

## REVIEW

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## Kinase signaling cascades: an updated mechanistic landscape

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Here, we shed physico-chemical light on major kinase signal transduction cascades in cell proliferation in the Ras network, MAPK and PI3K/AKT/mTOR. The cascades respond to external stimuli. The kinases are allosterically activated and relay the signal, leading to cell growth and division. The pathways are crosslinked, with the output of one pathway influencing the other. The effectiveness of their allosteric signaling relay stems from coordinated speed and precision. *These qualities are essential for cell life—yet exactly how they are obtained and regulated has challenged the community over four decades.* Here, we define their nature by their kinases' repertoires, substrate specificities and breadth, activation and autoinhibition mechanisms, catalytic rates, interactions, and their dilution state. The cascades are lodged in a dense molecular condensate phase at the membrane adjoining RTK clusters, where their assemblies promote specific, productive signaling. Aiming to shed further physico-chemical light, we ask (i) how starting the cascades with a single substrate and ending with hundreds is still labeled specific; (ii) what we can learn from their different number of mutations; and (iii) why B-Raf unique side-to-side inverse dimerization slows ERK activation and signaling. We point to the (iv) chemical mechanics of the distributions of rates of the crucial MAPK cascade: slower at the top and rapid at the bottom. Finally, the cascades provide inspiration for pharmacological perspectives. Collectively, our updated physico-chemical outlook provides the molecular basis of targeting protein kinases in cancer and spans mechanisms and scales, from conformational landscapes to membraneless organelles, cells and systems levels.

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## Introduction

Kinase signaling cascades underlie life processes, including diseases.<sup>1–6</sup> Through their interactions, allostery plays a major role.<sup>7–12</sup> We focus on the classical components of the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) cascades in the Ras signaling network and their small molecule pharmacology. Numerous excellent reviews have been written on kinases and their signaling, especially focusing on these pathways, including by us (*e.g.*, ref. 13–23). Here, we consider the attributes and characteristics of kinases. We consider their mechanisms, roles, organization and positions in their cascades, and whether their collective differential characteristics can seed a new drug outlook.

MAPK is a complex interconnected kinase signaling cascade (Fig. 1). Its multiple kinases are commonly mutated and targeted in cancer. Drug resistance is a major problem, primarily

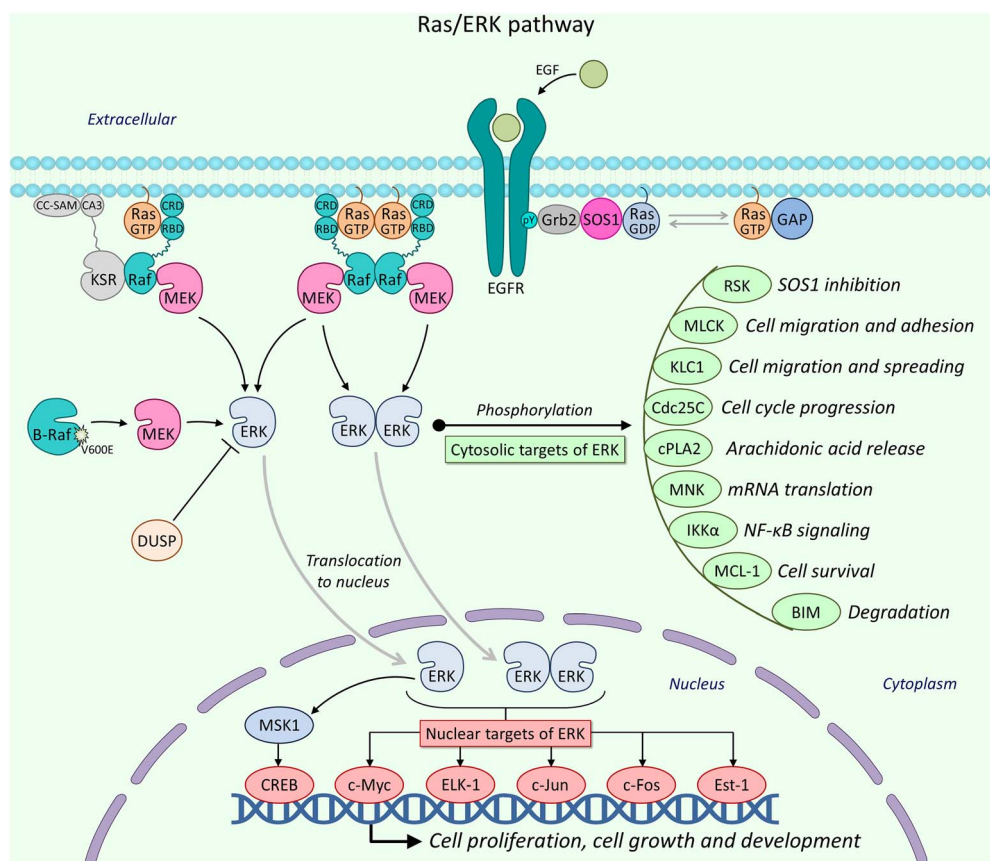
because of pathway crosstalk and bypasses.<sup>14,24–26</sup> Growth factors (*e.g.*, epidermal growth factor, EGF) bind the extracellular domains of receptor tyrosine kinases (RTKs, *e.g.*, epidermal growth factor receptor, EGFR, and platelet-derived growth factor receptor, PDGFR) spanning the cell membrane, stimulating their signal transduction cascades.<sup>27–32</sup> The MAPK cascade includes the Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway.<sup>14,33,34</sup> Ras, a key signaling protein, is activated by growth factors binding to an RTK. Stimulated RTK recruits the adaptor protein, growth factor receptor bound protein 2 (Grb2). Grb2 recruits the Ras guanine nucleotide exchange factor (GEF), such as Son of Sevenless 1 (SOS1), translocating SOS1 to the plasma membrane. At the membrane, it binds membrane-anchored Ras, activating it by exchanging GDP for GTP.<sup>35</sup> Active Ras activates Raf, a serine/threonine kinase that relays signals from Ras to the MAPK cascade.<sup>36,37</sup> Raf activates MEK, which then activates ERK. ERK phosphorylates proteins in the cytoplasm and nucleus.<sup>15,33,34,38,39</sup> Translocating to the nucleus, ERK at the bottom of the pathway promotes the transcription of genes by phosphorylating and activating transcription factors, culminating in the transcription of target genes acting downstream of the RTKs.<sup>40,41</sup> Key among them are proliferation, differentiation, and survival.<sup>14,42</sup> MAPK signaling initiates with

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single, specific substrates and peaks in activating multiple specific cellular programs.

How can single specific substrates culminate in activating multiple, specific cellular programs? MAPK signals in immensely diverse cell types, each with a large number of different cell states. Chromatin structures vary, and the environments differ too, influencing the expression levels of specific proteins and thus the protein–protein interactions in their respective cellular networks. Cell types and states—over developmental time, disease, and broadly changing environments—influence the relative protein concentrations, thus homeostasis through complex, regulated signaling crosstalks.<sup>43–46</sup> The temporal concentration of the substrates (ligands) in the cell type- and

state-specific environment is vital. Extracellular ligands preferentially select specific RTKs and allosterically stimulate specific phosphorylation sites, thereby activating pathways. Pathway propagation depends on the presence of multiple regulatory proteins, including specific kinases and phosphatases. MAPK and PI3K/AKT/mTOR kinases preferentially locate at the outer surface of membrane-bound organelles<sup>44</sup> and in dynamic, membraneless biomolecular condensates.<sup>47</sup> A decade ago, we described them as transient ‘inter-connected nanocluster assemblies with gel-like properties’ spanning over nano- to micrometers.<sup>26,48</sup> *In vivo*, regulated multimolecular condensates are far from equilibrium. They enhance target proximity and increase local concentration.<sup>49–52</sup> High dilution, *e.g.*, upon rapid



