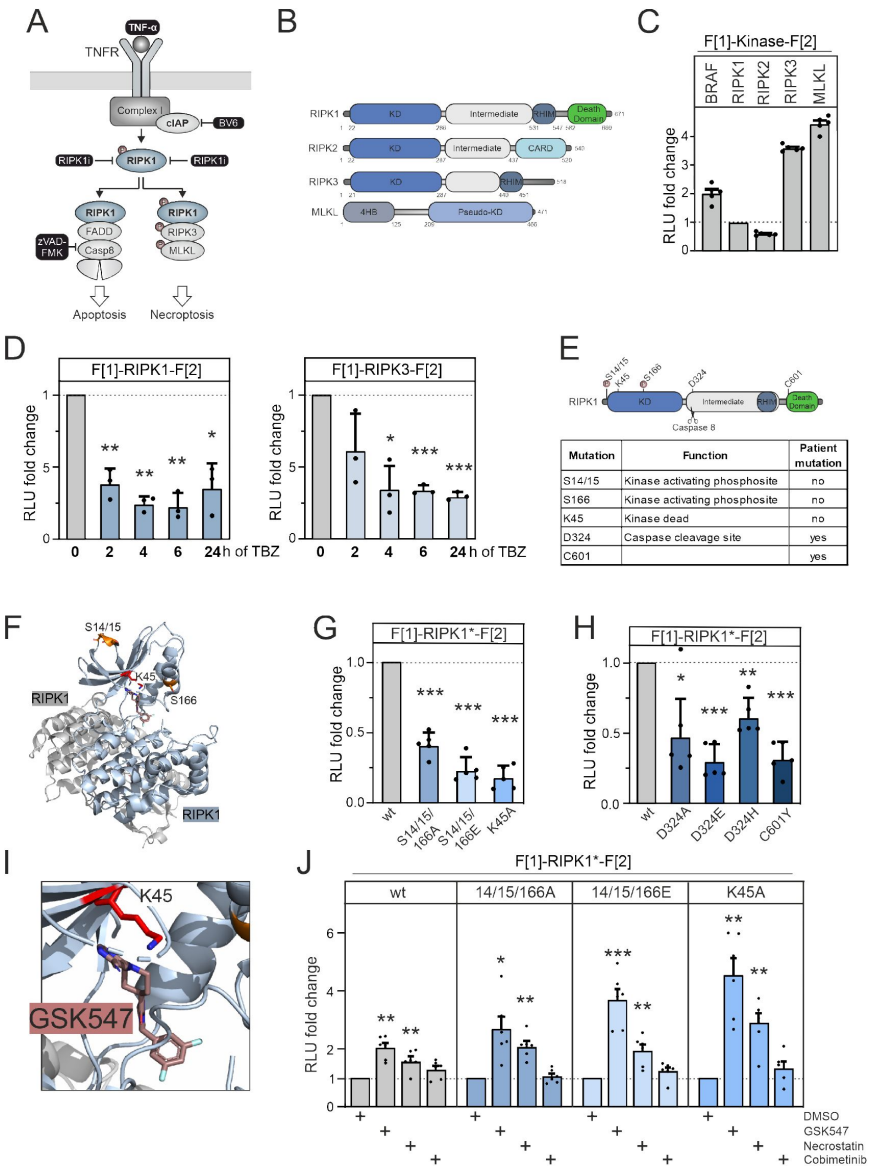

Figure 3.

RIPK1 conformation dynamics.

A) Simplified schematic representations of the activation pathways for apoptosis and necroptosis. Highlighted in black is the combination treatment termed TBZ (10pg/ml TNF*α*, 10nM BV-6, and 20nM zVAD-FMK) that induce necroptosis. **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, (expression corrected) (mean ±SD, n=5 ind. experiments). **D**) Time-dependent treatments using TBZ of HEK293T cells transiently expressing wt RIPK1 (left) and wt RIPK3 (right) KinCon reporters (expression corrected)(mean ±SD, n=3 ind. experiments). **E**) Domain organization of RIPK1 displaying missense mutation sites. **F**) 3D structure of RIPK1 dimers with functional mutations highlighted (PDB code: 6HHO (Wang et al. (2018))). GSK547 is depicted as brown sticks **G**) KinCon reporter signals with/without mutations (S14/15/166A, S14/15/166E, K45A) were measured in a HEK293T RIPK1 knock-out cell line (expression corrected)(mean ±SEM, n=5 ind. experiments). **H**) KinCon reporter signals of RIPK1 (patient loci: D324A, D324E, D324H, C601Y) were measured in HEK293T RIPK1 KO cells (expression corrected) (mean ±SD, 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 (PDB code: 6HHO (Wang et al. (2018))). **J**) RIPK1 reporter signals with indicated mutations (described in G) upon exposure to GSK547 and Necrostatin 1μM, and the MEKi Cobimetinib (1μM, control experiment) or DMSO for 1h (mean ±SD, n=6 ind. experiments, HEK293T RIPK1 KO). Statistical significance for C to J: One-sample t-test (*p<0.05, **p<0.01, ***p<0.001)

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)**. It needs to be noted that additional kinases 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/S15/S166 (referred to as 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 kinasedeficient 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 notion 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 kinasedeficient mutation (K45A) of the KinCon biosensor displayed the strongest opening of the kinase conformation. We used the RIPK1-K45A KinCon to benchmark the time dependent effects of Necrostation and GSK547 on the conformation changes (Figure Supplement 2C). Application of both compounds showed engagement with all the tested RIPK1 KinCon reporters tested. An increase of bioluminescence for all RIPK1 inhibitors was evident reflecting the closing of the full length kinase conformation. Even the phosphorylation mimetic (S14/15/166E) and phosphorylation preventing (S14/15/166A) KinCon reporters showed these phenomenon of promoting a more closed configuration when compared to the unrelated control, the MEK1 kinase inhibitor Cobimetinib (**Figure 3J**, Figure Supplement 2C, 3A). This data underline that application of both allosteric kinase blockers alter the conformations and as we assume scaffolding functions of active and inactive RIPK1 protomers.

