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RAS Function in Cancer Cells: Translating Membrane Biology and Biochemistry into New Therapeutics

Walaa E. Kattan[1,2], **John F. Hancock**[1,2,*]

[1]Department of Integrative Biology and Pharmacology, McGovern Medical School University of Texas Health Science Center at Houston, TX 77030, USA

[2]The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences, TX 77030, USA

Abstract

The three human RAS proteins are mutated and constitutively activated in ~20% of cancers leading to cell growth and proliferation. For the past 3 decades, many attempts have been made to inhibit these proteins with little success. Recently; however, multiple methods have emerged to inhibit KRAS, the most prevalently mutated isoform. These methods and the underlying biology will be discussed in this review with a special focus on KRAS-plasma membrane interactions.

1. RAS Isoforms, Structure and Posttranslational Modifications

RAS proteins are part of a superfamily of more than 150 low molecular weight GTP binding proteins [1, 2] that include the RAB, RAN, RHO and ARF families [3]. RAS proteins oscillate between an active GTP-bound and inactive GDP-bound state to modulate cell survival, proliferation and differentiation [4]. RAS is activated by guanine nucleotide exchange factors (GEFs) that facilitate GDP unloading; this promotes GTP binding because GTP concentrations in the cell are 10-fold higher than GDP [5]. RAS proteins possess a slow intrinsic GTPase activity [6, 7] which is enhanced 10^5-fold by GTPase-activating proteins (GAPs). GTP hydrolysis returns active GTP-bound RAS to the inactive GDP-bound ground state.

There are 3 RAS genes ubiquitously expressed in human cells. HRAS and KRAS were first identified as oncogenes in the Harvey and Kirsten Rat Sarcoma retroviruses [8]. NRAS was identified in transformation assays using DNA from a Neuroblastoma [9]. Due to alternative splicing, the KRAS gene generates two protein isoforms, KRAS4A and KRAS4B (hereafter KRAS), the latter of which is generally accepted to be expressed at higher levels [10]; however, some studies have shown that KRAS4A is equally expressed in a variety of cancer cell lines and human colorectal cancers [11]. The RAS isoforms share 90% sequence homology in the G domain, which binds the guanine nucleotide [12, 13]. The G domain comprises an effector lobe (residues 1–86) and an allosteric lobe (residues 87–165). The effector lobe interacts with RAS effectors such as RAF and PI3K and has two regions called

*To whom correspondence should be addressed: John F. Hancock, Department of Integrative Biology and Pharmacology, McGovern Medical School University of Texas Health Science Center at Houston, Houston, TX 77030; john.f.hancock@uth.tmc.edu.

Switch I (residues 30–40) and Switch II (60–76) [12] that undergo major conformational reorganization on GTP binding [14, 15]. The GTP-bound form exists in two states: state 1 is an open conformation that promotes nucleotide exchange and discourages effector binding; while state 2 is a closed conformation that encourages GTP hydrolysis and effector binding [16]. GTP binding also reorients the RAS protein with respect to the membrane to allow for effector protein interaction [15, 17, 18]. Despite extensive sequence homology in the G-domain, the RAS isoforms differ substantially in their C-terminal hypervariable region (HVR) (residues 166–189) which regulates subcellular localization, trafficking and plasma membrane (PM) spatiotemporal organization [19, 20].