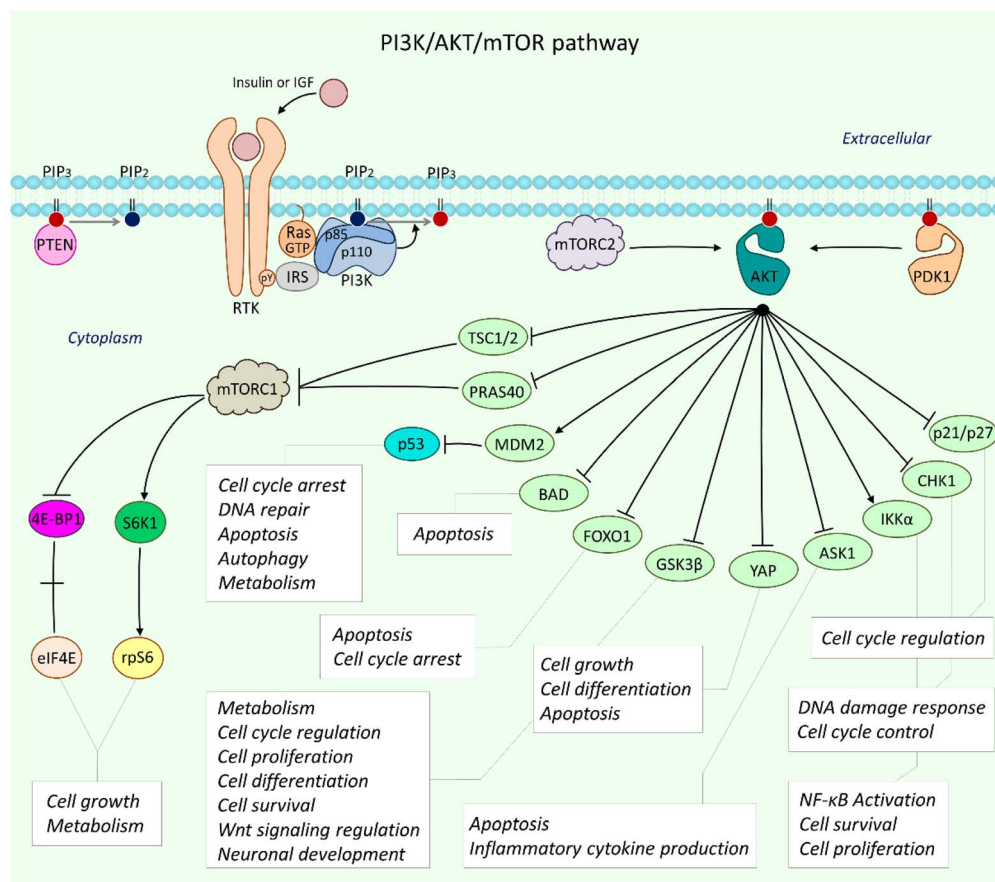
**Fig. 1** The MAPK pathway. When stimulated by EGF, the EGFR recruits Grb2–SOS1 complexes, which activate Ras. This leads to the phosphorylation of kinase cascades, including Raf, MEK, and ERK. The scaffolding protein, KSR, is also involved in the MAPK pathway. Active ERK exists as either a monomer or a dimer that can phosphorylate cytoplasmic targets. ERK phosphorylation activates or inhibits these targets, thereby regulating their functions. Upon phosphorylation by ERK, RSK negatively regulates the Ras/ERK pathway by inhibiting SOS1 or positively regulating it by phosphorylating CREB; MLCK increases MLC phosphorylation and cell motility; KLC1 acts as a cargo adaptor crucial for cell motility and spreading; Cdc25 activates CDK1 to progress through the G<sub>2</sub>/M checkpoint; cPLA2 releases arachidonic acid and other fatty acids involved in cellular processes of inflammation and cell growth; MNK phosphorylates eIF4E, a key factor in mRNA translation and cell growth; IKK $\alpha$  is inhibited by ERK, which leads to the suppression of NF- $\kappa$ B-dependent inflammatory genes; MCL-1 is an anti-apoptotic protein that is stabilized by phosphorylation, thus promoting cell survival; BIM is a pro-apoptotic protein that is degraded by phosphorylation, thus promoting cell survival. DUSP is a member of the MAPK phosphatase family that dephosphorylates and deactivates ERK *via* negative feedback. Active, monomeric, or dimeric ERK translocates to the nucleus and activates transcription factors through phosphorylation. ERK directly activates the transcription factors, such as c-Myc, ELK-1, c-Jun, c-Fos, and Est-1, leading to cell proliferation, cell growth and development. ERK indirectly activates CREB by phosphorylating MSK1. Abbreviations: BIM, Bcl-2 interacting mediator of cell death; cPLA2, cytosolic phospholipase A2; CREB, cAMP-responsive element binding protein; DUSP, dual-specificity phosphatase; IKK $\alpha$ , I $\kappa$ B kinase  $\alpha$ ; KLC1, kinesin light chain 1; MCL-1, myeloid cell leukemia 1; MLCK, myosin light chain kinase; MNK, MAPK-interacting kinase; MSK1, mitogen- and stress-activated protein kinase 1; NF- $\kappa$ B, nuclear factor-kappa B; RSK, ribosomal S6 kinase.



mutant cell growth, degrades relay efficiencies, deteriorating control and risking senescence. The large mammalian target of rapamycin complex 1 (mTORC1) multimolecular assembly in the PI3K/AKT/mTOR pathway can control phase separation by tuning crowding.<sup>53</sup>

The PI3K/AKT/mTOR cascade, also a major drug target in cancer,<sup>54–56</sup> is tasked with metabolic signaling and protein

synthesis in cell growth (Fig. 2). It too can be activated *via* RTKs and Ras,<sup>57</sup> also promoting cell survival, growth, and proliferation in response to RTK stimuli,<sup>58–60</sup> and with crosstalk with other pathways, including MAPK.<sup>61–63</sup> PI3K, a lipid kinase, phosphorylates the signaling lipid, phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>), an action reversed by phosphatase and tensin homolog (PTEN), both



**Fig. 2** The PI3K/AKT/mTOR pathway. RTKs recruit and activate PI3K when stimulated by insulin or IGF. Active PI3K then converts PIP<sub>2</sub> to PIP<sub>3</sub>. AKT is recruited to the membrane by PIP<sub>3</sub> and activated by PDK1 and mTORC2. Active AKT then phosphorylates a number of proteins, regulating their functions. AKT phosphorylation activates or inhibits these targets, thereby regulating their functions. Upon phosphorylation by AKT, TSC1/2 is inhibited, which increases the active form of RHEB, which then activates mTORC1 allosterically at the lysosomal membrane. mTORC1 phosphorylates S6K1 and 4E-BP1. S6K1 activates rpS6. The phosphorylation of 4E-BP1 removes its inhibitory role on eIF4E. S6K1 and eIF4E participate in translational activation and regulate cell growth and metabolism; PRAS40 interacts with 14-3-3, inhibiting its function as a regulator of mTOR signaling; MDM2 is activated and translocates to the nucleus, where it subsequently degrades p53 through ubiquitination. This results in the inhibition of apoptosis and the promotion of cell survival and proliferation. p53 is a tumor suppressor that regulates cell cycle arrest, DNA repair, apoptosis, autophagy, and metabolism; BAD binds to 14-3-3, thereby preventing its interaction with Bcl-xL and inhibiting apoptosis; FOXO1 is inhibited, which prevents its translocation to the nucleus and its function as a transcription factor for apoptosis and cell cycle arrest; GSK3β is inhibited, which has a broad impact on various cellular processes, including the promotion of cell survival, growth, and proliferation, and the inhibition of apoptosis. GSK3β, a serine/threonine kinase, plays a crucial role in metabolism, cell cycle regulation, cell proliferation, cell differentiation, cell survival, Wnt signaling regulation, and neuronal development; YAP is inhibited by interacting with 14-3-3, which prevents its translocation to the nucleus and functions as a transcriptional coactivator. YAP inhibition affects cell growth, differentiation, and apoptosis; ASK1 is inhibited, and its activity is decreased, which promotes cell survival and inhibits apoptosis. ASK1 is involved in the activation of JNK and p38 pathways, as well as inflammatory cytokine production; IKKα is activated, which subsequently activates NF-κB. This leads to cell survival and proliferation. In contrast, ERK phosphorylation inhibits IKKα; CHK1 is inhibited and moves into the cytoplasm. When DNA is damaged, CHK1 inhibits CDC25 phosphatase activation on CDK1 but activates Wee1 kinase inhibition on CDK1, resulting in G<sub>2</sub>/M checkpoint arrest; both p21 and p27 are inhibited and move into the cytoplasm. These proteins (p21<sup>Cip1</sup>, or p21<sup>Waf1</sup>, and p27<sup>Kip1</sup>) are primarily CDK2 inhibitors, arresting the G<sub>2</sub>/S checkpoint. Abbreviations: 4E-BP1, eukaryotic translation initiation factor 4E (eIF4E)-binding protein; ASK1, apoptosis signal-regulating kinase 1; BAD, Bcl2-associated agonist of cell death; CDC25, cell division cycle 25; CHK1, cell cycle checkpoint kinase 1; FOXO1, forkhead box protein O1, a.k.a. FKHR; IGF, insulin-like growth factor; IKKα, IκB kinase α; IRS, insulin receptor substrate; MDM2, murine double minute 2; NF-κB, nuclear factor-κB; PRAS40, proline-rich AKT substrate 40 kDa; RHEB, Ras homolog enriched in brain; TSC1/2, tuberous sclerosis complex 1/2; YAP, Yes-associated protein.



catalytic actions at the membrane.<sup>64</sup> In turn, phosphoinositide-dependent protein kinase 1 (PDK1) binds to PIP<sub>3</sub> through its C-terminal Pleckstrin homology (PH) domain, with high affinity. This binding is essential for PDK1 to phosphorylate and activate AKT kinase, which also binds PIP<sub>3</sub>, through its PH domain.<sup>65</sup> AKT is phosphorylated by PDK1 and mTORC2, the next kinase in the cascade. Thus, PI3K, PTEN, PDK1, and AKT are all recruited to the membrane through the signaling lipid—unphosphorylated (PIP<sub>2</sub>; PI3K) or phosphorylated (PIP<sub>3</sub>; PTEN, PDK1, and AKT). PDK1 activation was proposed to involve *trans*-autophosphorylation by a PIP<sub>3</sub>-mediated face-to-face dimer.<sup>66</sup> Finally, both kinase cascades enter the cell cycle, which also involves cascading cyclin-dependent kinases (CDKs) interacting with their cyclins in each of the phases.<sup>67–69</sup>

Below, we first describe the spatial structure of the cell signaling systems, which permits efficiency and specificity. We then ask *how cascades, which start with single-substrate kinases, and end at their bottoms with tens, or hundreds of substrates, can still be designated specific*. We discuss their mutations, asking why some have multiple, or many mutations, whereas others may have few or practically none. We further investigate the kinases' activation mechanisms and autoinhibition, which has evolved for some kinases, but not for others, raising questions about why the difference exists, why activation rates differ, and how these could have arisen. We discuss kinase signal transduction in the chemical framework of molecular condensates and the risk of high dilution resulting from signaling overdrive in cancer due to rapid cell growth. Finally, we survey available drugs for the main kinases in these major cell proliferation cascades, asking whether the cascades' distinct properties, organization and environment can spawn new therapeutic strategies.

Altogether, our review stands apart in its innovative framework, which we hope will help inspire new, creative therapies.