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), Scheicher et al. (2015)) Kollmann and Sexl (2013)). These activities are modulated by its molecular interaction partners, particularly cyclins and proteinogenic inhibitors (Goel et al. (2018), Giacinti and Giordano (2006), Ortega et al. (2002)). CDK4/6 deregulation is caused by alterations of

protein expression levels and/or by mutations within 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)).

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 arginine at the position 31 of CDK6 with cysteine (R31C) alters the binding affinities for $p16^{INK4a}$. 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 /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 the tested 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. 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**, Figure Supplement 1C).

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 over-expressed 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. $P16^{INK4a}$ binding deficient CDK4 and CDK6 mutants exhibited a more opened conformation, as indicated by a decrease in bioluminescence (**Figure 4E**). In the following, all KinCon reporters have been

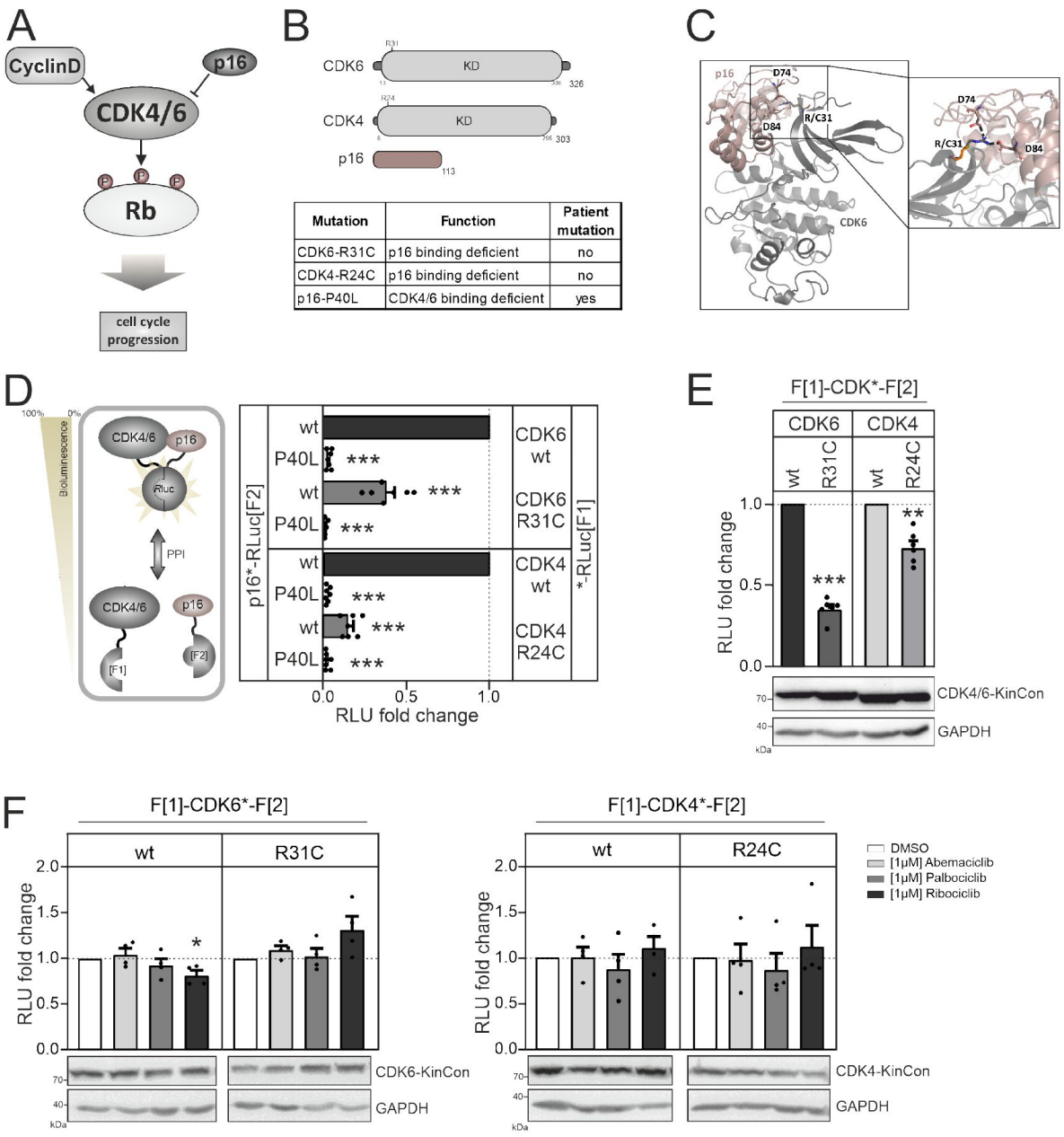

Figure 4.

CDK4/6 interactions and conformations.

A) 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 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 (expressed for 48h in HEK293T cells; expression corrected signals) (mean ±SEM, n=6 ind. experiments). **F**) Quantification of alterations of CDK4/6 KinCon reporter bioluminescence signals (HEK293T, expression for 48h) upon exposure to indicated CDK4/6i (1μM) or DMSO for 3h (mean ±SEM, n=4 ind. experiments). Statistical significance for D-F: One-sample t-test (*p<0.05, **p<0.01, ***p<0.001)

subjected to analyzes upon CDK4/6 inhibitor exposure (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.

Effect of different kinase inhibitor types on the KinCon reporter system

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, 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) 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 mediated phosphorylation of the reporter (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 PKA-C-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.

