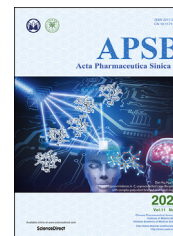




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REVIEW

Targeting RAS phosphorylation in cancer therapy: Mechanisms and modulators



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Abstract RAS, a member of the small GTPase family, functions as a binary switch by shifting between inactive GDP-loaded and active GTP-loaded state. RAS gain-of-function mutations are one of the leading causes in human oncogenesis, accounting for ~19% of the global cancer burden. As a well-recognized target in malignancy, RAS has been intensively studied in the past decades. Despite the sustained efforts, many failures occurred in the earlier exploration and resulted in an ‘undruggable’ feature of RAS proteins. Phosphorylation at several residues has been recently determined as regulators for wild-type and mutated RAS proteins. Therefore, the development of RAS inhibitors directly targeting the RAS mutants or towards upstream regulatory kinases supplies a novel direction for tackling the anti-RAS difficulties. A better understanding of RAS phosphorylation can contribute to future therapeutic strategies. In this

Abbreviations: ABL, Abelson; APC, adenomatous polyposis coli; CK1, casein kinase 1; CML, chronic myeloid leukemia; ER, endoplasmic reticulum; GAPs, GTPase-activating proteins; GEFs, guanine nucleotide exchange-factors; GSK3, glycogen synthase kinase 3; HVR, hypervariable region; IP3R, inositol trisphosphate receptors; LRP6, lipoprotein-receptor-related protein 6; OMM, outer mitochondrial membrane; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PPIs, protein–protein interactions; RIN1, RAB-interacting protein 1; SHP2, SRC homology 2 domain containing phosphatase 2; SOS, Son of Sevenless; STK19, serine/threonine-protein kinase 19; TKIs, tyrosine kinase inhibitors.

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review, we comprehensively summarized the current advances in RAS phosphorylation and provided mechanistic insights into the signaling transduction of associated pathways. Importantly, the preclinical and clinical success in developing anti-RAS drugs targeting the upstream kinases and potential directions of harnessing allostery to target RAS phosphorylation sites were also discussed.

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1. Introduction

The RAS family, which consists of small GTPases (guanine triphosphatases), is a pivotal component in the network of signal transduction, cell differentiation, proliferation, and survival^{1–5}. RAS proteins operate as binary molecular switches, which cycle between a GDP-bound inactive state and a GTP-bound active state^{6–8}. RAS activation is mediated by guanine nucleotide exchange-factors (GEFs), such as SOS or RAS guanyl nucleotide-releasing protein, which catalyze the release of GDP and promote the binding of the more dominant GTP^{5,9}. In the GTP-loaded active state, RAS proteins interact with various effectors, including RAF kinase and phosphatidylinositol 3-kinase (PI3K), leading to a wide range of cellular processes^{10–13}. To accelerate the intrinsic hydrolysis of GTP and therefore return RAS to the stable, inactive state, GTPase-activating proteins (GAPs), such as p120GAP or neurofibromin, function as negative regulators to expedite GTP hydrolysis and turn off the downstream signaling¹⁴.

Aberrantly activated RAS mutants were first identified in human cancer back in 1982, while recent studies have unraveled that malfunctions of RAS proteins are among the most common in tumorigenesis, taking up approximately 19% of the global cancer burden^{15–17}. Most oncogenic mutations concentrate at three hotspots, including Gly12, Gly13, and Gln61, which impair GTPase activity and GAP-mediated GTP hydrolysis, thus shifting the conformational equilibrium of RAS toward the GTP-loaded active state^{1,18–22}. Accordingly, the excessively activated RAS mutants initiate constitutive engagement and activation of the downstream effectors signaling through pathways such as mitogen-activated RAF/MEK/ERK or PI3K/AKT, leading to aberrant cell growth and survival.

In humans, the RAS family includes four different yet highly homologous RAS proteins (H-RAS, N-RAS, and two K-RAS splice variants K-RAS4A and K-RAS4B), encoded by three human RAS genes (*H-RAS*, *N-RAS*, and *K-RAS*)^{23,24}. Although all three genes are potential harbors of oncogenic mutations, they are not mutated at equivalent frequencies. K-RAS mutants are responsible for ~85% of all RAS-driven human cancers, followed by N-RAS (~12%) and H-RAS (~3%) mutations (COSMIC v80)²⁵. Moreover, the frequency imbalance is also reflected by the association that the mutated isoforms and codons vary by different tumor types¹⁸. For example, K-RAS mutations account for a large percentage of pancreatic ductal adenocarcinoma (86%), colorectal adenocarcinoma (41%), and lung adenocarcinoma (32%), which predominantly occur at position 12. While the prevailing N-RAS mutations are detected in melanomas (29%) and other hematological tumors, which commonly occur at position 61. H-RAS mutations are relatively rare in oncogenesis, but they also show a link with head and neck cancer (5%) and bladder urothelial carcinoma (6%), which occur at either position 12 or 61.

Given their central role in oncogenesis, RAS has been spotlighted as an intriguing target for anticancer drug development, and the

inhibition of RAS proteins has been considered a promising direction in oncology^{17,18,26–28}. However, many failures occur in the earlier exploration, in large part ascribed to the picomolar affinity of RAS for guanine nucleotide and lack of deep hydrophobic pockets amenable for small molecule ligands, leading to an ‘undruggable’ portrait of RAS^{3,18}. On the other hand, it was assumed that different isoforms of RAS were identical in function. Driven by the convenience of available reagents, early approaches mainly focus on H-RAS and ignored the features of other isoforms, rendering the efforts to develop farnesyltransferase inhibitors as anti-RAS modulators fruitless⁴. With accumulating evidence of functional differences among distinct isoforms, K-RAS, especially the predominant splice variant K-RAS4B, has attracted an ever-growing interest since it’s one of the most important targets in cancer research. In recent decades, the renewed impetus has promoted innovative attempts to tackle the elusive enigma, encompassing inhibition of the orthosteric site of RAS, RAS–GEF protein–protein interactions (PPIs), RAS-effector PPIs, cellular localization of RAS, and various post-translational modification such as acetylation, ubiquitylation, and nitrosylation^{9,29–37}. Due to nearly three decades of sustained attempts, AMG 510, a K-RAS^{G12C} inhibitor, exhibited anti-tumor activity and represents a potentially transformative therapy for patients harboring K-RAS^{G12C} mutation^{35,36,38–41} in clinical trials and has recently submitted its new drug application to FDA. Despite the recent advances in taming K-RAS^{G12C}, so far other RAS mutations, including K-RAS^{G12V} and K-RAS^{G12D}, still lack efficient targeting strategy, thus threatens the global public health and poses great challenges to pharmacological researches. Hence, with this reality check, further efforts to seek an effective anti-RAS strategy must become a priority, however difficult the task.

One of the emerging directions for tackling RAS-driven cancers is targeting the phosphorylation process of RAS. Phosphorylation is one of the most common mechanisms of protein regulation. Although the GTP/GDP loading switch is the major regulatory mechanism that controls the activity of RAS, with the ever-deepening knowledge of this family, phosphorylation at several residues has been recently determined as another regulator for wildtype and mutated RAS activity, which presents an up-and-coming trend in anti-RAS therapeutics^{42–49}. Not only the identified upstream kinases represent decent targets for RAS modulation, allosteric modulators can also target RAS at the phosphorylation sites distal to the GTP-binding site, and thus circumvent the obstacles for orthosteric drug binding. Therefore, upon targeting the phosphorylation reaction, therapeutic ligands can either target kinases and repress their phosphorylation function, or disrupt the modification by targeting the phosphorylation sites at RAS instead, as well as directly inhibit the phosphorylated RAS, thus creating lots of opportunities.

To date, targeting the upstream kinases in phosphorylation has already achieved some success in the campaign against RAS-driven oncogenesis^{50–53}. Quintessential examples include SRC

homology 2 domain containing phosphatase 2 (SHP2) inhibitors that preserve the phosphorylated state of Tyr32/64 and thus silence RAS, serine/threonine-protein kinase 19 (STK19) inhibitors targeting Ser89 phosphorylation to desensitize downstream effectors, and protein kinase C (PKC) inhibitors attenuating Ser181 phosphorylation to avoid RAS redistribution, etc. In addition, some of these successful attempts have progressed into clinical trials, which again highlights the great therapeutic potential of targeting RAS phosphorylation, against one of the most critical problems in clinic and pharmaceuticals-anti-RAS drug development ([ClinicalTrials.gov](https://clinicaltrials.gov) Identifiers: NCT03114319, NCT03565003, NCT03634982, NCT00031694 and NCT00006389).