## The dynamic spatial structure of cell signaling systems

The common, simplified representation of the cell and signaling pathways is helpful.<sup>48</sup> Pathway diagrams depict single protein nodes connected by edges, linking extracellular domains of membrane-spanning receptors through the cytoplasm to the nucleus. From the biophysical standpoint such diagrams may be misleading, obscuring cell coordination.<sup>70</sup> Signaling requires coordinated, transient physical interactions, which are not captured in the classical MAPK and PI3K/AKT/mTOR diagrams. They do not discern, or epitomize, exactly how a signal is regulated and relayed. In reality, the kinases do not lie as rigid bodies on a two-dimensional surface. A high level of cellular organization requires signaling that imparts homeostasis, with the internal environment varying in different cell types and cell states. That is, the interactions (edges) between the proteins should be transient, likely with certain time frames. For kinases, the time is commonly short. The phosphorylation reaction often occurs within seconds or minutes,<sup>71</sup> typically between 13 and 35 seconds for the receptors and between 25 and 200 seconds for downstream kinases, underscoring the gap between simplified

diagrams and cell coordination. Our view of cell signaling has been in terms of dynamic, short-lived, allosteric interactions within and among distinct, spatially organized transient clusters.

Clustering is often at the membrane, with some cluster members anchored. This is the case for MAPK (Fig. 1) and Ras/PI3K/AKT/mTOR (Fig. 2). While MEK and ERK are not membrane anchored, Ras activation is, as is Raf's, and their activation is in response to signals received at the membrane, making them functionally linked to the membrane and transducing membrane-transduced signals. As to PI3K/AKT/mTOR, PI3K binds the PIP<sub>2</sub> signaling lipid which recruits it, and PIP<sub>3</sub> recruits PDK1, AKT, and PTEN. mTOR is also at the cluster, phosphorylating AKT. Nawrocki *et al.*<sup>72</sup> offered an additional advantage to dynamic clustering at the membrane. Their molecular dynamics simulations suggested that nonspecific protein-membrane interactions create a water-rich protein depletion zone between the membrane and the crowded environment, leading to an increased propensity of proteins to aggregate in bulk, but also allow for accelerated diffusion on the surface of the membrane when proteins occasionally come closer to the surface. Considering the crowded cytosols and membrane surfaces, this provides a tantalizing hypothesis. Their results further suggested that crowding near the membrane could constitute a nonspecific mechanism for protein-induced membrane curvature formation. At the same time, considering the rapid cell growth in cancer, there is a risk of high dilution in the membraneless condensates. The sparser protein interactions in the highly diluted environment can stall physiological processes, blocking the cell cycle, and precipitating senescence.<sup>73,74</sup> The key pathway in cell growth is PI3K/AKT/mTOR, consistent with the observation that highly active mTORC1 is a key player.<sup>75</sup>

Mesoscale assemblies are favored by active crowded environments.<sup>76</sup> Mechanistically, dynamic clusters can be viewed as membraneless assemblies, formed by phase separation at the membrane and extending into the cell, enhanced by scaffolding proteins and the cytoskeleton.<sup>26,48</sup> Membrane-associated proteins participate in the phase separation.<sup>77</sup> They maintain the relevant homeostasis of their protein-protein interaction networks.<sup>78</sup> Membraneless molecular condensates involving specific interactions appear to be an apt description for specific and efficiently regulated kinase cascades.<sup>47</sup> Their site-specific localizations at the RTK (*e.g.*, EGFR and PDGFR) oligomer clusters, and the crosslinked signaling that they stimulate upon growth factor binding, could be a prime example of how basic physical chemistry guides efficient biological processes. We propose that site-specific kinase condensates and the chemically specific interactions of their assemblies, as their core composition, promote allostery and phosphorylation reactions, regulating signaling and gene expression.

## The cascades: number of substrates and of mutations

The functions of the kinases clarify their associated substrates and mutation numbers. As to substrates, both cascades start with single, specific substrates for upstream kinases and





culminate with many, coinciding with their diverse functions. As to the number of mutations, the situation appears more complex. *While each of the PI3K/AKT/mTOR kinases evidences multiple mutations, that is not the case for the MAPK components.*

### What we can learn from the number of substrates

Considering the number of substrates, B-Raf has one primary substrate, which is MEK, specifically the isoforms MEK1 and MEK2.<sup>79,80</sup> C-Raf (Raf-1) and A-Raf also primarily have only MEK. MEK also phosphorylates a single substrate, ERK. This is its sole function. ERK can phosphorylate hundreds of proteins (key target proteins of ERK are shown in Fig. 1), with estimates reaching over 1000 substrates.<sup>81</sup> However, several kinases can phosphorylate B-Raf, including AMP-activated protein kinase (AMPK), which phosphorylates B-Raf at Ser729 and ERK at Ser151 and Thr401, in a negative feedback loop. AMPK also negatively regulates mTOR signaling.<sup>82</sup> MEK is mainly phosphorylated by MEK kinase (MEKK1 or MAP3K1) and Raf, both phosphorylate MEK on specific serine residues in its activation loop.<sup>83</sup> ERK is activated by MEK. As to PI3K/AKT/mTOR, PI3K phosphorylates a single substrate PIP<sub>2</sub>. PDK1 is estimated to phosphorylate approximately 23 proteins.<sup>84</sup> AKT is estimated to phosphorylate over 100 proteins<sup>58,85</sup> (key target proteins of AKT are shown in Fig. 2) and mTOR is estimated to phosphorylate several hundred proteins.<sup>86</sup> PI3K is not directly phosphorylated by a single protein but activated mostly by conformational changes triggered through interactions with RTKs. PDK1 is activated by autophosphorylation.<sup>66,87</sup> While mTORC2 and mTORC1 phosphorylate AKT and S6K1, respectively, leading to activation, active S6K1 can directly phosphorylate mTOR,<sup>88</sup> indicating a bidirectional relationship.

*So how does the specificity of the cascades work when starting with a single substrate and ending with hundreds and still being labeled specific?* The numbers above tell the story, and they are supplemented by considering cell types and cell states. We believe that at least two main factors are at play. First, specific cell types and states may be associated with different functions. For example, cell differentiation occurs over developmental time, while cancer involves overexpression of certain proteins during evolution and metastasis. Second, not all possible substrates are available in the condensates, which limits the temporal repertoire and related regulatory mechanisms.

### What we can learn from the number of mutations

As to mutations, the situation varies but can be understood. PI3K $\alpha$  (encoded by *PIK3CA*) has multiple mutations, the most common hotspots at residues E542 and E545 in the helical domain and H1047 in the kinase domain,<sup>89–91</sup> as does PTEN,<sup>92–96</sup> which has over 110 germline and 332 somatic mutations identified. There is no count for PDK1, although some are documented.<sup>97</sup> Numerous possible mutations occur in the gene, with varying prevalence in AKT<sup>98,99</sup> and a significant number in mTOR.<sup>100,101</sup> As to the MAPK pathway, B-Raf has over 30 mutations, with the most prevalent being the V600E mutation, often observed in melanoma. It is also the most common in glioblastoma,<sup>102</sup> in addition to papillary thyroid cancer, colorectal

cancer, and serous ovarian cancer.<sup>103</sup> MEK has over 20 mutations.<sup>104–106</sup> However, ERK mutations are rare.

*So how to understand these numbers?* Overall, mutations in kinases in PI3K/PDK1/AKT/mTOR (including PTEN phosphatase) are more abundant than in MAPK. Both pathways feed into the cell cycle in the G<sub>1</sub> phase. We suggest that the numbers are consistent with PI3K/PDK1/AKT/mTOR being a cell growth pathway, and MAPK is the major pathway driving cell proliferation. Oncogenic driver mutations lead to stronger signaling.<sup>12,20,96</sup> Excessive cell growth causes cytoplasm dilution and contributes to senescence.<sup>74</sup> Signaling which is too strong can elicit OIS, oncogene induced senescence. The absence of driver mutations in ERK indicates that cells cannot sustain signaling, which is too strong. In line with this, the critical role of ERK in cell proliferation could be why it has no auto-inhibition mechanism and is the sole phosphorylation target of MEK, which is the sole target of Raf. The catalytic rate of ERK is high compared to many other protein kinases,  $\sim 5 \text{ mM}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$ ; that is, in its fully activated state one ERK molecule can catalyze the phosphorylation of a substrate at a rate of 5 millimoles per minute per milligram of enzyme protein. It is 5 to 6 orders of magnitude higher than ERK basal activity.<sup>33,107</sup> In addition to its single-substrate high selectivity, the catalytic rate of MEK is also high, which is why it is used commercially. Raf has a slower rate, likely due to its mechanism of activation involving dimerization and autophosphorylation.<sup>108</sup>

*Collectively, this informs us about the chemistry of the selectivity and the chemical mechanics of the distributions of rates of the MAPK cascade: slower at the top and rapid at the bottom.*