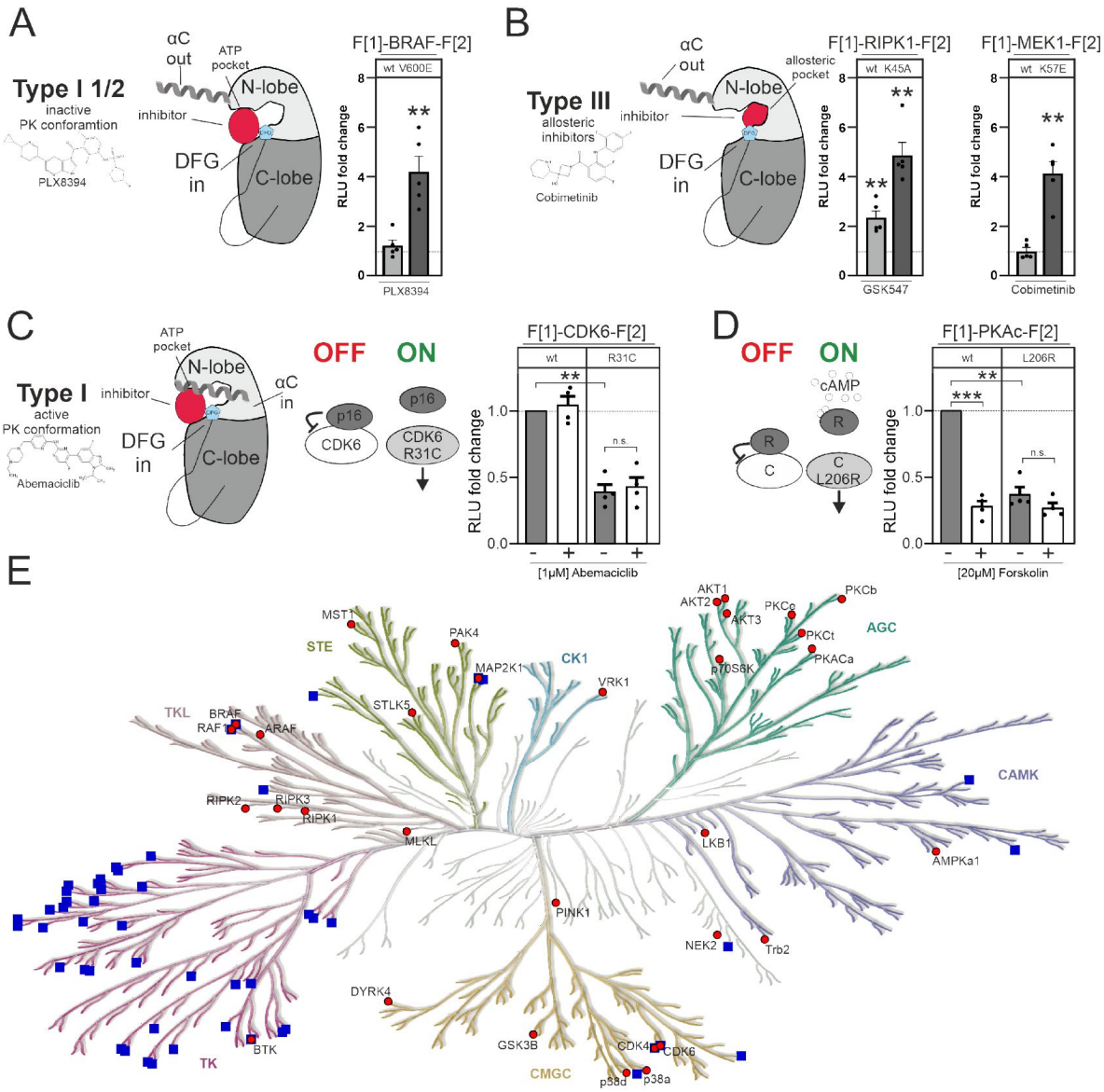

Figure 5.

Impact of small 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 on wt and mutated versions of BRAF, RIPK1 and MEK1 KinCon reporters. 48h post transfection HEK293T cells expressing respective reporter constructs were treated with indicated inhibitors for 1h (1μM) followed by Rluc PCA analyses (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 post transfection HEK293T cells expressing respective reporter constructs were treated with indicated inhibitors for 3h (1μM) followed by Rluc PCA analyses (mean ±SEM, n=4 ind. experiments). **D**) Bioluminescence measurement of PKAc wt and L206R KinCon reporters. HEK293T cells expressing the reporter were treated with 20 μM of Forskolin for 15 min followed by Rluc PCA analyses (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 kinases 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)

In this context of activating kinase mutations we showed that using PKAc (wt and L206R) and BRAF (wt and V600E) reporters as example we could not track alterations of cytoplasmic and nuclear localization (Figure Supplement 4).

Furthermore, subcellular localization of PKA-C KinCon reporters did not change when L206R mutant was introduced (Figure Supplement 4). As a control BRAF wt and V600E KinCon reporters were used and also no changes in localization were observed.

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 human kinases is targeted by approved drugs. 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. Many of these miss suitable tools/read outs for basic research analyses of physiological and pathological kinase functions. Such kinases have been referred to be part of the so called '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)**. These conformation reporters represent an extendable toolbox for gaining first and also deeper insights into alterations of kinase activity related conformation states induced by different means of inhibitors, activators, binders or mutations. Systematic implementation of this technology in kinase research programs would assist in advancing our understanding of kinase and pseudokinase regulations and functions.

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 successfully targeted by FDA-approved kinase blockers so far (Lahiry et al. (2010), Politi et al. (2015)). Moreover, 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 more extensive insights into cellular drug-target engagement, 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. So far, this relates to the tracking of kinase-scaffold and pseudo-kinase functions.