In this review, we comprehensively summarized the current advances in the knowledge of RAS regulation by phosphorylation in an order of residue numbers of the phosphorylation sites and provided mechanistic insights into the signaling transduction of associated pathways. Importantly, the preclinical and clinical success in developing anti-RAS drugs targeting the upstream kinases and future directions of harnessing allostery to target RAS phosphorylation sites were also discussed. We aimed to increase the awareness towards the strategy of targeting RAS phosphorylation to overcome the 'undruggable' strait and supply guidance for future relevant studies.

2. Phosphorylation: A novel promise for anti-RAS therapeutics

2.1. Tyr4 phosphorylation by JAK2, SRC, and EGFR represents a feedback mechanism to restrict RAS activation

Rabex-5, also known as RabGEF1, functions towards RAS as either a Rab5 guanine exchange factor (GEF) or an A20-like E3 ubiquitin ligase. Rabex-5 participates in RAS signaling regulation by promoting RAS mono- or di-ubiquitination, restraining RAS proteins in the early endosome instead of the plasma membrane or Golgi, thus decreases RAS activity^{54,55}. This inhibitory modulation of Rabex-5 was not only validated in wild-type RAS, but also extended into the constitutively active oncogenic mutant RAS^{G12V}.

Recently, Pflieger and colleagues⁵⁶ identified the phosphorylation at RAS Tyr4 as an indispensable factor required for the subsequent ubiquitination of RAS. *Drosophila* RAS, whose N-termini is identical to human H-, N- and K-RAS, was used to investigate the specific region of RAS that is related to RAS ubiquitination, suggesting a general effect of phosphorylation at Tyr4 across the RAS family. The authors mapped the ubiquitination signal in *Drosophila* RAS and narrowed down to the previously-discovered phosphorylation site Tyr4 that directs RAS for ubiquitination.

The phenylalanine substitution of Tyr4 (to prevent phosphorylation) renders RAS insensitive to Rabex-5-mediated ubiquitination, consistent with a gain-of-function of RAS discovered *in vivo*. In contrast, the glutamine substitution of Tyr4 (to mimic the negative charge of phosphorylation) led to an elevated ubiquitination activity of Rabex-5 in S2 cells, and the Y4E mutation at RAS^{G12V} also suppressed oncogenic RAS phenotypes *in vivo*, dependent on the presence of Rabex-5. To conclude, phosphorylation at Tyr4 of RAS promotes Rabex-5-mediated ubiquitination, which further down-regulates RAS activity by redistribution of RAS to prevent its signaling with downstream effectors. To date, the detailed mechanism of p-Tyr4 affecting the interaction between RAS and Rabex-5 remains vague, which is probably similar

to the mechanism of SCF cullin ring ligases binding to its phosphorylated substrates, or simply by recruiting another adaptor protein⁵⁷. The authors further demonstrated that JAK2 and SRC kinases can promote phosphorylation of both RAS^{WT} and RAS^{G12V} at Tyr4, while EGFR can only promote phosphorylation of RAS^{G12V} at Tyr4, as recognized by anti-pY4 antibodies.

Although it has been widely acknowledged that JAK2, SRC, and EGFR are positive-regulators of RAS signaling, these kinases have also been discovered to promote RAS ubiquitination and inhibit its biological effects, thus implying a feedback regulatory mechanism to fine-tune RAS activity and ensure precise pathway output (Fig. 1)⁵⁶.

Rather intriguingly, RAS^{Y4H} mutation was reported in human cerebellar glioblastomas, together with the identification of the gain-of-function RAS^{Y4F}, indicating that Tyr4 phosphorylation is important for maintaining appropriate limits on RAS activities⁵⁸. However, to target RAS for cancer therapies, from the aspect of controlling Tyr4 phosphorylation and/or Rabex-5 induced ubiquitination, calls for future investigation on how phosphorylation and ubiquitination influence RAS structures and interactions with other proteins.

2.2. Phosphorylation cycle at Tyr32/64 mediated by SRC and SHP2

SRC is the first confirmed oncogene, which plays key roles in cell growth, division, migration, and survival as a protein-tyrosine kinase⁵⁹. An elevated SRC activity has been found in cell lines harboring oncogenic RAS mutations as well as in pancreatic ductal adenocarcinomas that are closely related to aberrant K-RAS activation^{60,61}.

With the previously established evidence of the crosstalk between SRC and RAS, Bunda et al.⁴⁴ validated that SRC binds to and phosphorylates GTP-loaded H/N-RAS on a conserved Tyr32 residue, leading to RAF displacement and a concomitant increase in its binding with GAPs. Namely, the Tyr32 of H/N-RAS acts as an SRC-dependent regulatory site, whose phosphorylation promotes the GTP hydrolysis activity and ensures unidirectionality in the RAS GTPase cycle.

The authors further discovered that the ubiquitous tyrosine phosphatase SHP2 plays a converse role in the regulation of H/N-RAS phosphorylation⁶². SHP2 has been well-established as a compelling modulator in the RAS/MAPK signaling pathway, and its gain-of-function mutations or overexpression have been found in a growing list of oncogenic RAS-driven tumors, encompassing pancreatic ductal adenocarcinoma, glioblastoma, and lung cancer⁶³. Bunda et al.⁶² determined that SHP2 functions as a direct activator of H/N-RAS by dephosphorylation of Tyr32, thus restoring the binding of RAF and resuscitating the downstream signaling.

In a very recent study of this team, similar to previously described for H/N-RAS, K-RAS was shown to be phosphorylated *via* SRC at Tyr32 and Tyr64, leading to a conformational shift in switch I and switch II regions, respectively⁴⁷. The phosphorylation of K-RAS by SRC attenuates GAP-assisted GTP hydrolysis and GEF-mediated nucleotide exchange, eventually resulting in an accumulation of pK-RAS-GTP, which is not only resistant to activation, but also decoupled from upstream regulation (Fig. 2A). Moreover, the favored form of pK-RAS is impaired in its ability to engage and activate the downstream RAF, thus rendering it a 'dark state' in the GTPase cycle (Fig. 2A). While SHP2-dephosphorylation is indispensable to maintain the dynamic

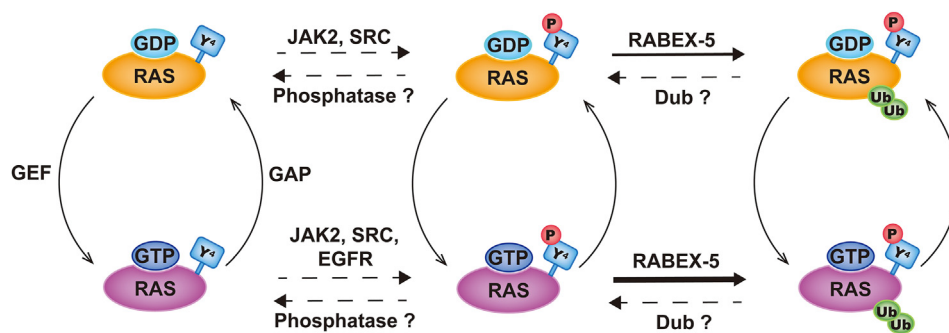


Figure 1 Proposed model of RAS^{pTyr4} ubiquitination by Rabex-5. Phosphorylation on RAS Tyr4 promotes ubiquitination of RAS-GDP and RAS-GTP. Tyr4 phosphorylation is potentially mediated by JAK2, SRC, and/or EGFR according to experimental observations.

cycle, or to unleash signaling-competent K-RAS from the ‘dark state’ of pK-RAS. Notably, this SRC- and SHP2-mediated phosphorylation cycle of K-RAS can be extended into oncogenic K-RAS with Gly12 mutations, whereby SHP2 similarly rescues the phosphorylated oncoproteins from silence, thus the therapeutic inhibition of SHP2 disrupts the balance and shifts the equilibrium of oncogenic K-RAS toward the silence ‘dark state’ (Fig. 2B).