## The kinase structural organization, activation and autoinhibition

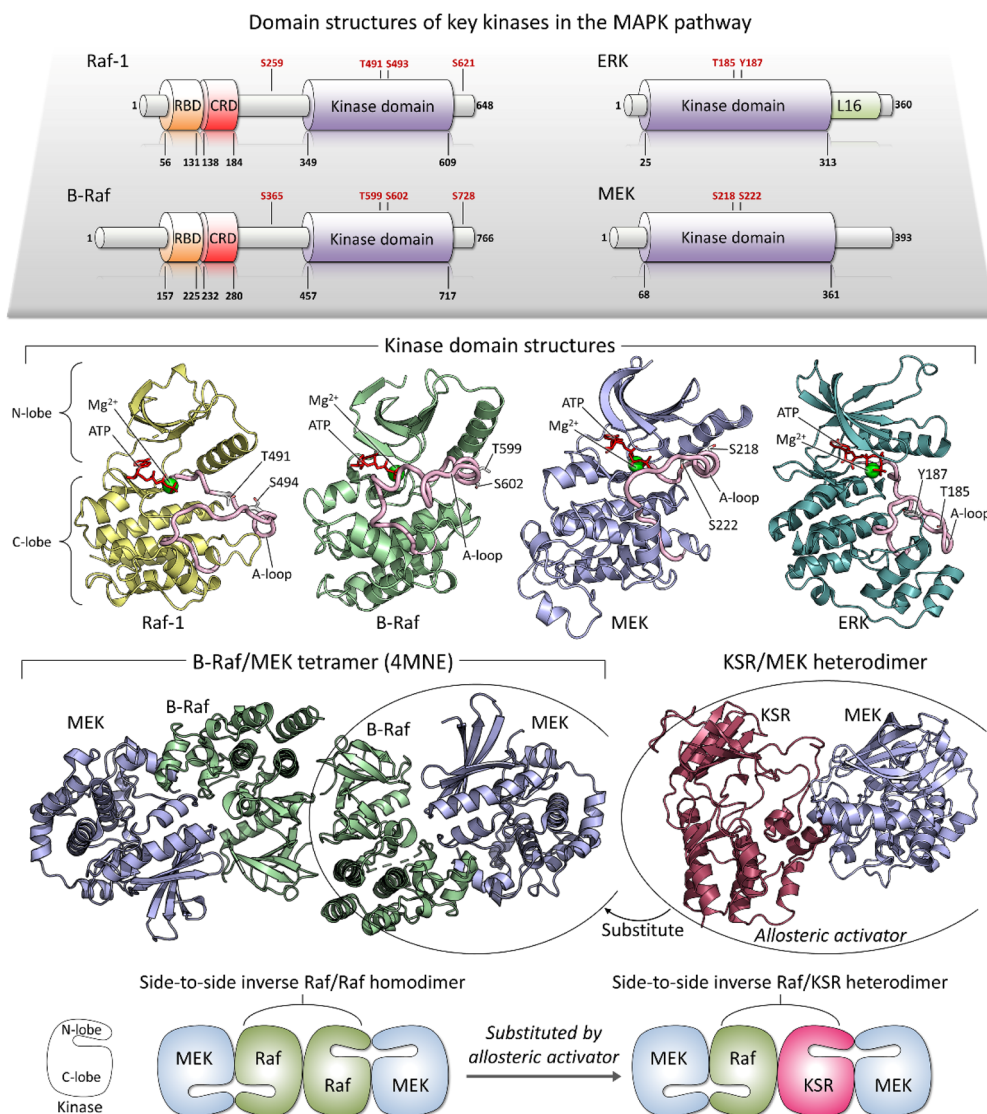
The MAPK signaling pathway involves a cascade of three main kinases, Raf, MEK, and ERK (Fig. 1). These kinases share some structural similarities in their conserved kinase domains but have distinct structural characteristics that allow them to perform their specific functions in the cascade (Fig. 3). These structural traits imply that kinases have different characteristics and that they exhibit context-dependent behaviors shaped by many different traits.<sup>109</sup> Raf and MEK are primarily cytoplasmic kinases, while ERK can be found both in the cytoplasm and in the nucleus. MAPK initiates with Raf.<sup>110–114</sup> B-Raf is auto-inhibited by its Ras binding domain (RBD) and cysteine-rich domain (CRD) interacting with its kinase domain and the 14-3-3 dimer. 14-3-3 interaction stabilizes the autoinhibited 'closed' state, interfering with the kinase domain dimerization, which is required for B-Raf activation.<sup>110</sup> For monomeric B-Raf, this autoinhibited conformation is highly populated, dominating the landscape. Activated monomeric B-Raf is in the 'open' state. Since this is an unstable conformation, its population is a minor species. Membrane-anchored, GTP-bound Ras interacts with the RBD, stabilizing the open B-Raf conformation, which is further stabilized by the high affinity CRD–membrane interaction. This allosterically shifts the equilibrium toward the now stable open state.<sup>111</sup> The released RBD–CRD



promotes fluctuations of the kinase domain, predisposing it for its *unique side-to-side inverse dimerization*.<sup>115,116</sup> We discovered that the driving force for the side-to-side inverse dimerization is the intermolecular  $\pi$ - $\pi$  stacking at the dimer interface, which replaces the intramolecular  $\pi$ - $\pi$  stacking, thereby stimulating the OFF-to-ON transition. Subsequent conformational events culminate in the ON-state kinase domain stabilized by the N-terminal basic motif in the dimer for Raf signaling.<sup>115</sup> *Why did evolution opt for this unique side-to-side transverse dimerization*

*scenario?* We suggest that *the main reason is slow kinetics in the first MAPK step, whose subsequent steps are fast, thereby reining proliferation*.

MEK1 is activated by phosphorylation of Ser218 and Ser222 in its activation segment catalyzed by Raf, with the kinase suppressor of Ras (KSR) proposed as a scaffold.<sup>117</sup> Still, key questions remain,<sup>112</sup> including *why, despite the similarity of the kinase domains of B-Raf and KSR1, B-Raf is the key activator of MEK—not KSR—and what the exact role of KSR is*. We observed



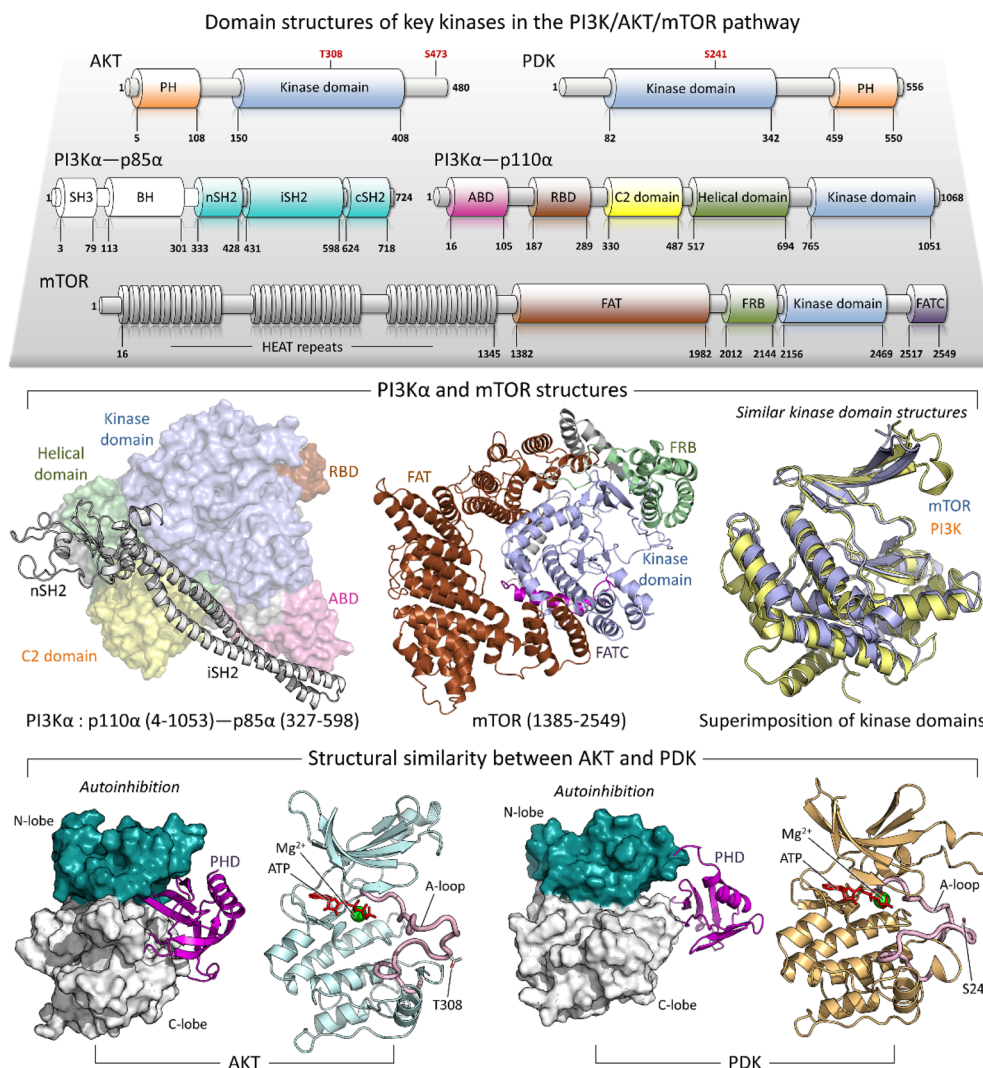
**Fig. 3** Kinases in the MAPK pathway. The domain structures of Raf-1, B-Raf, MEK (shown here for the MEK1 isoform), and ERK (shown here for the ERK2 isoform) are depicted (top panel). The phosphorylation sites are marked in red. Phosphorylation in the kinase domain occurs at the activation loop (A-loop). In the case of Raf, phosphorylation in the linker and C-terminal tail targets the 14-3-3 binding during activation. Kinase domain structures of Raf-1, B-Raf, MEK, and ERK (middle panel). The modeled kinase domain structures of MEK and ERK were derived from their respective crystal structures (PDB IDs: 9AYA and 6NYB). The modeled kinase domain structures of MEK and ERK were adopted from their respective crystal structures (PDB IDs: 7JUW and 8ZJV). Raf-1 exhibits active conformation, whereas B-Raf, MEK, and ERK exhibit inactive conformations. Crystal structures of the B-Raf/MEK tetrameric complex (PDB ID: 4MNE) and the modeled KSR/MEK heterodimer derived from the crystal structure (PDB ID: 7JUW) (bottom panel). The schematics of the Raf/MEK and Raf/KSR/MEK tetramers are provided below. Thus, Raf activation requires relieving its autoinhibited monomeric state by binding to active Ras and high affinity membrane attachment,<sup>110</sup> and formation of a side-to-side inverse homodimer.<sup>115</sup> MEK phosphorylation also requires the formation of a tetramer in a specific organization.<sup>112</sup> The KSR/MEK heterodimer acts as an allosteric activator for Raf. In the tetrameric complex, Raf and KSR form a side-to-side inverse heterodimer.<sup>112</sup> Formation of Raf dimers in this organization slows Raf activation. Formation of the Raf/MEK tetramer for MEK phosphorylation slows MEK activation. We suggest that these organizations were adopted by nature to slow MAPK signaling upstream.