Key advantages of the KinCon reporter technology is the robustness of the system to track kinase conformations at varying expression levels. However, in contrast to fluorescence-based reporter read-outs subcellular analysis and cell sorting are still challenging due to comparable low levels of light emission.

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 lossof-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 STRAD*α*-LKB1-MO25 was necessary for signal propagation to AMPK. In line with this, the LKB1 and STRAD*α* 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 STRAD*α* was introduced (**Figure 2G**). Further, we showed that tracking structural rearrangements induced by diverse types of mutations can be quantified using KinCon technology (**Figure 2I**). In this setting KinCon measurements of either the kinase (LKB1) or pseudokinase (STRAD*α*) report trimeric complex formation which is pivotal for cytoplasmic kinase pathway activation (**Figure 2E, F**). In contrast to MEK1 and BRAF KinCons, the closed STRAD*α* and LKB1 KinCon conformations seem to represent the active kinase conformations.

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. Additionally, we hypothesize that it could potentially impact the effectiveness of drug binding.

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 in the tested cell setting (**Figure 4F**).

In summary, we have extended the KinCon reporter scope of applications. Besides monitoring conformational changes induced by drugs and drug candidates, the reporter system can be applied to accurately track the formation of activated and kinase centered protein complexes. Besides its predictive value in assessing drug effectiveness, the KinCon technology helps consider or identify cellular factors that impact drug candidate binding. KinCon reporters provide unique insights into the molecular dynamics of kinase structure rearrangements. Understanding the molecular motion of kinases 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 minior 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. Transient transfections were performed with JetPRIME DNA and siRNA transfection reagent (Polyplus supplied by VWR, 101000046).

For the HEK293 RIPK1 KO cells HEK293 parental cells were transfected with pMA-T-gRNA-RIP1 targeting (synthetic construct expressing the guide) and Cas9-GFP (pSpCas9(BB)-2A-GFP-Addgene 48138). Both plasmids (one expressing the guide and the other cas9) were transfected with Lipofectamine LTX (Thermo Fisher Scientific). Cells were FACS (BD FACSymphony S6) sorted into single cell clones and analysed for RIPK1 expression by Western Blot analysis.

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-AMPK*α* (Thr172) (40H9) (Cell Signaling, 2532), rabbit-anti-Phospho-AMPK*α* (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 were 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), TNF*α* (MCE Med Chem Express, HY-P7090A), z-VAD(OMe)-FMK (MCE Med Chem Express, HY-16658), BV6 (MCE Med Chem Express, HY-16701).

Representation of data

In **Figure 1 E** and **F**, a representative experiment of n=4 independent experiments is shown. In these cases absolute bioluminescence values without any normalisation are shown. Otherwise, data was indicated as RLU fold change. This means the data was normalized on the indicated control condition (either with normalization of the western blot or without; as indicated).

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-STRAD*α*-MO25*α* (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

Patents

Aspects of the present study are subject of patents and pending patent application.

Author Contributions

Conceptualization, J.T., V.S., P.M., T.K., E.S.; methodology, E.S.; validation, formal analysis, investigation, data curation, V.K., S.S., S.S., A.F., F.E, J.F., T.K.; writing—original draft preparation, V.K., S.S., S.S,. E.S.; writing—review and editing, E.S., P.T., T.K.; supervision, project administration, funding acquisition, E.S. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

E.S and P.T. are co-founders of KinCon biolabs.

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Editors

Reviewer #1 (Public Review):

Summary:

This technical report by Kugler at al., expands the application of a fluorescence-based reporter to study the conformational state of various kinases. This reporter, named KinCon (Kinase Conformation), interrogates the conformational state of a kinase (i.e., active vs. inactive) based on engineering complementary fusion proteins that fluoresce upon interaction. This assay has several advantages as it allows studying full-length kinases, that is, the kinase domain and regulatory domains, inside the cell and under various experimental conditions such as the presence of inhibitors or activator proteins, and in wildtype and mutants involved in disease states.

Strengths:

One major strength of this study is that it is quite comprehensive. The authors use KinCon for four different kinases, BRAF, LKB1, RIP and CDK4/6. These kinases have very different regulatory elements and associated proteins, which the authors explore to study their conformational state. Moreover, they use small molecule inhibitors or mutations to further dissect how the conformational state of the kinase in disease states. The collective set of results strongly suggests that KinCon is a versatile tool that can be used to study many kinases of biomedical and fundamental importance. Given that kinases are extensively studied by researchers in academia or industry, KinCon could have a broad impact as well.

Weaknesses:

This manuscript, however, also has several weaknesses that I outline below. These weaknesses decrease the overall level of impact on the manuscript, as is.
• The manuscript is exceedingly long. For instance, the introduction provides background information for each kinase that is further expanded in the results section. I think the background information for each kinase in the Introduction and Results sections can be significantly reduced to highlight the major points. Otherwise, not only does the manuscript become too long, but also the main points get diluted.

• Similarly, the figure legends are very long, providing information that is already in the main text or in Methods. The authors should provide the essential information to understand the figure.

• A major concern throughout the manuscript is the use of the word "dynamics," which is used in the text in various contexts. The authors should clarify what they understand for dynamics of conformation. Are they measuring how the time-dependent process by which the kinase is interconverting between active and inactive states? It seems to me that the assays in this report evaluate a population of kinases that are in an open or close conformation (i.e., a particular state in each experimental condition) but there is not direct information how the kinase goes from one state to the other. In that sense, the use of dynamics is unclear. Also, the use of dynamics in different sentences in ambiguous. Here are a few examples but this should be revised throughout the manuscript:
- Line 27: dynamics of full-length protein kinases. Is this referred to dynamics of conformational interconversion between inactive and active states?
- Line 138: dynamic functioning of kinases. No clear what that means.
- Line 276: ... alters KinCon dynamics. Not clear if they are measuring time-dependent process or a single point.
- Figure legend 4F: dynamics of CDK4/6 reporters. Again, not clear how the assay is measuring dynamics.
Nonetheless, in my opinion the authors use proper terminology that describes their assay in which the term dynamics is not used: Title (... impact of protein and small molecule interactions on kinase conformations) and Line 89 (... reporter can be used to track conformational changes of kinases...)