The authors also provided structural insights into the phosphorylation results that might aid in understanding the underlying mechanism for the observed functional differences between K-RAS and its pTyr32 counterpart⁴⁷. A crystal structure of RAS in complex with GEF Son of Sevenless (SOS) revealed that Tyr32 and Tyr64 are key residues involved in the protein–protein interaction. Tyr32 binds directly with Asn944 of SOS to promote the insertion of an SOS helix into the RAS nucleotide-binding domain, inducing the opening up of the two switches and consequently destabilizing nucleotide binding (Fig. 3A). While Tyr64 extends into a hydrophobic pocket in SOS and forms a hydrogen bond with Gly931 (Fig. 3B). Therefore, the phosphorylation of Tyr32 and Tyr64 on K-RAS sabotages its interaction with SOS and other GEFs, due to an impaired hydrogen bond as well as steric hindrance and unfavorable electrostatics of the phosphate group. On the other hand, a bridging water molecule participates in both the intrinsic and GAP-catalyzed GTP hydrolysis process, which stabilizes the γ -phosphate of GTP and facilitates the attack by another water molecule. Tyr32 functions by directly binding the bridging water molecule, suggesting that its phosphorylation attenuates such stabilized structure for GTP hydrolysis and renders the resistance against GAPs (Fig. 3C). Meanwhile, Tyr64 forms a hydrophobic interface that facilitates the extensive interaction between K-RAS and GAP, highlighting its important role in GAP-assisted activation as well (Fig. 3D). Furthermore, since both residues locate in the effector binding site, it is predictable how phosphorylation reduces the affinity of RAS to RAF, thereby spoiling the RAF-MEK-ERK signaling pathway.

The discovery of an SRC- and SHP2-mediated phosphorylation cycle in K-RAS represents a significant step for the manipulation of RAS phosphorylation, based on which targeting SRC and SHP2 become a valid strategy for future anti-RAS efforts. Indeed, a series of preclinical and clinical trials have been initiated with SHP2 inhibitors, and several excellent reviews have discussed the inhibitor candidates and current trials⁵³. Herein, we highlight a list of newly-developed representative therapeutic agents targeting SHP2 at orthosteric or allosteric sites, including II-B08⁶², 11a-1⁴⁷, GS493⁶⁴, and SHP099⁴⁷ (Fig. 4). These SHP2

inhibitors block the GTP-loaded RAS mutant in its phosphorylated state and shift the equilibrium to silence the downstream effector RAF, leading to its anti-oncogenesis functions. On the other hand, despite the typical impression that SRC is a pro-oncogenic gene, its phosphorylation of RAS presents a distinct tumor-suppression function. Regarding the failures of SRC inhibitors in phase II trials, the unique RAS phosphorylation function of SRC provides us a clue that current trials may lack effective biomarkers to guide the selection of included patients⁶⁵. Accordingly, it would be prudent to take K-RAS mutational status into consideration for future clinical trials regarding SRC or SHP2 inhibitors.

2.3. A compelling yet controversial role of STK19 to phosphorylate Ser89 and mediate oncogenesis

As discussed above, the predominant N-RAS mutation in melanoma occurs at codon 61, where glutamine is replaced by arginine, lysine, or leucine¹⁸. Such substitution sabotages the intrinsic GTP hydrolysis and locks mutant N-RAS proteins in their GTP-loaded active state⁹. As a result, RAF proteins are constitutively recruited to the inner membrane for dimerization and activation, leading to uncontrolled proliferation of melanocytes and eventual transformation into melanoma.

Very recently, a study by Yin et al.⁵⁰ proposed that STK19 functions as a kinase for oncogenic N-RAS and initiates phosphorylation at Ser89, which enhances the binding between N-RAS and its downstream effectors, thus activating the malignancy transformation in oncogenic N-RAS-driven melanomagenesis. STK19 was reported as a potential cancer-driver gene, which has a statistically significant mutation burden in melanoma and skin basal cell carcinoma^{66,67}. It was originally reported to contain a protein kinase activity that phosphorylates α -casein at Ser/Thr residues and histone at Ser residues, and was further explored to participate in a transcriptional-related DNA damage response⁶⁸.

In their original study, Yin et al.⁵⁰ first screened a human kinome small interfering RNA library and identified STK19 as a positive N-RAS regulator, together with several previously reported N-RAS upstream kinases, including SRC⁴⁴ and EGFR⁶⁹. Biochemical, *in vitro*, and *in vivo* experiments ensued to demonstrate that STK19 phosphorylates N-RAS protein at Ser89 and enhances its binding affinity with the downstream effectors, contributing to the malignancy transformation in melanomagenesis. Importantly, a preferential phosphorylation of oncogenic N-RAS^{Q61R} and other N-RAS mutants compared to N-RAS^{WT} was observed by several *in vitro* assays, indicating that the STK19

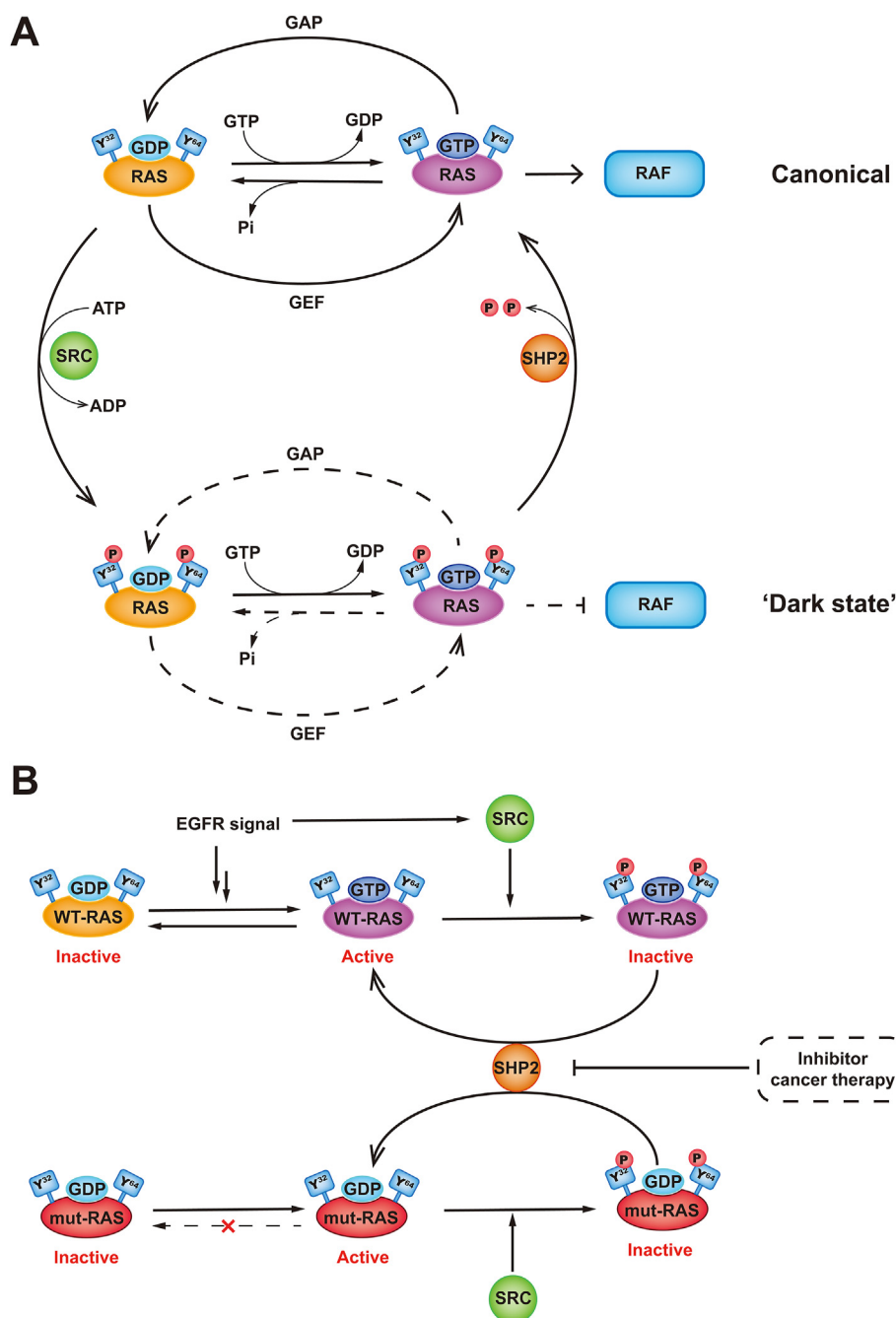


Figure 2 Model of RAS Tyr32/Tyr64 phosphorylation and its activity. (A) Impact of phosphorylation on the RAS GTPase cycle. SRC phosphorylates Tyr32 and Tyr64 of K-RAS, reduces GEF- and GAP-mediated nucleotide exchange and hydrolysis, and causes the accumulation of phosphorylated RAS-GTP. K-RAS^{pY32,pY64} has diminished affinity for RAF, thus shifting the RAS GTPase cycle to the 'Dark State'. SHP2 dephosphorylates RAS and reactivate the RAS GTPase cycle. (B) Wild-type and mutant RAS activity shift model mediated by upstream signal, SRC, and SHP2. Therapeutic inhibition of SHP2 disrupts the dephosphorylation of both wild-type and oncogenic K-RAS, shifting the equilibrium of RAS GTPase towards the silence 'Dark State'.