that if KSR1 were to adopt an active configuration with an extended A-loop resembling that in other protein kinases, then the MEK1 proline-rich loop (P-loop) would extend as in the active B-Raf/MEK1, triggering a more flexible MEK1 A-loop and rendering KSR1 B-Raf-like. KSR1/MEK1 can serve as a scaffold or an allosteric activator.<sup>112</sup> As a scaffold, in the heterodimer, KSR1 interacts with B-Raf through a side-to-side interface, resulting in the Raf/KSR1/MEK1 complex translocating to an active B-Raf dimer which phosphorylates it.<sup>118,119</sup> As an allosteric activator, KSR1/MEK1 blocks KSR1 autoinhibition, which promotes KSR/B-Raf side-to-side heterodimerization (refer to

the schematics in Fig. 3) with a B-Raf monomer that has already been recruited to the membrane by active Ras. Active B-Raf phosphorylates a second MEK1 kinase.<sup>120</sup> This mechanistic scenario can explain MEK1 activation.

ERK dynamics has been reviewed in detail.<sup>121–125</sup> Its activation mechanism poses a few questions, including *why two phosphorylation events occur on tyrosine and threonine residues in the activation loop* and *why there appears to be a preferred phosphorylation order*, first pY187 and then pT185,<sup>126–131</sup> and why no autoinhibition. As to the phosphorylation, our recent molecular dynamics simulations suggested that tyrosine is more



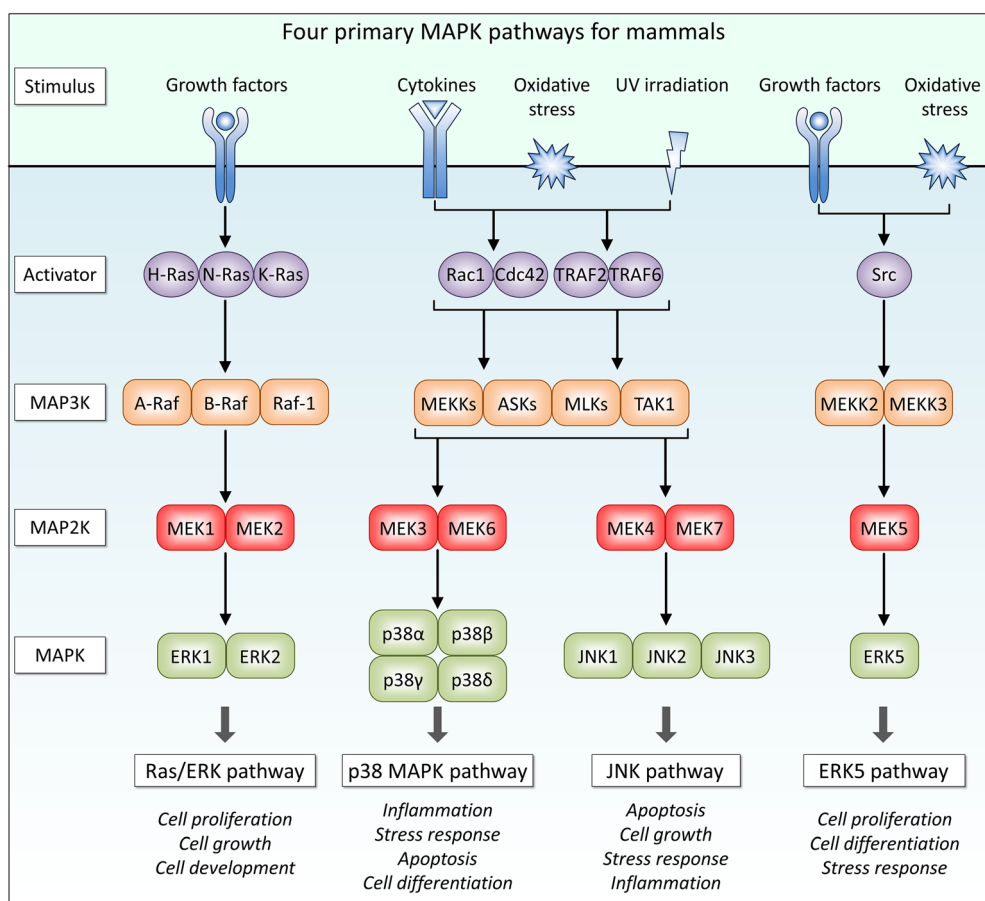
**Fig. 4** Kinases in the PI3K/AKT/mTOR pathway. The domain structures of AKT (shown here for the AKT1 isoform), PDK (shown here for the PDK1 isoform), PI3K $\alpha$ , and mTOR are depicted (top panel). As marked in red, phosphorylation in the kinase domains of AKT and PDK occurs at the activation loop (A-loop). Molecular structures of PI3K $\alpha$  and mTOR, and the superimposition of their kinase domains (middle panel). The *in silico* structures of PI3K $\alpha$  and mTOR were derived from their respective crystal structures (PDB IDs: 4OVV and 4JSP). Molecular structures of AKT and PDK (bottom panel). The modeled AKT structure in an autoinhibited state was derived from the crystal structure (PDB ID: 4EJN). The modeled PDK structure in an autoinhibited state adopted the AKT autoinhibition structure. Despite the structural similarity of PDK and AKT, the relative stabilities of their autoinhibited states differ.<sup>87</sup> AKT has been crystallized in its autoinhibited state, whereas PDK was not, due to the absence of specific variable loop-mediated interaction between the PH and kinase domains, resulting in a sparsely populated autoinhibited state. PDK is estimated to phosphorylate approximately 23 proteins, whereas AKT phosphorylates over 100. This evolutionary advantage of PDK is likely to have arisen from a weak interaction between the PH and kinase domains compared to AKT, ensuring that PDK effectively phosphorylates its substrate while binding PIP<sub>3</sub>, but phosphorylates S6K, SGK, and RSK kinases independently of PIP<sub>3</sub>. Abbreviations: PHD, Pleckstrin homology domain; FAT, FRAP, ATM, and TRRAP; FRB, FKBP–rapamycin binding.





accessible than threonine.<sup>132</sup> Phosphorylating it extends the activation loop favoring the successive phosphorylation of threonine by making it more accessible, establishing an *allosteric phosphorylation code in ERK activation*. ERK phosphorylation states—unphosphorylated, monophosphorylated, and dual phosphorylated—can effectively modify the strength of the interactions of the lobes and ATP binding and stabilize ERK active state, in which the catalytic domain can facilitate phosphoryl transfer. This is crucial for a kinase that activates over hundreds of substrates,<sup>81</sup> and whose sustained activation lasts several hours, with a transient activation peaking at ~20 min.<sup>121–125</sup> As we noted above, the apparent absence of an autoinhibited state underscores its critical role in cell proliferation, activating a very large number of substrates in different environments and spatial locations and over developmental time.

The major kinases in the cell growth pathway include PI3K, PDK1, AKT, and mTOR (Fig. 2). PI3K is a lipid kinase, whereas PDK1, AKT, and mTOR are protein kinases. The structure of the kinase domain of PI3K is similar to that of mTOR, as both are members of the PI3K-related kinase family (Fig. 4). The PI3K/AKT/mTOR pathway initiates with PI3K activation by an RTK, supported by active Ras at the membrane.<sup>133,134</sup> As to PDK1, its PH domain structure resembles that of AKT, as they belong to the AGC family of kinases. However, the linker and C-terminal region differ. We surmised that PDK1 samples AKT-like autoinhibited states. Consistently, the simulations identify a conformation resembling that of AKT.<sup>87</sup> As to why the autoinhibited PDK1 structure has not been captured by crystallography while AKT was,<sup>135</sup> unlike AKT, the monomeric autoinhibited state of PDK1 is relatively only stable, with low kinetic barriers that appear to further facilitate PDK1 PIP<sub>3</sub>-



**Fig. 5** Four primary MAPK pathways. The four major mammalian MAPK pathways include the Ras/ERK, p38 MAPK, JNK, and ERK5 pathways. These MAPK pathways are initiated by extracellular stimuli, such as growth factors, cytokines, UV irradiation, and internal or external processes of oxidative stress. These signals cascade to activators, including Ras family proteins, such as H-Ras, N-Ras, and K-Ras; Rho family proteins, such as Rac1 and Cdc42; adapter proteins, such as TRAF2 and TRAF6; and non-receptor protein tyrosine kinases, such as Src. These activators then convey the signal to downstream protein kinases, which activate a cascade involving at least three kinases: MAP3K, MAP2K, and MAPK. In the Ras/ERK pathway, Rafs (MAP3Ks) activate MEK1/2 (MAP2Ks), which then activate ERK1/2 (MAPKs). Other MAP3Ks, including MEKKs, ASKs, MLKs, and TAK1, activate MEK3/6 (or MKK3/6) and MEK4/7 (or MKK4/7), which then activate p38α/β/γ/δ and JNK1/2/3, respectively, in the p38 MAPK and JNK pathways. In the ERK5 pathway, Src activates MEKK2 and MEKK3, which then activate MEK5 (or MKK5) and subsequently ERK5. The MAPK pathways activate multiple cytoplasmic proteins and nuclear transcription factors, resulting in various biological functions. Abbreviations: ASK, apoptosis signal-regulating kinase; MEKK, MEK kinase; MLK, mixed lineage kinase; TAK1, transforming growth factor-β-activated kinase 1; TRAF, tumor necrosis factor receptor-associated factor.





mediated shift to its active state. PDK1 linker is the key player, with intramolecular interactions between the kinase domain, the PH domain, and the linker region.<sup>87</sup> The detailed activation mechanism of mTOR has been challenging, which is not surprising given its complexity. Simulations coupled with experimental data recently established how its motifs can allosterically govern its kinase activity.<sup>136</sup> The disordered negative regulator domain (NRD) is a key regulator. When in the catalytic cleft—it promotes a closed conformation; when outside—mTOR prefers an open state, which exposes the substrate-binding site on the FRB domain. mTOR's mechanism has been dubbed “active-site restriction”. This mechanism features protein domains partially blocking the catalytic site, acting as a lever in permitting substrate access thereby controlling its activity. Full activity requires specific signals unleashing a conformational change to open the catalytic site allowing substrate access.