• The authors use the phrase that KinCon has predictive capabilities (abstract and line 142). What do the authors refer to this?

• The authors indicate that KinCon is a highly sensitive assay. Can the authors elaborate on what high sensitivity means? For example, can they discuss how other fluorescence-based approaches that are less sensitive would not be able to accomplish the same type of results or derive similar conclusions? Can they provide a resolution metric both in space and time? Given that the authors state that this is a technical report, this information is of relevance.

• The authors nicely describe how KinCon works in Figure 1B and part of 1C. I do think that the bottom of panel 1C needs to be revised, as well as the text describing the potential scenarios of potency, efficacy and synergism.
- One issue with this part of Figure 1C is that it is not clear what the x-axis in the 3 plots refer to. Is this time? Is this concentration of a small molecule, inhibitor or binding partner? This was confusing also in the context of the term dynamics used throughout the text. The terms potency, efficacy and synergism should be subtitles or the panels and the x-axis should be better defined, especially for a non-specialized reader.
- Related to this part of Figure 1C is the text. The authors mention potency, effectiveness and synergy (Line 195). Can the authors use more fundamental terminology related to these three scenarios, for example, changes in activation constant, percent of protein activates? Also, why synergy is only related to effectiveness? Can synergy also be associated to potency?
- Lastly, the use of these three cartoons gives the impression that the experimental results to come will follow a similar representation. Instead, the results are presented in bar plots for many different conditions. I think this will lead to confusion for a broad audience.

• For a non-expert reader, can the authors clarify the use of tracking basal conformations vs. transient over-expression of the various KinCon constructs? Moreover, the authors use the term transient over-expression for 10, 16, 24 and 48 h (Line 203). This, to a non-expert reader, seems not transient.

• Regarding Figure 1E and similar graphical representations: Why is the signal (RLU) non-linear with time? If the fluorescence of the KinCon construct is linearly related with its expression or concentration inside the cell, one would expect a linear increase. Have the authors plotted RLU/Expression band intensity to account for changes in protein

concentration? For instance, some of the results within Figure 3 are normalized to concentration on the reporter expression level.

• For the results with LKB1, the authors claim that intermediate fold change in fluorescence (Figure 2E) is due to a partially closed intermediate state (Line 262). Can the authors discard the possibility by which there is a change in populations of active and inactive that on average give intermediate values?

• The authors claim in Line 274 that mutations located at the interface of the LKB1/STRADalkpha complex affect interactions and hypothesize that allosteric communication between LKB1 and STRADalpha is essential for function. Given that this mutations are at the interaction interface, why would the authors postulate an allosteric mechanism that evokes an effect distant to the interaction/active site? Could it be that function requires surface contacts alone that are disrupted by the mutations?

• I was unable to find text to explain the following: Figure 2I shows the mutation R74A as n.s., but in the text only W308C is mentioned to not change fluorescence. Could the authors clarify why R74A is not discussed in the text? Maybe this reviewer missed the text in which it was discussed. Similarly, the author states in line 326 that the study included an analysis of RIPK2. However, I was unable to find results, graphs or additional text discussing RIPK2.

• Some figures of RLU use absolute values, percentages and fold change. Is there a reason why the authors use different Y-axis values? These should be explained and justified in Methods. Similarly, bars for wt in Figures 3D, G, or 4D, E,F show no errors. How are the authors normalizing the data and repeats so that there is no error, and are they treating the rest of the data (i.e., mutants and/or treated with small molecules) in the same way?

• Lastly, the section starting in Line 472 reads more like a discussion of results from different type of inhibitors used in this study that results on its own. The authors should consider a new subtitle as results or make this section a discussion.

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Reviewer #2 (Public Review):

Summary:

Protein kinases have been very successfully targeted with small molecules for several decades, with many compounds (including clinical drugs) bringing about conformational changes that are also relevant to broader interactions with the cellular signaling networks that they control. The authors set out to develop a targeted biosensor approach to evaluate distinct kinase conformations in cells for multiple kinases in the context of incoming signals, other proteins and small molecule binding, with a broad goal of using the KinCon assay to confirm (and perhaps predict) how drug binding or signal perception changes conformations and outputs in the presence of cellular complexes; this work will likely impact on the field with cellular reporters of kinase conformations a useful addition to the toolbox.

Strengths:

The KinCon reporter platform has previously been validated for well-known kinases; in this study, the team evaluate how to employ a full-length kinase (often containing a known pathological mutation). The sensitive detection method is based on a Renilla luciferase (RLuc)protein fragment complementation assay, where individual RLuc fragments are present at the N and the C terminus of the kinase. This report, which is both technical and practical in nature, co-expresses the kinase with known interactors (at low levels) in a high throughput format and then performs pharmacological evaluation with known small

molecule kinase modulators. This is explained nicely in Figure 1, as are the signaling pathways that are being evaluated. Data demonstrate that V600E BRAF iexposed to vemurafenib is converted to the inactive conformation, as expected. In contrast, the more closed STRADα and LKB1 KinCon conformations appear to represent the more active state of the complexed kinase, and a W308C mutation (evaluated alongside others) reverses this effect. The authors then evaluated necroptotic signaling in the context of RIPK1/3 under conditions where RIPK1 and RIPK3 are active, confirming that the reporters highlight the active states of both kinases. Exposure to compounds that are known to engage with the RIPK1 arm of the pathway induce bioluminescence changes consistent with the opening (inactivation) of the kinase. Finally, the authors move to an important drug target for which clinical drugs have arrived relatively recently; the CDK4/6 complexes. These are of additional importance because kinase-independent functions also exist for CDK6, and the effects of drugs in cells usually relies on a downstream marker, rather than demonstration of direct protein complex engagement. The data presented are interpreted as the formation of complexes with the CDK inhibitor p16INK4a; reducing the affinity of the interaction through mutations drives an inactive conformation, whilst the application of CDK4/6 inhibitors does not, implying binding to the active conformation.