is critical for mutation-driven malignancy in N-RAS. Through additional kinase assay using N-RAS preloaded with GDP, GTP, or GTP γ S in the presence of STK19, the researchers confirmed that this difference in phosphorylation activity of STK19 stemmed from its preference for GTP-loaded active N-RAS compared to the GDP-loaded inactive form, suggesting a promising target for selective anti-cancer drugs.

Since the key residue Ser89 is conserved among all four RAS proteins, suggesting that other mutated isoforms may share similar

effects upon STK19 phosphorylation, STK19 was assumed to exhibit attractive potential for anti-RAS therapies in general. Accordingly, the researchers screened an in-house library of small molecule compounds, based on a combination of optimized biochemical ADP generation assays and structure-activity relationship studies. After iterative rounds of medicinal chemistry optimization, ZT-12-037-01 was yielded to boost potent inhibitory activity ($IC_{50} = 27.2 \pm 3.2$ mol/L), coupled with impressive selectivity among the human kinome (Fig. 5A). In a following study,

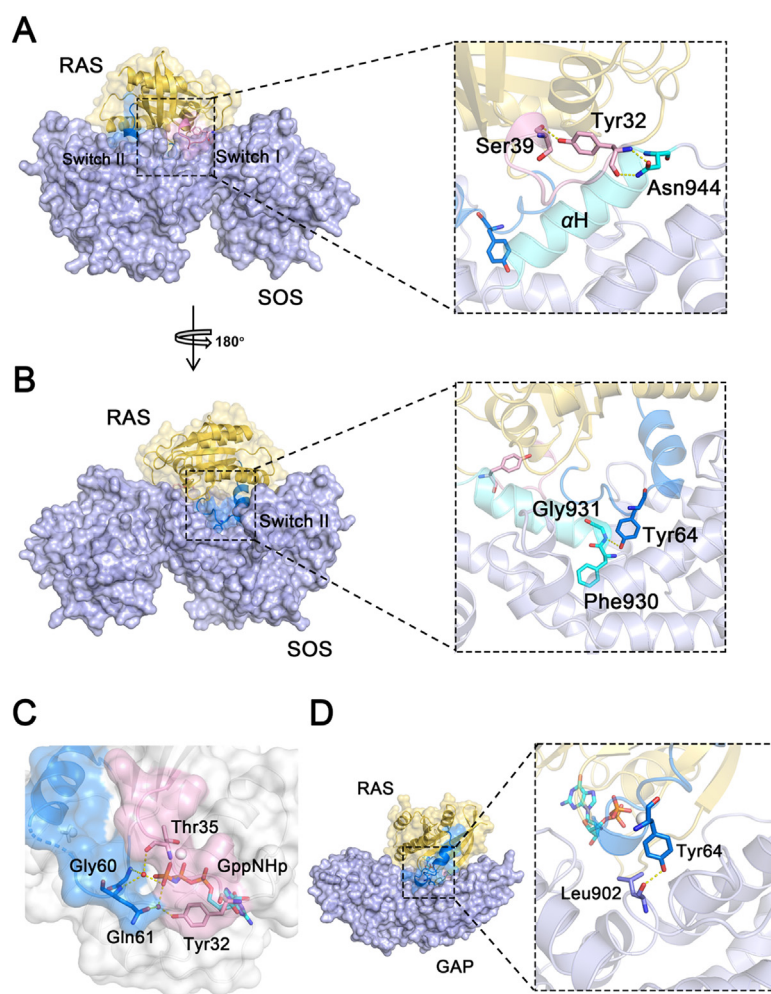


Figure 3 Crystal structure of RAS and its regulatory proteins SOS and GAP. (A) Front view of RAS–SOS complex (PDB ID 1BKD). Switch I and Switch II of RAS are shown in pink and marine, respectively, while the α H helix of SOS is shown in cyan. Yellow dashed lines represent hydrogen-bond interactions. RAS Tyr32 forms hydrogen bonds with Asn944 of SOS, promoting the insertion of SOS helix α H into the RAS nucleotide-binding domain. (B) Back view of RAS–SOS complex. RAS Tyr64 forms a hydrogen bond with SOS Gly931 of helix α H. (C) Proposed conformation of RAS Switch I and Switch II in intrinsic GTP hydrolysis (PDB ID 4G0N). Tyr32 binds to a bridging water molecule (depicted as red spheres), thus stabilizes GTP γ -phosphate and facilitates the attack by another water molecule. (D) Crystal structure of RAS–GAP complex (PDB ID 1WQ1). RAS Tyr64 interacts with GAP Leu902, thereby facilitates the GAP-catalyzed GTP hydrolysis.

Qian et al.⁵¹ screened a natural compound library based on a similar phosphorylation assay and identified chelidonine as a potential selective inhibitor of STK19 ($IC_{50} = 125.5 \pm 19.3$ mol/L), showing inhibitory potency both *in vitro* and *in vivo* (Fig. 5B). These validated STK19 inhibitors function by restraining RAS phosphorylation at Ser89, therefore hindering its affinity with the downstream effectors. In conclusion, targeting Ser89 phosphorylation presents another avenue for the development of anti-RAS therapies, and as a novel hope, STK19 inhibition warrants further exploration in preclinical and clinical studies.

Despite the inspiring success, Rodríguez-Martínez et al.⁷⁰ challenged the conclusion on STK19 reported by Yin et al.⁵⁰ and suggested it to be reconsidered. Their major concern stems in great part from their failure to detect the 41 kDa STK19 isoform, which includes the 110 N-terminal amino acids encoded by the first exon within the full-length gene. Instead, they only validated a 29 kDa isoform STK19 excluding the Asp89 residue, which was located in the nucleus and showed no apparent kinase function in MEK-AKT signaling. In response to this query, Yin et al.⁷¹ attributed their

failure to culture conditions and knockdown methods. They once again illustrated the expression of the 41 kDa STK19 isoform in melanocytes, which might be regulated by microenvironment such as ultraviolet radiation. Despite a relatively low abundance, the regulated expression seems sufficient to modulate N-RAS for melanomagenesis *in vitro* and *in vivo*. However, since the original kinase assays were conducted with STK19 derived from cell extracts, they acknowledged that STK19 may not possess intrinsic kinase activity, in agreement with Rodríguez-Martínez et al.⁷⁰. Instead, it is more likely that STK19 modulates N-RAS indirectly *via* an associated *bona fide* kinase. Under the circumstances, ZT-12-037-01 and chelidonine would probably function as the inhibitor targeting the associated kinase.