Finally, above, we referred to the ‘classical’ MAPK cascade. In mammals, MAPK has four cascades<sup>137</sup> (Fig. 5) including Ras/ERK, p38, JNK, and ERK5 pathways (details in ref. 138). Some feature alternatively spliced isoforms.<sup>139</sup> Some have different modes of regulation.<sup>140</sup> They may be stimulated by different signals and have distinct primary roles, although they may complement each other under pharmacological regimes. The ERK1/2 cascade is the key in proliferation, differentiation (in development, including neurodevelopmental disorders<sup>141,142</sup>), and migration;<sup>143</sup> p38 in immune responses;<sup>143</sup> JNK in apoptosis;<sup>144</sup> and ERK5 in cancer (proliferation),<sup>145</sup> and known to play a role in drug resistance to Raf.<sup>146,147</sup>

## Learning the cascades to generate pharmacological perspectives

### Drugs approved in the pipeline

We learn the kinase cascades. We survey available drugs in these cascades and then ask whether the pathways' properties can spawn new strategies. Starting with Ras, approved K-Ras-targeting drugs<sup>148–152</sup> include sotorasib (Lumakras) and adagrasib (Krazati) for K-Ras<sup>G12C</sup>. Ras clinical trials include RMC-6236, RMC-6291, and RMC-9805, and preclinical development includes RMC-5127, RMC-0708, and RMC-8839 (Table 1), which are being developed by Revolution Medicines, Inc.<sup>153</sup> Recently, RMC-7977 was developed as a broad-spectrum inhibitor targeting both the mutant and wild type forms of multi-selective Ras (ON).<sup>150,154,155</sup> These Ras (ON) drugs are small molecule inhibitors that act as molecular glues, forming a tricomplex with Ras and cyclophilin A (CypA), preventing effectors from binding to Ras and thereby disrupting downstream signaling.

There are several kinase inhibitors that target different points along the MAPK pathway (Table 2), including B-Raf, MEK, and ERK inhibitors (Fig. 6). B-Raf inhibitors prescribed for *BRAF* mutations include vemurafenib (Zelboraf), dabrafenib (Tafinlar), and encorafenib (Braftovi). The combination of dabrafenib with trametinib (Mekinist) has been approved for solid tumors.<sup>156</sup> The combination of encorafenib with cetuximab (Erbix) and mFOLFOX6 (leucovorin calcium (folinic

Table 1 Drugs targeting mutant Ras (ON, active state) in the MAPK pathway<sup>a</sup>

Compound name (generic name)	Target	Drug type	Mechanism of action	ClinicalTrials.gov identifier (status)	Condition or disease
RMC-6236 (daraxonrasib)	Ras (ON) multi-selective	Non-covalent, non-degrading molecular glue	Oral, tri-complex formation with CypA	NCT05379985 (phase 1)	NSCLC, CRC, PDAC, advanced solid tumors
RMC-6291 (elironrasib)	K-Ras (ON) G12C	Covalent modification at G12C, non-degrading molecular glue	Oral, tri-complex formation with CypA	NCT05462717 (phase 1)	NSCLC, CRC, PDAC, advanced solid tumors
RMC-9805 (zoldonrasib)	K-Ras (ON) G12D	Covalent modification at G12D, molecular glue	Oral, tri-complex formation with CypA	NCT06040541 (phase 1)	NSCLC, CRC, PDAC, advanced solid tumors
RMC-5127	K-Ras (ON) G12V	Non-covalent, non-degrading molecular glue	Oral, tri-complex formation with CypA	—	NSCLC, CRC, PDAC, brain metastases
RMC-0708	K-Ras (ON) Q61H	Non-covalent, non-degrading molecular glue	Oral, tri-complex formation	—	NSCLC, CRC, PDAC, multiple myeloma
RMC-8839	K-Ras (ON) G13C	Covalent modification at G13C, non-degrading molecular glue	Oral, tri-complex formation	—	NSCLC, CRC

<sup>a</sup> Abbreviations: CRC, colorectal cancer; CypA, cyclophilin A; NSCLC, non-small cell lung cancer; PDAC, pancreatic ductal adenocarcinoma.



Table 2 Small molecules targeting the Ras/ERK pathway<sup>a</sup>

Drug name [brand name] (synonyms)	Drug type	PubChem CID	Target disease	Mechanism of action	Route of administration
Vemurafenib [Zelboraf] (RO5185426, PLX-4032, RG-7204)	Orthosteric inhibitor	42611257	Metastatic melanoma	B-Raf inhibitor binding to the ATP-binding site, specifically targeting cancers with the V600E mutation	Oral
Dabrafenib [Tafinlar, Finlee] (GSK-2118436A)	Orthosteric inhibitor	44462760	Melanoma, NSCLC, thyroid cancer	B-Raf inhibitor binding to the ATP-binding site and used in combination with trametinib, targeting cancers with V600E/K mutations	Oral
Encorafenib [Braftovi] (LGX818, NVP-LGX818)	Orthosteric inhibitor	50922675	Metastatic melanoma	B-Raf inhibitor binding to the ATP-binding site and used in combination with binimetinib, targeting cancers with V600E/K mutations	Oral
Clatrafenib (PF-07799933, ARRY-440)	Orthosteric inhibitor	165150001	Solid tumors	Class II (dimeric mutations) pan-mutant B-Raf inhibitor binding to the ATP-binding site and tested in combination with binimetinib or cetuximab	Oral
Trametinib [Mekinist, Spexotras] (GSK-1120212, JTP-74057)	Allosteric inhibitor	11707110	Melanoma, NSCLC, thyroid cancer	Allosteric MEK1/2 inhibitor used in combination with dabrafenib, targeting cancers with B-Raf V600E/K mutations	Oral
Cobimetinib [Cotellic] (GDC-0973, RG-7420, XL-518)	Allosteric inhibitor	16222096	Metastatic melanoma	Allosteric MEK1 inhibitor used in combination with vemurafenib, targeting metastatic melanoma with B-Raf V600E/K mutations	Oral
Binimetinib [Mektovi] (ARRY-162, ARRY-438162, MEK162)	Allosteric inhibitor	10288191	Metastatic melanoma, NSCLC	Allosteric MEK1/2 inhibitor used in combination with encorafenib, targeting cancers with B-Raf V600E/K mutations	Oral
Selumetinib [Koselugo] (ARRY-142886, AZD-6244)	Allosteric inhibitor	10127622	NF1	Allosteric MEK1/2 inhibitor targeting the treatment of NF1	Oral
Avutometinib [Avmapki]	Allosteric inhibitor	16719221	KRAS-mutated recurrent LGSOC	Allosteric inhibitor targeting Raf and MEK used in combination with defactinib to inhibit FAK	Oral
Fakzinja Co-pack (946128-88-7, RO-5126766)	Allosteric inhibitor	9826528	NF1	Highly selective, allosteric MEK1/2 inhibitor preventing aberrant glioblastoma cell growth	Oral
Mirdametinib [Gomeldi] (391210-10-9, PD0325901)	Orthosteric inhibitor	118045847	NSCLC, pancreatic, colorectal, and ovarian cancers	ERK1/2 inhibitor preventing its activation and also inhibiting Raf	Oral
Rineterkib (LTT 462)	Orthosteric inhibitor	—	—	Highly selective ERK1/2 inhibitor preventing substrate phosphorylation	Oral
HH2710	Orthosteric inhibitor	121408882	AML, CLL	Highly selective ERK1/2 inhibitor blocking the MAPK pathway with B-Raf, N-Ras, and K-Ras mutations	Oral
Temuterkib (LY-3214996)	Orthosteric inhibitor	—	—	Highly selective ERK1/2 inhibitor as an ATP-competitive inhibitor, preventing the phosphorylation of ERK1/2	—
SCH77298	Orthosteric inhibitor	—	—	—	—

<sup>a</sup> Abbreviations: AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; LGSOC, low-grade serous ovarian cancer; NF1, neurofibromatosis type 1; NSCLC, non-small cell lung cancer.

acid) + fluorouracil (5-FU) + oxaliplatin) has also been approved for metastatic colorectal cancer.<sup>157</sup> More are in clinical trials, including the pan-mutant *BRAF* inhibitor clatrafenib.<sup>158</sup> Allosteric drugs for MEK mutations include trametinib (Mekinist), cobimetinib (Cotellic), binimetinib (Mektovi), selumetinib (Koselugo), avutemetinib (Avmapi Fakzyňa Co-pack), and mirdametinib (Gomekli). MEK inhibitors are often combined with B-Raf inhibitors. Targeting ERK directly is challenging,<sup>13</sup> although several inhibitors are available, including rineterkib, HH2710, temuterkib, and SCH77298. Its multiple functions, regulatory mechanisms, and complex feedback loops make it difficult, leading to harnessing MEK drugs.