Weaknesses:

(1) The work is very solid, and uses examples from the literature and also extends into new experimental space. An obvious weakness is mentioned by the authors for the CKDK data, in that measurements with Cyclin D (the activating subunit) are not characterised, although Cyclin D might be assumed to be present?
(2) The work with the trimeric LKB1 complex involves pseudokinase, STRADalpha, whose conformation is also examined as a function of LKB1 status; since STRAD is an activator of LKB1, a future goal should be the evaluation of the complex in the presence of STRAD inhibitory/activating small molecules.

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Author response:

The following is the authors' response to the original reviews.

We would like to thank you and the two Reviewers for the thoughtful evaluation of the manuscript and the support for publication. We have addressed all points raised by the two Reviewers.

- We have extensively streamlined the manuscript. Repetitive passages regarding the respective kinase cascades have been removed.

- We improved the presentation of the main Figures (mainly labeling and font size):

- Figure 1: C, D, E, F o Figure 2: C, E, F, G, I, o Figure 3: D o Figure 4: F

- Figure 5: A, B, C, D, E

- We integrated new SI-data related to kinase functions, expression and the 'cell-type comparisons' of the KinCon reporter system (Figure Supplement 4, 5).

Below you will find a detailed point-by-point response.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Regarding the issue of the use of the word "dynamics," as described in the public review, here are a few examples of ambiguous use in different sentences: o Line 27: dynamics of*

> *full-length protein kinases. Is this referring to the dynamics of conformational interconversion between inactive and active states?*
>
> *- Line 138: dynamic functioning of kinases. It is not clear what this means. o Line 276: … alters KinCon dynamics. Not clear if they are measuring time-dependent process or a single point.*
>
> *- Figure legend 4F: dynamics of CDK4/6 reporters. Again, not clear how the assay is measuring dynamics.*
>
> *In my opinion, the authors use proper terminology that describes their assay in which the term dynamics is not used: Title: "… impact of protein and small molecule interactions on kinase conformations" and Line 89 "… reporter can be used to track conformational changes of kinases…".*

We have replaced the “dynamics” sections.

- Line 27: The understanding of the structural dynamics of…

- Line 91: This reporter can be used to track dynamic changes of kinases conformations…

- Line 139: Conventional methods often fall short in capturing the dynamics of kinases within their native cellular environments…

- Line 146: Such insights into the molecular structure dynamics of kinases in intact cells…

- Line 199: In order to enhance our understanding of kinase structure dynamics…

- Line 276: These findings underline that indeed the trimeric complex formation alters….

- Figure Legend 4F: Quantification of alterations of CDK4/6 KinCon reporter bioluminescence signals…

> *The authors state that KinCon has predictive capabilities (abstract and line 142). What do the authors mean by this?*

Previously we have benchmarked the suitability of the KinCon reporter for target engagement assays of wt and mutated kinase activities. With this we determined specificities of melanoma drugs for mutated BRAF variants (Mayrhofer 2020, PNAS).

> *The authors indicate that KinCon is a highly sensitive assay. Can the authors elaborate on what high sensitivity means?*

With sensitivity we mean that we can detect conformation dynamics of the reporter at low expression levels of the hybrid protein expressed in the cell line of choice.

- Line 209: Immunoblotting of cell lysates following luminescence measurements showed expression levels of the reporters in the range and below the endogenous expressed kinases (Figure 1E). …

- Line 219: 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).

- Line 221: These data underline that at expression levels far below the endogenous kinase, protein activity conformations can be tracked in intact cells. …

> *For example, can they discuss how other fluorescence-based approaches that are less sensitive would not be able to accomplish the same type of results or derive similar conclusions? Can they provide a resolution metric both in space and time? Given that the authors state that this is a technical report, this information is of relevance.*

We highlight the key pros & cons of the KinCon reporter technology in following sections:

-Line 529: 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. So far, this relates to the tracking of kinase-scaffold and pseudo-kinase functions.

- Line 535: Key advantages of the KinCon reporter technology is the robustness of the system to track kinase conformations at varying expression levels. However, in contrast to fluorescence-based reporter read-outs subcellular analysis and cell sorting are still challenging due to comparable low levels of light emission

> *The authors nicely describe how KinCon works in Figure 1B and part of 1C. I do think that the bottom of panel 1C needs to be revised, as well as the text describing the potential scenarios of potency, efficacy, and synergism.*
>
> *One issue with this part of Figure 1C is that it is not clear what the x-axis in the 3 plots refers to. Is this time? Is this concentration of a small molecule, inhibitor, or binding partner? This was confusing also in the context of the term dynamics used throughout the text. The terms potency, efficacy, and synergism should be subtitles, or the panels and the x-axis should be better defined, especially for a non-specialized reader.*
>
> *Related to this part of Figure 1C is the text. The authors mention potency, effectiveness, and synergy (Line 195). Can the authors use more fundamental terminology related to these three scenarios, for example, changes in activation constant, and percent of protein activates? Also, why synergy is only related to effectiveness? Can synergy also be associated with potency?*

Thank you for bringing this up, we have revised Figure 1C to better reflect the mentioned effects of potency. To avoid confusion, we removed the illustration for drug synergism. Accordingly, we have integrated the axis descriptions for the presented dose-response curves.