2.4. ABL phosphorylate Tyr137 to allosterically enhance effector binding

The RAS- and RAB-interacting protein 1 (RIN1) is a RAS effector that transmits activated signals from RAS and orchestrates

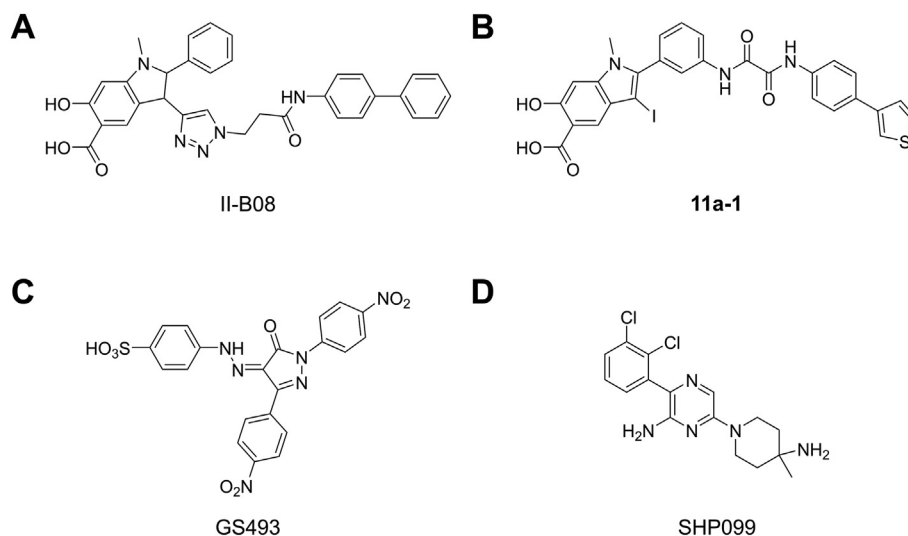


Figure 4 Small molecule SHP2 inhibitors. (A) II-B08, orthosteric inhibitor; (B) **11a-1**, orthosteric inhibitor; (C) GS493, orthosteric inhibitor; (D) SHP099, allosteric inhibitor.

downstream signaling *via* its own effectors, including Abelson (ABL) tyrosine-protein kinases, to modulate multiple cellular behaviors such as receptor endocytosis, cell adhesion, and cell migration^{45,72}. Recently, Ting et al.⁴⁵ reported a feedback mechanism that upon forming a ternary complex with activated RAS and ABL, RIN1 stimulates ABL and enables it to phosphorylate RAS on Tyr137, leading to an allosteric effect that attenuates the intrinsic GTP hydrolysis of RAS. The phosphorylation of Tyr137 by ABL2 were discovered in all four RAS isoforms, and both ABL isoforms (ABL1 and ABL2) were able to phosphorylate H-RAS, implying a functional conservation across different isoforms. It was proposed that RIN1 binds to the activated GTP-loaded H-RAS *via* its C-terminal, while its N-terminal binds to ABL SH3 and SH2 through a proline-rich domain (PxxP) and pTyr36, respectively. Consequently, RIN1 stably tethers RAS and ABL, further facilitating RAS phosphorylation by bringing it in close proximity to the catalytic domain of ABL (Fig. 6A). Although ABL was proved capable of phosphorylating both active and inactive conformations of H-RAS, according to the feedback mechanism, the oncogenic mutants of H-RAS, such as H-RAS^{G12V}, activates the downstream effector RIN1 more frequently, leading to a preference of Y137 phosphorylation in the cells expressing mutated H-RAS.

Phosphorylation on Tyr137 induces a higher affinity to RAF and prolongs the GTP-bound active state of RAS, thus promoting RAF-associated downstream signals. Although no direct structural data of H-RAS^{pTyr137} could be obtained due to difficulties in purification, crystal structures of H-RAS Tyr137 mutants, including Y137E and Y137F, provide mechanistic insights into the allosteric effects of phosphorylation on Tyr137. RAS Tyr137 was putatively recognized as a hotspot allosteric site that is connected with the active lobe through an intramolecular hydrogen bond network⁷³. In wild-type H-RAS, Tyr137 forms a hydrogen bond with His94 to bridge helices 3 and 4, while simultaneously packs along the Arg97 side-chain and participates in a hydrophobic pocket in the protein core (Fig. 6B). Upon RAF binding, the outward motion of Arg97 to the solvent reduces packing in the hydrophobic pocket, and allows helix 3 to shift towards helix 4, thus stabilizing an ordered Switch II (R state) and accelerates the hydrolysis rate. By contrast, in the H-RAS^{Y137E} mutant, Arg97 stretches deeper into a cavity occupied by the Tyr137 phenyl ring in wild-type RAS,

forming hydrogen bonds with Glu137 and Lys101 (Fig. 6C). As a result, His94 in helix 3 becomes less ordered and induced the repositioning of helix 4 to accommodate the reorganization. During RAF binding, since Arg97 is stuck deeper in the hydrophobic core, the shift of helix 3 towards helix 4 and Switch II is impeded, leading to a poorly ordered Switch II (T state) with attenuated GTP hydrolysis activity. However, H-RAS^{Y137F} mutant shows no significant structural difference with wild-type H-RAS (Fig. 6D). The structural information above is consistent with the experimental findings that Y137E mutant has an impaired GTP hydrolysis rate upon RAF binding than wild-type or Y137F H-RAS.

The revelation of the feed-back phosphorylation mechanism mediated by the RAS–RIN1–ABL complex provides a broad prospect in regulating RAS activities. Through inhibition of the ABL kinase, the phosphorylation of Y137 can be constrained and the allosteric signaling pathway to attenuate intrinsic GTP hydrolysis can be obstructed, thus promoting the conformational shift of RAS towards its GDP-loaded inactive state and blocking the aberrant activation of signal transduction in cancer cells. Tyrosine kinase inhibitors targeting ABL or/and BCR-ABL fusion protein have been well investigated, including imatinib, nilotinib, and dasatinib, in the effort to treat chronic myeloid leukemia (CML, Fig. 7)^{74,75}. Targeting ABL to regulate RAS phosphorylation states creates possibilities in extending anti-RAS therapies.

Intriguingly, H-RAS^{pTyr137} also showed interactivity with the SH2 domain of GAP RASA1, suggesting another mechanism of phosphorylation that impact RAS activities, which likely involves potential targets on RAS modulation. Therefore, the structural features and allosteric regulation mechanism of RAS^{pTyr137} is yet to be more deeply examined for targeting phosphorylation sites of RAS.

2.5. RAS stabilization or degradation mediated by WNT/ β -catenin signaling and Thr144/148 phosphorylation

It has been well received that canonical WNT signaling plays a critical role in both oncogenesis and tumor development^{76–78}. In the WNT/ β -catenin pathway, the absence of WNT ligands leads to phosphorylation of β -catenin by the destruction complex containing scaffold protein Axin, glycogen synthase kinase 3 (GSK3),

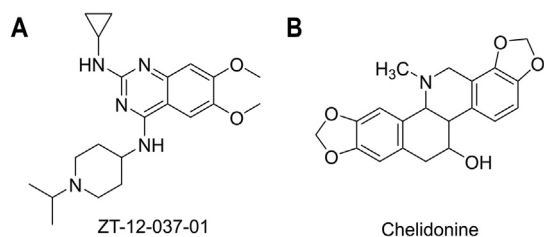


Figure 5 Small molecule inhibitors targeting STK19 kinase. (A) ZT-12-037-01; (B) chelidonium.

casein kinase 1 (CK1), and adenomatous polyposis coli (APC). In this WNT inactive state, β -catenin is phosphorylated by GSK3 β to form a phosphodegron, which is followed by ubiquitination *via* β -TrCP200 and targeted for proteasomal degradation. The canonical pathway is activated upon binding of WNT ligands to their co-receptors, including low-density lipoprotein-receptor-related protein 6 (LRP6) and frizzled proteins. LRP6 are then phosphorylated and induce the recruitment of dishevelled proteins to the plasma membrane for polymerization and activation, thus inactivating the destruction complex. Accordingly, the activation of WNT results in the stabilization and translocation of β -catenin, which functions as transcriptional coactivator to initiate downstream gene expression. Besides β -catenin, GSK3 has many other protein substrates⁷⁹. Therefore, the inhibition of WNT/ β -catenin signaling contributes to the polyubiquitin-dependent degradation of these proteins after their phosphorylation by GSK3 and the subsequent recruitment of β -TrCP–E3 ligase, leading to a phenomenon called WNT-stabilization of proteins (WNT-STOP)^{79,80}.