Kinase inhibitors that target different points along the PI3K/AKT/mTOR pathway (Table 3) include PI3K, PDK1, AKT, and mTOR inhibitors (Fig. 7). Drugs that treat PI3K include alpelisib (Piqray), copanlisib (Aliqopa), duvelisib (Copiktra), idelalisib (Zydelig), and inavolisib (Itovebi).<sup>159</sup> Allosteric drugs for PI3K mutations include tertsolisib, RLY-2608, and LOXO-783.<sup>159–161</sup> As to PDK1, there are no direct FDA-approved drugs, but several are in clinical trials, including GSK2334470, BX-795, leelamine, OSU-03012, and PS210.<sup>162</sup> Drugs that target

AKT in cancer include capivasertib (Truqap), ipatasertib, MK-2206, perifosine, and miransertib.<sup>163</sup> For mTOR, rapamycin and its analogs are the main inhibitors, including ridaforolimus (Taltorvic), sirolimus (Rapamune), everolimus (Afinitor), and temsirolimus (Torisel). Sirolimus, everolimus, and temsirolimus have already been approved by the FDA. Roskoski has recently updated the kinase-targeting small molecule drugs, including their molecular weight, number of hydrogen bond donors/acceptors, polar surface area, potency, solubility, lipophilic efficiency, and ligand efficiency.<sup>162</sup> Combinations of drugs listed above often target mutants of the same proteins, as well as different kinases in the same and in complementary pathways.<sup>148,164</sup>

### The cascades offer pharmacological perspectives

Drug resistance may emerge, and temporal drug combinations are expected to have better outcomes than consecutive single drugs. Our chemical framework underscores several points when planning a pharmacological regimen. (i) Regulation of the signal is at least on two levels: efficient kinase activation and inhibition at the protein level, and homeostasis at the systems

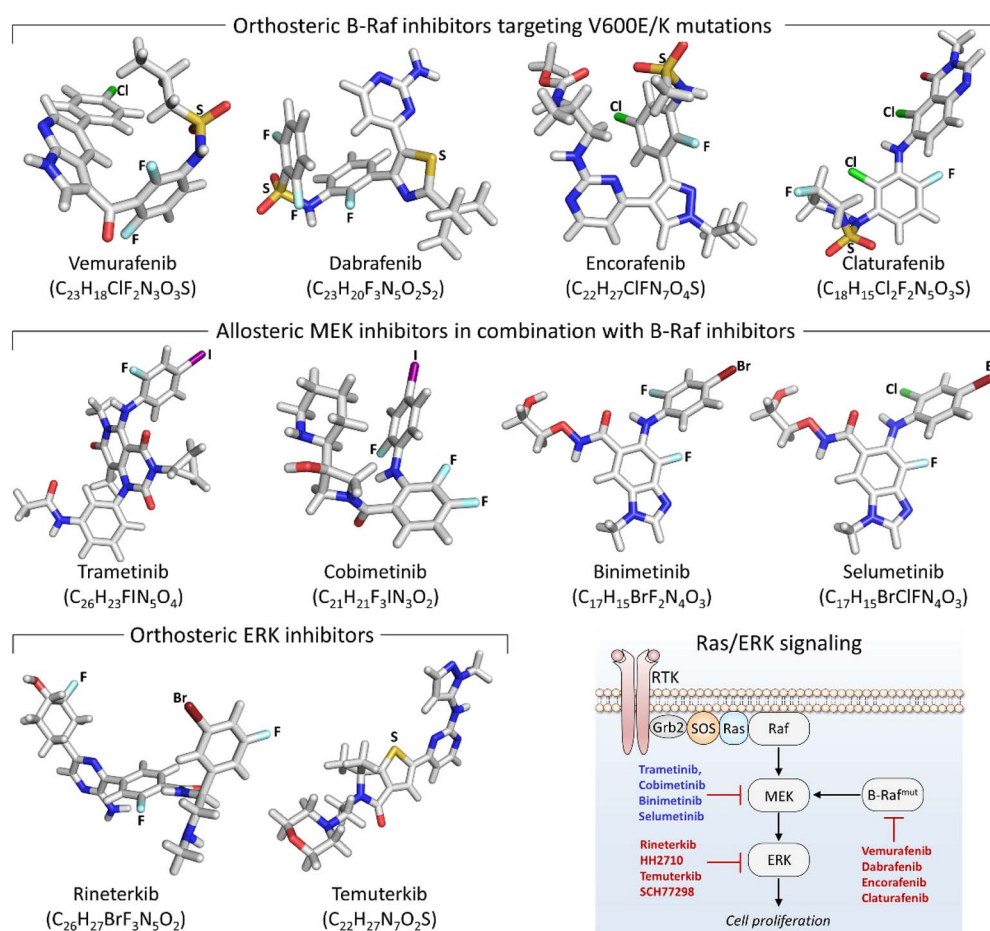


Fig. 6 Molecular structures of drugs targeting the Ras/ERK pathway. Examples of drugs targeting the B-Raf, MEK, and ERK kinases in the cell proliferation pathway. The molecular formula of each drug is given in parentheses. The three-dimensional drug structures were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>), a public chemical database of the National Library of Medicine (NLM). In the pathway diagram, orthosteric drugs are colored red and allosteric drugs are colored blue. Table 2 summarizes the details of drugs in the Ras/ERK pathway.







Table 3 Small molecules targeting the PI3K/AKT/mTOR pathway<sup>a</sup>

Drug name [brand name] (synonyms)	Drug type	PubChem CID	Target disease	Mechanism of action	Route of administration
Alpelisib [Piqray] (BYL-719, NVP-BYL719)	Orthosteric inhibitor	56649450	Metastatic breast cancer, PROS, ovarian and colorectal cancers	Selective class I PI3K $\alpha$ inhibitor used in combination with fulvestrant, targeting metastatic breast cancer	Oral
Copanlisib [Alicopa] (BAY-80-6946)	Orthosteric inhibitor	135565596	FL	Selective class I pan-PI3K inhibitor, preferentially inhibiting PI3K $\alpha$ and PI3K $\delta$ isoforms	Intravenous
Duvelisib [Copiktra] (INK-1147, INK-1197, IPI-145)	Orthosteric inhibitor	50905713	CLL, SLL	Selective PI3K $\delta$ and PI3K $\gamma$ inhibitor, restricting the activity to hematopoietic cells and inhibiting BCR signaling	Oral
Idelalisib [Zydelig] (CAL-101, GS-1101)	Orthosteric inhibitor	11625818	CLL, FL, SLL	Selective PI3K $\delta$ inhibitor used in combination with rituximab, inducing apoptosis of malignant cells and inhibiting BCR and C-X-C chemokine receptor signaling	Oral
Inavolisib [Iktoveli] (GDC-0077, RG-6114, RO-7113755)	Orthosteric inhibitor	124173720	HR+/HER2– breast cancer	Mutant-selective PI3K $\alpha$ inhibitor used in combination with palbociclib and fulvestrant	Oral
Tersolisib (STX-478, AGX9NKC8M9)	Allosteric inhibitor	166532451	Metastatic breast cancer, other solid tumors	Mutant-selective PI3K $\alpha$ H1047X inhibitor, suppressing cancer cell growth and inducing apoptosis	Oral
RLY-2608	Allosteric inhibitor	166822065	HR+/HER2– breast cancer	Selective pan-mutant PI3K $\alpha$ inhibitor	Oral
LOXO-783 (LOX-22783, LY-3849524)	Allosteric inhibitor	—	Breast cancer, other solid tumors	Mutant-selective PI3K $\alpha$ H1047X inhibitor	Oral
GSK2334470	Orthosteric inhibitor	46215815	—	PI3K $\delta$ inhibitor preventing AKT activation and used in combination with proteasome inhibitors	Oral
BX-795	Orthosteric inhibitor	10077147	OSCC, PDAC, neuroblastoma	PI3K $\delta$ inhibitor preventing AKT activation, also inhibiting other kinases such as TBK1 and IKK $\epsilon$	Oral, topical
Leelamine (dehydroabietylamine, NSC 2955)	Natural compound	118215	Melanoma, prostate cancer	Disrupting intracellular cholesterol transport and key signaling pathways such as PI3K/AKT, MAPK, and STAT	Oral, intravenous, intraperitoneal injection
OSU-03012 (AR-12)	Allosteric inhibitor	10027278	Glioblastoma, CLL, and gastric, pancreatic, breast, and esophageal cancers	Potent PDK1 inhibitor inducing apoptosis and autophagy. Also, enhancing endoplasmic reticulum stress and activating PERK that leads to cell death	Oral
PS210	Allosteric modulator	—	—	Potent PDK1 activator binding to the PIF-binding pocket <i>in vitro</i> . But in cells, its prodrug PS423 inhibits S6K phosphorylation by PDK1	—
Capivasertib [Truqap] (AZD-5363)	Orthosteric inhibitor	25227436	HR+/HER2– breast cancer	Pan-AKT (AKT1, AKT2, and AKT3) inhibitors used in combination with fulvestrant, suppressing phosphorylation of downstream AKT substrates	Oral
Ipatasertib (GDC-0068, RG-7440)	Orthosteric inhibitor	24788740	Neoplasm, solid cancer, triple-negative breast cancer, gastric cancer	Pan-AKT (AKT1, AKT2, and AKT3) inhibitors targeting the active, phosphorylated form of AKT	Oral



Table 3 (Contd.)