Thus, we have further streamlined the text in the introduction – examples are shown below:

- Line 195: 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)

- Figure 1C: 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. Expression of the genetically encoded reporter in indicated 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 respective endogenous kinase, interlinked

kinases or proteinogenic regulators of the respective pathway. After systematic administration of pathway modulating drugs or drug candidates, analyses of KinCon structure dynamics may reveal alterations in potency, efficacy, and potential synergistic effects of the tested bioactive small molecules (schematic dose response curves are depicted)

> *Lastly, the use of these three cartoons gives the impression that the experimental results to come will follow a similar representation. Instead, the results are presented in bar plots for many different conditions. I think this will lead to confusion for a broad audience.*

The bottom panel of Figure 1C is not the depiction of real experiments but rather an illustration of fitted dose-response curves. We would like to present previous demonstrations of doseresponse curves using BRAF KinCon data and ERK phosphorylation (Röck 2019, Sci. Advances)

We further agree with the reviewer and have therefore added a new part in the methods section addressing the evaluation of data extensively.

- Line 668: In Figure 1 E and F, a representative experiment of n=4 independent experiments is shown. In these cases, absolute bioluminescence values without any normalization are shown. Otherwise, data was indicated as RLU (relative light unit) fold change. This means the data was normalized on the indicated control condition (either with normalization of the western blot or without; as indicated.

> *For a non-expert reader, can the authors clarify the use of tracking basal conformations vs. transient over-expression of the various KinCon constructs? Moreover, the authors use the term transient over-expression for 10, 16, 24, and 48 h (Line 203). This, to a non-expert reader, does not seem transient.*

We have revised the manuscript to clarify it:

- Line 207: We showed that transient over-expression of these KinCon reporters for a time frame of 10h, 16h, 24h or 48h in HEK293T cells delivers consistently increasing signals for all KinCon reporters (Figure 1E, Figure Supplement 1A).

- Figure 1E) Representative KinCon experiments of time-dependent expressions of indicated KinCon reporter constructs in HEK293T cells are shown (mean ±SEM). Indicated KinCon reporters were transiently over-expressed in 24-well format in HEK293T cells for 10h, 16h, 24h and 48h each.

> *Regarding Figure 1E and similar graphical representations: Why is the signal (RLU) nonlinear with time? If the fluorescence of the KinCon construct is linearly related to its expression or concentration inside the cell, one would expect a linear increase. Have the authors plotted RLU/Expression band intensity to account for changes in protein concentration? For instance, some of the results within Figure 3 are normalized to concentration on reporter expression level.*

Out intention was to show that varying expression levels can be used for the illustrated target engagement assays.Indeed, the represented elevations of RLU might be due to factors such as:

- Doubling times of cells

- Cell density

- Media composition (which changes over time)

- Reporter protein stabilities

- Abundance of interactors of kinases

> *For the results with LKB1, the authors claim that intermediate fold change in fluorescence (Figure 2E) is due to a partially closed intermediate state (Line 262). Can the authors discard the possibility by which there is a change in populations of active and inactive that on average give intermediate values?*

Based on our experience with KinCon reporter conformation states of kinases we tested so far, we assume that the presented data reflects an intermediate state. We agree that it needs further validation. We have changed the text accordingly:

- Line 264: Upon interaction with LKB1 this conformation shifts to a partially closed intermediate state.

> *The authors claim in Line 274 that mutations located at the interface of the LKB1/STRADalpha complex affect interactions and hypothesize that allosteric communication between LKB1 and STRADalpha is essential for function. Given that these mutations are at the interaction interface, why would the authors postulate an allosteric mechanism that evokes an effect distant from the interaction/active site? Could it be that function requires surface contacts alone that are disrupted by the mutations?*

We agree with the reviewer and changed our argumentation for this point:

- Line 276: These findings underline that indeed the trimeric complex formation alters the opening and closing of the tested full-length kinase structures using the applied KinCon reporter read out

> *I was unable to find text to explain the following: Figure 2I shows the mutation R74A as n.s., but in the text, only W308C is mentioned to not change fluorescence. Could the authors clarify why R74A is not discussed in the text? Maybe this reviewer missed the text in which it was discussed.*

We adapted the manuscript and include the R74A mutation as followed:

- Line 296: Among these mutations, only the W308C and R74A mutation prevented significant closing of the LKB1 conformation when co-expressed with STRADα and MO25 (Figure 2I).

In Figure 2I where the individual measurements of the LKB1-R74A KinCon are highlighted in red to better emphasize the deviations. In the case of the R74A mutation the effect seen might be due to the high deviation between the experiments (Highlighted in red). These deviations are much higher when compared to either the wt or the W308 mutant, and can also be seen in the LKB1-R74A-KinCon only condition (white). Even though no significant closing of the LKB1 conformation could be observed in the case of R74A, we believe, since the trend of the conformation closing upon complex formation is still visible that the effect is still there. Further replicates would be necessary to validate this theory.

> *Similarly, the authors state in line 326 that the study included an analysis of RIPK2. However, I was unable to find results, graphs, or additional text discussing RIPK2.*

The RIPK2 conformation was analyzed in Figure 3C (page 12).

> *Some figures of RLU use absolute values, percentages, and fold change. Is there are reason why the authors use different Y-axis values? These should be explained and justified in Methods. Similarly, bars for wt in Figures 3D, G, or 4D, E, F show no errors. How are the authors normalizing the data and repeats so that there is no error, and are*

> *they treating the rest of the data (i.e., mutants and/or treated with small molecules) in the same way?*

We have changed the Y-axis values. Now, throughout the manuscript we show that there is a RLU fold-change. Except are selected experiments when solely absolute RLU values are shown (such as Figure 1E, F). We have also decided to integrate a paragraph into the methods section (Line 655). Figure 3D was changed as well.