Jeong et al.⁴³ reported that H-RAS is a direct substrate of GSK3, containing two threonine residues (Thr144 and Thr148) for phosphorylation. This post-translation modification recruits the β -TrCP–E3 ligase to H-RAS and mediates its polyubiquitylation and degradation by the proteasome (Fig. 8). Whereas RAS lacks the conserved β -TrCP binding motif, the recruitment of β -TrCP–E3 indicates that the phosphorylated H-RAS presents a novel binding site. In addition, this GSK3-mediated phosphorylation of H-RAS at Thr144/148 can be inhibited by WNT3 and is facilitated by Axin and APC. They also provided *in vivo* evidence that excessive H-RAS stabilization resulting from the aberrant activation of WNT/ β -catenin signaling, is positively associated with oncogenesis in colonic tumor cells. In addition, consistent with the location of Thr144 and Thr148 in H-RAS at a GSK3 β phosphorylation consensus motif, S/TXXXS/T, the amino acids ¹⁴⁴TSAKT¹⁴⁸ are conserved among all RAS isoforms. Therefore, the GSK3 β -induced ubiquitylation and degradation was also discovered in K-RAS and H-RAS.

Since H-RAS phosphorylation at Thr144/148 has been validated as a pivotal step for degradation and the failure of phosphorylation results in colorectal oncogenesis, it is proposed that the inhibition of the aberrant WNT/ β -catenin signaling may play an anti-cancer role through the recovered degradation pathway, and the destabilization of RAS by the destruction complex represents an appealing therapeutic target.

A sufficient elaboration of anti-cancer drug development targeting the WNT/ β -catenin pathway has been conducted by Krishnamurthy et al.⁸¹. These WNT/ β -catenin pathway inhibitors promote the GSK3 β -induced phosphorylation at Thr144/148, which represent a critical driver for β -TrCP-mediated polyubiquitylation. Thus, the over-expressed and aberrantly activated RAS can be

degraded by the ubiquitin-proteasome system to impede cell growth. For example, LGK974 and ETC-159 were proved to be able to inhibit porcupine, which is crucial for WNT ligands secretion, therefore blocks WNT signaling (Fig. 9). In addition, NVP-TNKS656 as a tankyrase inhibitor, constrains the degradation of Axin in the WNT/ β -catenin pathway, could help overcome the drug resistance to PI3K/AKT/mTOR inhibition (Fig. 9).

2.6. Ser181 phosphorylation influences K-RAS4B membrane localization and affects RAS oncogenicity

The C-terminal hypervariable region (HVR) of RAS, consists of residues 167-188/189, varies across four human RAS isoforms, and is associated with RAS membrane targeting. In K-RAS4B, the HVR contains a polylysine motif and a CAAX motif modified by farnesylation, responsible for the electrostatic interaction with negatively charged phospholipids of the plasma membrane. Ser181, a residue within HVR, has been well established as a phosphorylation site of K-RAS4B by PKC, representing one of the best-known examples for RAS phosphorylation⁴².

Bivona et al.⁴² first suggested a ‘farnesyl-electrostatic switch’ mechanism that upon Ser181 phosphorylation mediated by PKC, the polybasic region of K-RAS HVR is partially neutralized, therefore induces K-RAS translocation from the plasma membrane to the endoplasmic reticulum (ER), Golgi apparatus, and outer mitochondrial membrane (OMM). Recently, a more detailed mechanistic association between RAS phosphorylation and relocalization was revealed by Zhang et al.⁸². They reported that the phosphoryl group leads to weakened electrostatic interactions between K-RAS4B and membranes in a membrane fluidity-dependent manner, instead of complete inhibition towards the binding.

Sung et al.⁸³ further reported significantly-enhanced cytotoxicity observed in K-RAS^{G12V,S181E}, a p-Ser181-mimicking oncogenic RAS mutant. The K-RAS/BCL-X_L interaction, which is found to be enhanced by PKC agonists, was proposed to account for the stimulated cell death in S181E mutants. The GTP-loaded, phosphorylated K-RAS4B binds to inositol triphosphate receptors (IP3R) in a BCL-xL-dependent fashion and forms a ternary complex, thereby attenuates the ability of BCL-xL to potentiate the IP3R-mediated Ca²⁺ flux from ER to mitochondria, causing respiration inhibition and cell apoptosis (Fig. 10).

Moreover, differed from other RAS isoforms, the unique HVR of K-RAS4B contains a single farnesyl modification and a positively charged polylysine sequence, which modulate the binding of K-RAS and the anionic phospholipids, as well as the farnesyl membrane orientation. Upon phosphorylation, the strong electrostatic interaction of the phosphoryl with the HVR’s highly positive side-chains bends the HVR and collapses the surrounding C-terminal residues, thus generating electrostatic repulsion with phospholipid headgroups and releasing the farnesyl-plasma membrane interaction. Accordingly, phosphorylation reduces but does not inhibit membrane binding of K-RAS4B, which explains the isoform-specific signaling at the membrane⁸⁴.

However, discordant opinions are held by Barceló et al.^{85,86} that Ser181 phosphorylation of K-RAS4B up-regulates RAS downstream signaling by affecting its distribution within plasma membrane, instead of suppressing cell survival. They found that Ser181 phosphorylation decreases K-RAS’ susceptibility to GAP and maintains its ability to activate AKT and MAPK, therefore

enhancing the functionality of both wild-type and oncogenic K-RAS. They also demonstrated that upon phosphorylation, the majority of K-RAS is still located at the plasma membrane, but a distinct K-RAS nanocluster favoring the activation of RAF-1 and PI3K is formed, which could explain the increased RAS activity due to Ser181 phosphorylation.

Although the general effects of phosphorylation on RAS localization remains unclear, Ser181 phosphorylation in K-RAS has been reported to promote tumorigenesis of human colon cell lines and is essential to the oncogenic function of mutant K-RAS *in vivo*, leading to the constantly emerging pharmacologic treatment trials targeting PKC⁸⁵. PKC inhibitors, such as bryostatin-1 and edelfosine, have been validated to suppress tumor growth in K-RAS^{G12V}, which may partly result from the reduced phosphorylation level at Ser181 and the subsequent the translocation of K-RAS towards ER, Golgi apparatus, and OMM, promoting apoptosis of cancer cells. In addition, these inhibitors failed in non-phosphorylatable K-RAS^{G12V,S181D}, further confirming the anti-cancer potential of inhibiting Ser181 phosphorylation by targeting PKC (Fig. 11)⁸⁵. Despite the deepening knowledge, we are still at the beginning of developing anti-PKC drugs for clinical approval. Most academic and pharmaceutical efforts to regulate PKC are faced with challenges in overcoming the low selectivity and inevitable toxicity during clinical trials⁵². A combination of paclitaxel with bryostatin-1

for pancreatic cancer therapy or cisplatin with bryostatin-1 for stomach cancer therapy, for example, has failed phase II study for lack of response and inevitable adverse effects⁷¹. In addition to improving the pharmaceutical performances of drugs targeting PKC, direct inhibition of the RAS phosphorylation site around Ser181 may provide a novel opportunity to enhance selectivity and avoid toxicity.

3. Conclusions and perspectives

In the past decades, taming RAS mutations, one of the most common genetic lesions in human malignancy, has become an urgent need for anti-cancer therapy. Recent progress to reveal RAS phosphorylation mechanisms has aroused the interest to revisit this long-pursued target, bringing new hope and potential to the attempts for conquering RAS-driven cancers^{18,34}.

Relevant kinases and phosphatases have been proven decent targets for RAS modulation, such as STK19, PKC, and SHP2. This alternative strategy avoids the difficulties of exploring hydrophobic pockets in RAS amenable for ligand binding, and shifts the research focus from the long-puzzling RAS towards the better-understood cell signaling proteins. In this paper, we have discussed the on-going trials and potential directions of the development of small molecule regulators targeting these cell signaling proteins, including tyrosine kinase inhibitors (TKIs), aurora kinase