Drug name [brand name] (synonyms)	Drug type	PubChem CID	Target disease	Mechanism of action	Route of administration
MK-2206	Allosteric inhibitor	24964624	Breast, pancreatic, thyroid, endometrial, and colorectal cancers	Non-ATP competitive AKT inhibitor inducing apoptosis	Oral
Perifosine (KRX-0401, D-21266)	Alkylphospholipid, allosteric inhibitor	148177	Multiple myeloma and colorectal, lung, prostate, and brain cancers	AKT inhibitor targeting the PH domain and interfering with AKT-lipid interaction	Oral
Miransertib (ARQ 092, MK-7075)	Allosteric inhibitor	53262401	Solid tumor, relapsed lymphoma	Pan-AKT (AKT1, AKT2, and AKT3) inhibitors with non-ATP competitive binding	Oral
Sirolimus [Rapamune, Fyarro, Hyftor] (Rapamycin, AY-22989, WY-090217)	Allosteric inhibitor	5284616	LAM, PEComa	mTOR inhibitor, specifically targeting mTORC1, binding to FKBP12 and forming a complex that then interacts with and inhibits mTOR's activity, and suppressing T-cell and B-cell proliferation	Oral, topical, intravenous
Ridaforolimus [Taltorvic] (deforolimus, AP-23573, MK-8669)	Allosteric inhibitor	11520894	Solid tumor, sarcoma, endometrial, prostate cancer, bone metastases	mTOR inhibitor, specifically targeting mTORC1, leading to cell cycle arrest, and inhibiting tumor cell growth and proliferation	Oral, intravenous
Everolimus [Afinitor, Torpenz, Votubia, Zortress] (RAD-001, RAD-666, SDZ-RAD)	Allosteric inhibitor	6442177	RCC, NET, SEGA	mTOR inhibitor, specifically targeting mTORC1, binding to FKBP12 and forming a complex that then interacts with and inhibits mTOR's activity, leading to cell cycle arrest in the G <sub>1</sub> phase, and reducing the production of VEGF, and inhibiting T-cell proliferation	Oral
Temsirolimus [Torisel] (CCI-779, WAY-CCI 779)	Allosteric inhibitor	6918289	RCC, breast cancer, lymphoma, rheumatoid arthritis, multiple myeloma	mTOR inhibitor, specifically targeting mTORC1, binding to FKBP12 and forming a complex that then interacts with and inhibits mTOR's activity, leading to cell cycle arrest in the G <sub>1</sub> phase, and reducing the production of VEGF	Intravenous

<sup>a</sup> Abbreviations: BCR, B-cell receptor; CLL, chronic lymphocytic leukemia; FKBP12, FK506-binding protein 12; FL, follicular B-cell non-Hodgkin lymphoma; IKK $\epsilon$ , I $\kappa$ B kinase epsilon; LAM, lymphangioleiomyomatosis; NET, neuroendocrine tumor; OSCC, oral squamous cell carcinoma; PDAC, pancreatic ductal adenocarcinoma; PEComa, perivascular epithelioid cell tumor; PERK, protein kinase R-like endoplasmic reticulum kinase; PROS, *PIK3CA*-related overgrowth spectrum; RCC, renal cell carcinoma; SEGA, subependymal giant cell astrocytoma; SLL, small lymphocytic lymphoma; TBK1, TANK-binding kinase 1; VEGF, vascular endothelial growth factor.

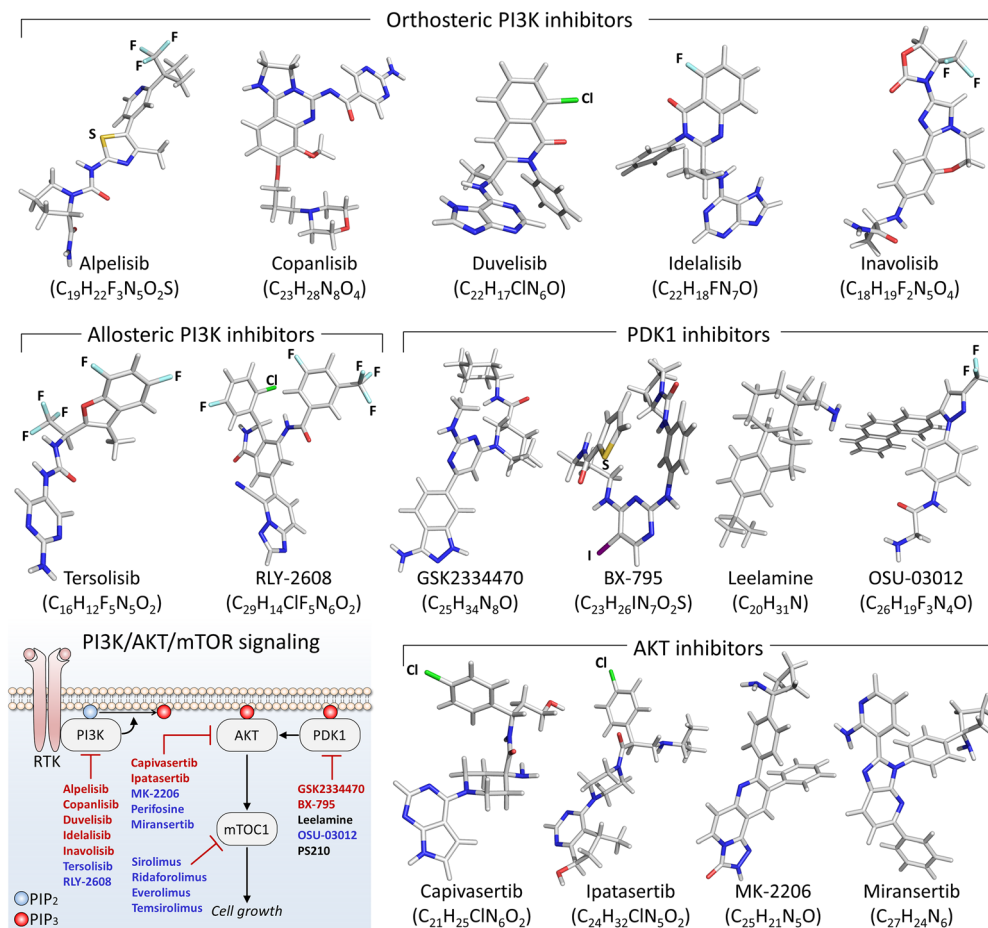


Fig. 7 Molecular structures of drugs targeting the PI3K/AKT/mTOR pathway. Examples of drugs targeting the PI3K, PDK1, and AKT kinases in the cell growth pathway. The molecular formula of each drug is given in parentheses. The three-dimensional drug structures were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>), a public chemical database of the National Library of Medicine (NLM). In the pathway diagram, orthosteric drugs are colored red and allosteric drugs are colored blue. Table 3 summarizes the details of drugs in the PI3K/AKT/mTOR pathway.

level. (ii) Considering cell types and cell states is vital, as is (iii) tumor evolution. To overcome drug resistance, learning single-cell transcriptomes over time could offer valuable insights into tumor evolution. Learning single cell data helps address the tumor heterogeneity challenge. The variability in tumor behavior is not random.<sup>165</sup> Cell types and states are determined by their networks and their transitions.<sup>43,166</sup> The network of a skin cell differs from that of a liver cell. Cells metastasize to tissues where their normally expressed proteins are overexpressed. Melanomas overexpress the highest number of brain-selective genes and this may contribute to melanoma metastasis to the brain.<sup>167</sup> Combining experimental and clinical data with computational (AI) tools can unravel how these complex data change over time. (iv) Position in the pathway matters: single substrate B-Raf is upstream of the MAPK pathway and its activation is slow. ERK downstream is challenging due to its feedback loops, large number of substrates, fast kinetics and continuous ON state,<sup>168–170</sup> suggesting their pharmacological combination. However, (v) kinases with high activation rates are commonly considered better drug targets because their dysregulation can have a more significant impact on cellular

processes.<sup>171–173</sup> (vi) Accounting for pathway crosslinks, feed-back loops and connectors.<sup>164,174</sup> Finally, (vii) kinase cascades exist in biomolecular condensates associated with LLPS, liquid-liquid phase separation. The condensates can be drug targets.<sup>175</sup> The condensates may also enrich and prolong the retention of small-molecule drugs.<sup>176</sup> Even though the principles and functions of condensate modifying drugs have been considered,<sup>177</sup> development of specific drugs is challenging.

## Conclusions

A single, constitutively active kinase can transform a healthy cell into an oncogenic cell;<sup>178</sup> pharmacology can vanquish kinase activity, decimating oncogene ‘addicted’ cancer cells, while sparing others. The active—but not the autoinhibitory—kinase conformation has a flawlessly organized structure. Next-generation inhibitor development requires knowledge of the activation mechanism and an insight into how activating mutations transform a kinase into its constitutive state, and importantly, foretell the emerging mechanism of drug resistance. Acquired relapse mutations interfere with drugs that





block signaling by compensating for mutational lesions in the same kinase or by an aberrant kinase hijacking an alternative pathway, vertically or horizontally bypassing the blockade. The future challenge of small molecule kinase inhibitors relies on combinations of optimized drugs to target individual learned cancer subtypes. It should also benefit from innovative perspectives.

Here, we learn kinase cascades to foster such innovations. Formalizing and computing a biological multivariable system, as here, is a complex challenging task. A possible avenue could include a protein language model, which includes a protein sequence model and its 3D structure, integrated with a computer vision model to image information about the cell, such as its type, localization, and spatial features.<sup>179</sup>

## Author contributions

R. Nussinov: conceptualization, formal analysis, funding acquisition, investigation, project administration, supervision, validation, and writing – original draft. C. Regev: validation and writing – review & editing. H. Jang: conceptualization, data curation, investigation, methodology, resources, validation, visualization, and writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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