- Line 668: In Figure 1 E and F, a representative experiment of n=4 independent experiments is shown. In these cases absolute bioluminescence values without any normalisation are shown. Otherwise, data was indicated as RLU fold change. This means the data was normalized on the indicated control condition (either with normalization of the western blot or without; as indicated).

The data is generally normalized on wt or untreated conditions, when the cells were treated with small molecules for target engagement assays.

> *Lastly, the section starting in Line 472 reads more like a discussion of results from different types of inhibitors used in this study that results on its own. The authors should consider a new subtitle such as results or make this section a discussion.*

We agree with the reviewer and this part of the results was split into a new section of the result:

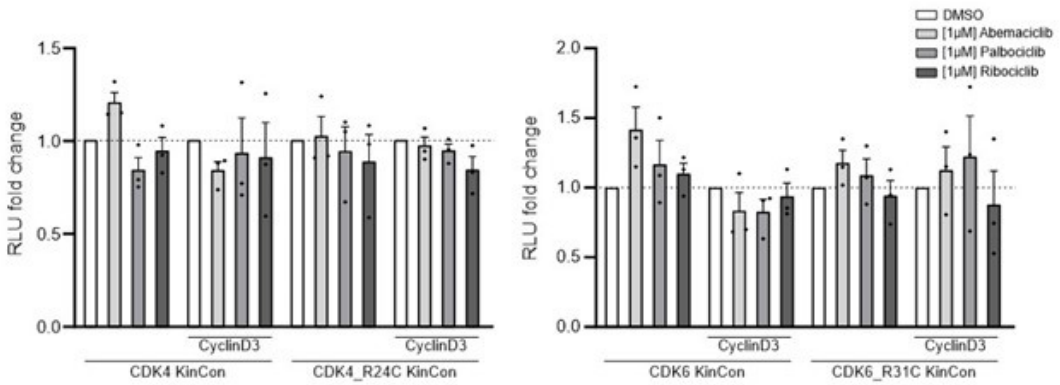
- Line 455: “Effect of different kinase inhibitor types on the KinCon reporter system”.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *I have a few suggestions, since the paper is a distillation of a vast amount of work and tells a useful story.*
>
> *(1) The work is very solid, uses examples from the literature, and also extends into new experimental space. An obvious weakness is mentioned by the authors for the CKD data, in that measurements with Cyclin D (the activating subunit) are not characterized, although Cyclin D might be assumed to be present.*

We performed experiments with the CDK4/6 KinCon reporters and co-expressed CyclinD with a ratio of 1:3 (HEK293T cells, expression for 48h). However, in the context of inhibitor treatments we could not track conformation changes in these initial experiments. The cells were treated with the indicated CDK4/6i [1µM] for 3h. This seems to not impact the conformation of CDK4/6 wt or mutated KinCon reporters. There is a tendency that CyclinD co-expression promotes CDK4/6 conformation opening (data not shown).

Author response image 1.

Bioluminescence signal of CDK4/6 KinCon reporters with co-expressed CyclinD3 (HEK293T, expression for 48h) upon exposure to indicated CDK4/6i [1µM] or DMSO for 3h (mean ±SEM, n=3 ind. experiments). No significant changes using the current setting.



> *(2) The work with the trimeric LKB1 complex involves pseudokinase, STRADalpha, whose conformation is also examined as a function of LKB1 status; since STRAD is an activator of LKB1. A future goal should be the evaluation of the complex in the presence of STRAD inhibitory/activating small molecules.*

Thank you for this great idea, we are currently compiling a FWF grant application to get support for such a R&D project.

> *Minor points*
>
> *• Have any of the data been repeated in a different cell background? This came to mind because HeLa cells lack LKB1, which might be a useful place to test the LKB1 data in a different context.*

This experiment was performed and we show it in Figure Supplement 5. Further, we followed the advice of the reviewer and performed suggested experiments. We integrated the colon cancer cell line SW480 into the experimental setup. Overall, three cell settings showed the same pattern of KinCon reporter analyses for LKB1-STRADα-MO25 complex formation utilizing the LKB1- and STRADα-KinCon reporters.

> *• The study picks up the PKA Cushings Syndrome field, which makes sense, and data are presented for L206R. PMID 35830806 explains how different patient mutations drive different signaling outcomes through distinct complex formations, and it would be interesting to discuss how mutations in KinCon complexes, especially those with mutations, could affect sub-cellular localization. Could the authors explain if this was done for any of the proteins, whose low experimental expression is a clear advantage, but is presumably hard to maintain across experiments?*

The feedback of the reviewer motivated us to perform subcellular fractionation experiments. They were performed with PKAc wt and L206R KinCon reporters as well as BRAF wt and V600E reporters. We were not able to see major differences between the wt and mutated reporter constructs in respect to their nucleus: cytoplasm localizations (Figure Supplement 4). For your information, in a R+D project with the mitochondrial kinase PINK1 we see localization of the reporter as expected almost exclusively at the mitochondria fraction.

- Line 495: In this context of activating kinase mutations we showed that using PKAc (wt and L206R) and BRAF (wt and V600E) reporters as example we could not track alterations of cytoplasmic and nuclear localization (Figure Supplement 4). Furthermore, subcellular localization of PKAc KinCon reporters did not change when L206R mutant was introduced

(Figure Supplement 4). As a control BRAF wt and V600E KinCon reporters were used and also no changes in localization was observed.

> • *I suggest changing PMs (Figure 2 and others) simply to mutation, I read this as plasma membrane constantly.*

We agree and we have changed it to “patient mutation” in Figure 2C, Figure 3E, Figure 4B.

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