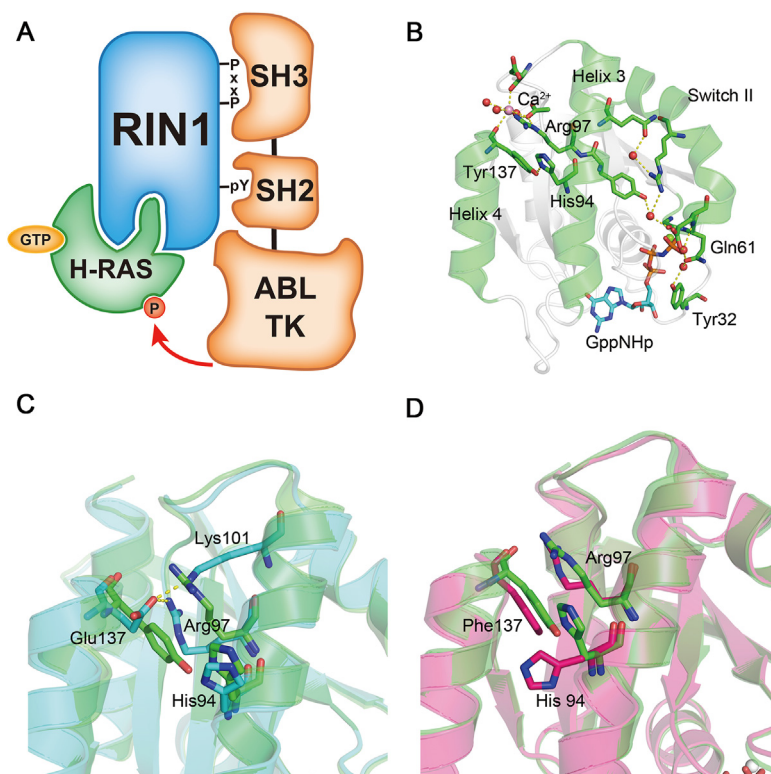


Figure 6 (A) Model of H-RAS/RIN1/ABL ternary complex. The C-terminal of RIN1 binds to H-RAS while its N-terminal binds to ABL. Activated ABL phosphorylates H-RAS on Tyr137. (B) Crystal structure of wild-type H-RAS in complex with GTP analog GppNHp (PDB ID 3K8Y). Switch II, helix 3, and helix 4 are shown in green. Gln61 is stabilized in a precatalytic conformation (R state) by a water-mediated hydrogen-bond network. Waters and residues that participate in the network are depicted as red spheres and sticks, respectively. Yellow dash lines represent hydrogen-bond interactions. (C) Crystal structure of H-RAS^{Y137E} (cyan) (PDB ID 4XVQ) superimposed on H-RAS^{WT} (green). Mutated residue Glu137 forms hydrogen bonds with Arg97 and Lys101, dragging Arg97 deeper into the hydrophobic core of H-RAS. (D) Crystal structure of H-RAS^{Y137F} (magenta, PDB ID 4XVR) superimposed on H-RAS^{WT} (green). The orientations of R97 and F137 side-chains showed no significant differences with wild-type H-RAS.

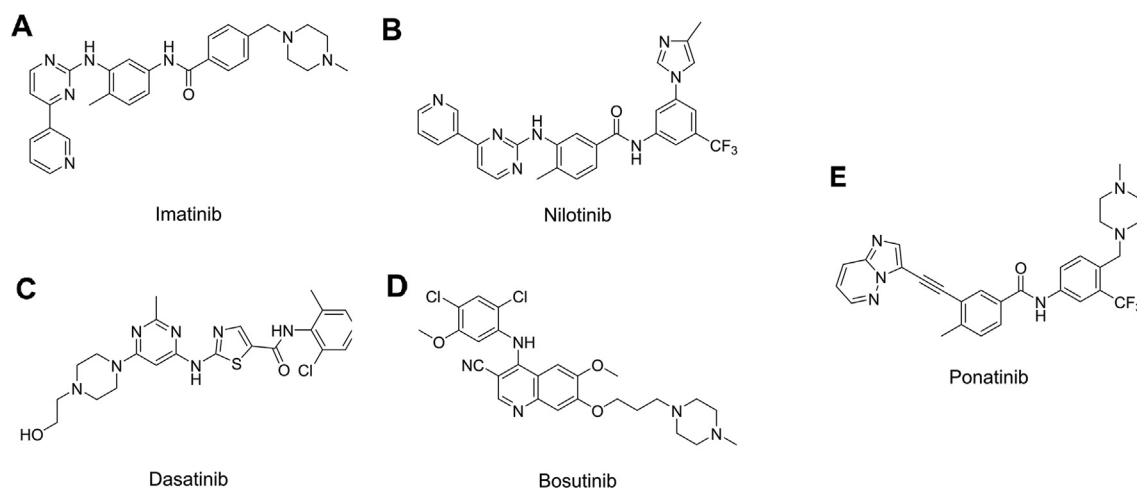


Figure 7 Clinical approved small molecule BCR-ABL inhibitors. (A) Imatinib; (B) nilotinib; (C) dasatinib; (D) bosutinib; (E) ponatinib.

inhibitors (serine/threonine kinase inhibitors), phosphatase inhibitors and WNT signaling inhibitors, in the efforts of anti-RAS cancer therapies.

In spite of the convenience of targeting RAS phosphorylation regulatory proteins, the poor selectivity of these inhibitors represents another marked obstacle in anti-cancer therapies. As previously discussed, the corresponding kinases and phosphatases exhibit relatively low preference for either mutant protein or a specific isoform, which may lead to side-effects or systemic toxicity *in vivo*. In the aspect of mutant form selectivity, STK19 prefers to phosphorylate Ser89 in GTP-loaded active N-RAS compared to the GDP-loaded inactive form, thereby circumventing inhibition of the needed wild-type RAS and reducing side-effects. However, the selectivity of other corresponding kinases and phosphatases is insignificant or only reflects the strength of feedback regulation, which lacks pharmaceutical value of selectivity. Meanwhile, in terms of selectivity across the RAS family, Ser181 phosphorylation induced by PKC is notably feasible in K-RAS and outstands all other isoforms, owing to the divergencies in the HVR of different proteins. Therefore, modulators targeting Ser181 in the HVR provides potential solution for isoform-specific inhibitors targeting K-RAS. On the contrary, other

phosphorylation sites are located in the conserved regions of the RAS family, leading to unsatisfying selectivity for a single isoform. To identify the phosphorylation selectivity on a molecular level, intramolecular signals, such as allosteric signaling pathways throughout the proteins, may provide structural details and subtle distinctions among different RAS mutants or isoforms. Additionally, despite the potential side-effects, pan-RAS inhibitors (inhibition of activated RAS signaling regardless of isoform or mutation) are still considered therapeutically beneficial based on *in vivo* experiments⁸⁷. Thus, the assumption of targeting RAS phosphorylation process is promising while at the same time calling on further investigations on the selectivity and safety.

On the other hand, the development of RAS inhibitors targeting the phosphorylation sites also presents a potential direction, which still has a long way to go. Targeting the topologically distal allosteric sites of RAS phosphorylation features subtle but accurate orchestrations among a series of biological events, and thus circumvent the obstacles of orthosteric ligand binding^{37,88}. Targeting RAS phosphorylation site with allosteric modulators not only disrupts the phosphorylation process and regulates RAS activities, as inspired by the accumulating cryptic allosteric sites detected in wild-type and mutant RAS, it also presents a

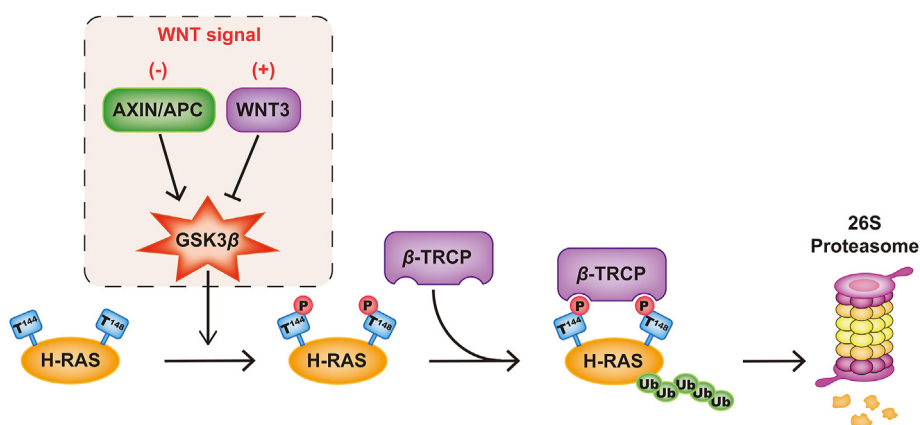


Figure 8 Model of phosphorylation-induced H-RAS degradation. GSK3 β mediates H-RAS to phosphorylate Thr144 and Thr147, followed by polyubiquitylation *via* β -TrCP and degradation *via* 26S proteasome. GSK3 β -mediated phosphorylation is inhibited by WNT3 and is facilitated by Axin and APC.