All RAS proteins have a C-terminal “CAAX” sequence where C is cysteine, A is an aliphatic amino acid and X is Methionine or Serine. Nascent RAS proteins synthesized in the cytosol are processed by farnesyl transferase (FTase) that catalyzes the addition of a 15-carbon (farnesyl) isoprenoid to the C-terminal cysteine [21] (Figure 1). This modification targets RAS to the cytosolic surface of the endoplasmic reticulum (ER) where the endoprotease RAS converting enzyme 1 (RCE1), cleaves the “AAX” tripeptide from the prenylated cysteine [22–26]. Finally, isoprenylcysteine carboxyl-methyltransferase (ICMT) methyl-esterifies the a-carboxyl group of the now C-terminal farnesylated cysteine [27–33]. Following CAAX processing, the RAS isoforms differ in membrane trafficking and localization due to different “second signals” in the HVR upstream of the processed CAAX motif [34]. The HVR of NRAS and HRAS, contain one or two, respectively, cysteine residues that are palmitoylated by the heterodimeric Golgi palmitoyl acyltransferase DHHC9 and GCP16 [35, 36] and are then transported to the inner leaflet of the plasma membrane (PM) via the exocytic pathway [27, 37, 38]. The HVR of KRAS, contains a polybasic domain of 6 contiguous lysine residues that targets KRAS to the largely negatively-charged inner leaflet of the PM via the endosome, bypassing the Golgi [38]. Hence, the HVR contributes to differential signaling between RAS proteins due to different post-translational modifications these regions undergo that dictate RAS membrane trafficking and localization [5, 19]. This can clearly be seen in studies showing that KRAS but, not N- or HRAS, is essential for normal development in mice [24, 39–44] and that each isoform activates a common set of effectors with varying efficiencies *in vitro* [42].

RAS proteins undergo constant cycles of solubilization and membrane binding that are required to maintain the fidelity of PM localization. Following endocytosis, HRAS and NRAS undergo de-palmitoylation by acyl-protein thioesterase (APT) releasing the proteins into the cytosol for recycling back to the Golgi where they are repalmitoylated and returned to the PM by vesicular transport via the exocytic pathway (Figure 1). PM-bound KRAS also undergoes endocytosis but has different outcomes depending on the phosphorylation state of the KRAS HVR at Ser181 by PKC and PKG2 [24, 45–49]. The endocytic vesicle is initially rich in the anionic phospholipid phosphatidylserine (PtdSer) but loses this asymmetry, decreasing the negative charge after endocytosis [50–53], releasing KRAS into the cytosol. Soluble KRAS is then captured by the prenyl-binding protein phosphodiesterase delta (PDEδ) which unloads KRAS at the perinuclear region where the Arl2 GTPase is concentrated. Arl2 interacts with PDEδ inducing a conformational change that promotes KRAS release [49]. Non-phosphorylated KRAS is then loaded onto PtdSer-rich recycling endosomes (REs) by electrostatic trapping for forward transport to the PM by vesicular

transport (Figure 1). By contrast, electrostatic repulsion likely operates to prevent phosphorylated KRAS from binding to the negatively charged membrane of the RE [54], leading to the accumulation of phosphorylated KRAS in the cytosol and other endomembranes [5, 48, 55]. Therefore, phosphorylation of membrane-bound KRAS does not directly trigger dissociation from the PM but rather decreases KRAS PM content by inhibiting recycling back to the PM [48].

2. RAS in Cancer

2.1 Mutational Profiles

The RAS genes are the most frequently mutated oncogenes in cancer [13, 56] and as such have long been considered a drug target [5, 57, 58]. The prevalence of mutations varies substantially across the isoforms with KRAS, NRAS and HRAS being implicated in 85%, 12% and 3% of cancers, respectively [5, 59]. Additionally, mutational bias between the isoforms also exists with the majority of mutations occurring at codons 12 and 61 for KRAS and NRAS, respectively, while HRAS is mutated at both these sites at a similar frequency. Overall, the 3 oncogenic mutational hotspots in RAS genes are G12, G13 and Q61 [13]. G12 and G13 mutations displace crucial interactions between RAS and GAPs required to form a transition-state complex, without which an increase in GTPase activity cannot occur, effectively maintaining KRAS in its “on” state leading to constitutive downstream signaling [60–62]. The Q61 mutation inhibits GTP hydrolysis of GTP-RAS by interfering with a catalytic water molecule essential for this process [60, 61]. Together, these mutations render RAS primarily GTP-bound and relatively insensitive to GAPs [62], with a 10-fold decrease in the rate of intrinsic GTP hydrolysis [7, 63–65].

KRAS is the isoform predominantly mutated in human cancers and occurs in some of the more deadly cancers in the U.S: pancreatic cancer (~90%), colorectal cancer (~50%) and non-small cell lung cancer (~25%). These cancers originate from the endodermal layer which correlate with comparative studies that showed that KRAS G12V mutations lead to enhanced proliferation and inhibited differentiation of endodermal stem cells, increasing the pool of progenitor cancer cells in these organs [66–68]. Since KRAS represents the critical clinical concern, it will be the focus of this review.