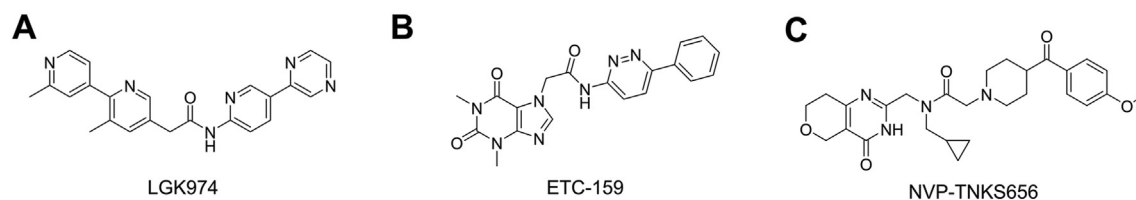


Figure 9 WNT/β-catenin pathway inhibitors. (A) PORCN inhibitor LGK974; (B) PORCN inhibitor ETC-159; (C) Tankyrase inhibitor NVP-TNKS656.

promising strategy to explore potential druggable pockets on the surface of phosphorylated RAS. Compared with traditional orthosteric pockets, allosteric cavities are less conserved in evolution, contributing to higher selectivity and reduced off-target toxicity⁸⁹. Moreover, cooperatively modulating the affinity or efficacy of guanine nucleotide instead of directly competing for binding, allosteric modulators targeting phosphorylation sites are no longer subject to the strict limits of binding affinity, and present more favorable pharmacological performances such as less adverse effects and a lower chance for resistance, since they subtly tweaking the protein activity rather than completely switching it on or off. Recent successful K-RAS^{G12C} specific inhibitor AMG 510, or sotorasib, has been proved to bind K-RAS^{G12C} on its 'cryptic allosteric site' and forms a covalent bond with the mutated residue³⁹. The development and validation of AMG 510 inspires the exploration of allosteric drugs directly targeting RAS phosphorylation sites. Also, it gave us a clue to achieve mutant and isoform selectivity by directly targeting the mutated RAS isoform, instead of the upstream or downstream proteins. Moreover, since the cryptic allosteric cavities on the surface of K-RAS^{WT} and K-RAS^{G12C} are mutant-distinct^{39,90,91}, it is quite possible that phosphorylation may induce novel cryptic sites for allosteric drug development.

Despite the impressive advantages, the trapped development of allosteric agents targeting RAS phosphorylation sites reflects the

difficulties in identifying suitable pockets for ligand binding in RAS, which is the pre-requisite of allosteric drug discovery^{92,93}. Once based on serendipitous discovery, the identification of allosteric pockets has greatly benefited from the rapid progress in bioinformatics methodologies over the latest years^{92–94}. The application of several recently-reported computational tools, such as AlloFinder, AlloSitePro, and CavityPlus, which aid not only allosteric pocket detection, but also ligandability and druggability evaluation, as well as AlloDriver and AlloMAPS, which enable the evaluation of mutations' allosteric effects, greatly facilitate the structure-based rational allosteric drug development^{95–100}. Moreover, a workflow integrating multi-disciplinary techniques has been validated to detect cryptic allosteric cavities throughout protein dynamic ensembles, and has achieved considerable success in detecting allosteric pockets in K-RAS⁹¹. Consequently, these bioinformatics methods provide promising aids to harness allosteric inhibitors targeting RAS phosphorylation sites.

Furthermore, the inevitable issues of drug resistance to the traditional RAS inhibitors remains an urgent need to be addressed. In the campaign to tackle this refractory problem, the combination of modulators targeting RAS phosphorylation together with traditional anti-RAS drugs provides a novel strategy for overcoming acquired resistance¹⁰¹. Both the upstream kinase regulators and the RAS allosteric inhibitors targeting the phosphorylation sites, function synergistically with the traditional

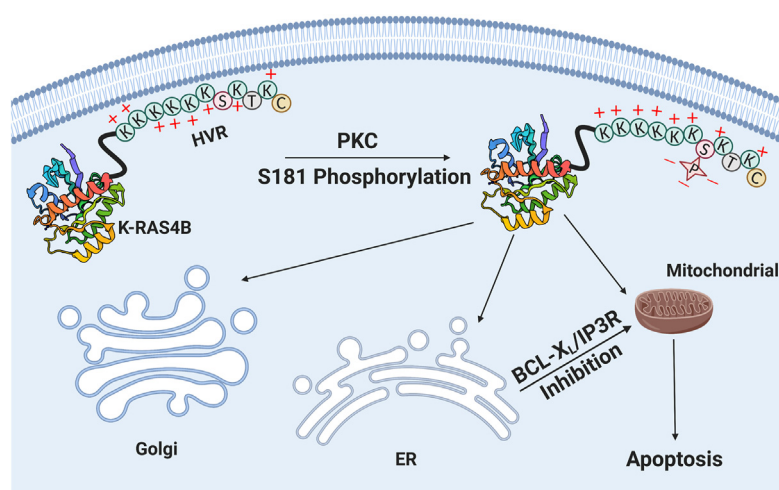


Figure 10 Model of phosphorylation-induced K-RAS4B redistribution and cell apoptosis. Protein kinase C (PKC) phosphorylates K-RAS4B on Ser181 of hypervariable region (HVR) and induces K-RAS4B to translocate towards ER, Golgi, and OMM. K-RAS4B^{Ser181} attenuates BCL-X_L/IP3R-mediated calcium transfer from ER to mitochondria, thus promoting cell apoptosis.

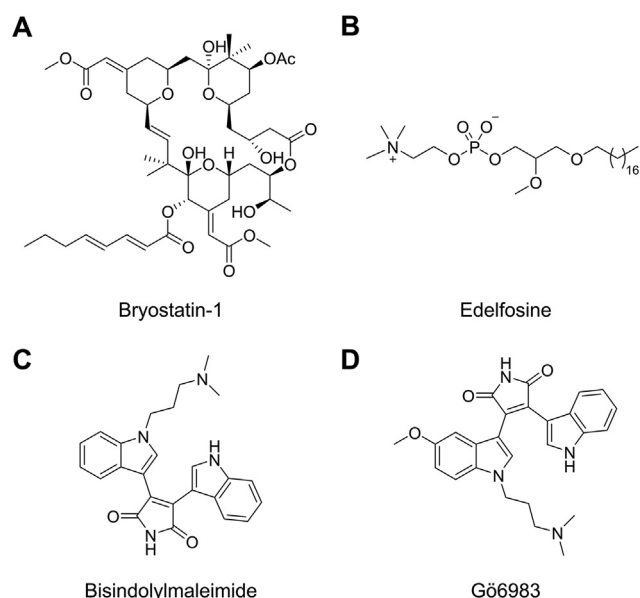


Figure 11 Small molecules targeting PKC activity. (A) Bryostatatin-1; (B) Edelfosine; (C) Bisindolylmaleimide (BIM); (D) Gö6983.

anti-RAS drugs through a novel and different mechanism. Therefore, with the coadministration strategy utilizing kinase regulators, RAS proteins are faced with less selectivity pressure, leading to a delay of acquired drug resistance. Meanwhile, with the double-targeting application towards RAS, two distinct ligands co-bind with the recalcitrant target. The phosphorylation regulator fine-tunes the residue network to favor a conformation prone to the other ligand, or subtly shifts the resistance-related pathway within RAS, thus resensitizing the target¹⁰¹. Accordingly, combinational therapy may represent a major solution to eventually overcome this clinically insuperable obstacle.

To summarize, although the understanding of RAS phosphorylation needs to be further deepened, targeting this intriguing regulation pathway to tackle RAS-related oncogenesis; that is, one of the critical bottlenecks of modern anti-cancer research, creates lots of opportunities and has achieved some success. Given the compelling discoveries and substantial advantages of targeting RAS phosphorylation, we express cautious optimism that it might pave a promising avenue for future anti-RAS therapeutics.

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Author contributions

Shaoyong Lu conceived and designed the study. Yuran Qiu, Yuanhao Wang, Zong-Tao Chai, Duan Ni, Xinyi Li, Jun Pu, Jie Chen, Jian Zhang, Chuan Lv, and Mingfei Ji analyzed the results. Yuran Qiu and Yuanhao Wang wrote the original manuscript. All authors discussed the results and reviewed the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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