2.2 KRAS Effector Signaling

Multiple RAS effectors that bind GTP-RAS proteins via a RAS-binding domain (RBD) or a RAS association (RA) domain have been described [69]. Among these, the most important in KRAS oncogenesis are components of the RAF-MEK-ERK (MAPK) pathway and the PI3K-AKT-mTOR pathway that promote cell proliferation and survival, respectively [3, 13, 70].

The RAF proteins (CRAF, BRAF and ARAF) are serine/threonine kinases that, on activation, phosphorylate and activate MEK1 and MEK2 which in turn phosphorylate and activate ERK1 and ERK2 [71, 72]. Activated ERK1/2 then phosphorylate >200 substrates including members of the ETS family of transcription factors and c-FOS which activates the AP1 transcription factor. Together, these events lead to the expression of D-type cyclins and

other cell cycle regulatory proteins to promote cell cycle progression through the G1 phase [73–77].

Interaction of GTP-KRAS with the catalytic subunits of type I phosphatidylinositol 3-kinase (PI3K), p110α/δ/γ [78, 79], recruits the kinase to the PM where it phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [80, 81]. PIP_3 then binds to and activates the serine/threonine kinase AKT, which phosphorylates and activates many downstream targets including the NF-κB transcription factor involved in cell survival, and FOXOs involved in DNA damage repair [69, 82–85]. RAS activation of PI3K is important because it mimics a PI3K-dependent survival signal normally induced by extracellular matrix (ECM) attachment, which is lost in cancer cells [85] (reviewed in [86]). Under normal physiological conditions, loss of ECM attachment induces BAX translocation to the mitochondria and cytochrome c release, leading to apoptosis [87]. PI3K signaling inhibits cytochrome c release through AKT-mediated phosphorylation of I-kB kinase (IKK), releasing NF-κB, leading to the production of anti-apoptotic proteins [88]. NF-κB activation through PI3K-RAC signaling can attenuate the toxic effects of reactive oxygen species, modulate oxidative stress [87, 89–91] and lead to the production of IAPs (inhibitor of apoptosis proteins) (reviewed in [92, 93]).

In vitro experiments show that HRAS and KRAS more effectively activate the PI3K and MAPK pathways, respectively [94, 95]. Several lines of evidence also suggest that KRAS oncogenesis predominantly depends on the MAPK pathway: First, *KRAS*-WT pancreatic ductal adenocarcinomas (PDACs) have BRAF mutations, which phenocopy KRAS rather than PI3K pathway mutations [96–98]. Second, PI3K mutations co-occur with KRAS mutations indicating that KRAS does not potently activate the PI3K/AKT pathway [99]. Finally, only activated RAF and MEK can restore the growth of RASless mouse embryonic fibroblasts (MEFs) [100].

3. KRAS localization and nanoclustering

KRAS signaling occurs predominantly on the PM to where effectors are recruited and activated. A prerequisite for effector recruitment is the formation of transient (t<1s) GTP-KRAS nanoclusters, containing 5–6 KRAS proteins which selectively sort PM lipids [101–104]. Though it has historically been thought that the KRAS polybasic domain (PBD) associates with anionic PM phospholipids purely through electrostatic interactions, recent work has shown that the PBD-farnesyl anchor actually exhibits exquisite binding specificity for PtdSer. Indeed, the precise amino acid sequence of the PBD and the prenyl group define a cryptic code for lipid binding specificity [102, 104]. Differential interactions between different lysine side chains and PM lipids lead to a realized tertiary structure on the PM with a very specific binding preference for PtdSer [105]. Moreover the KRAS anchor binds specifically to asymmetric PtdSer containing one saturated and one desaturated tail, thus the anchor also recognizes the acyl chain structure of PtdSer [106]. In consequence, KRAS PM binding and spatial organization is highly sensitive to PM PtdSer content such that decreasing levels of endogenous PtdSer decreases KRAS PM levels, nanoclustering, and signaling [104, 106–108]. A role for G-domain homodimerization in KRAS spatial organization has also been proposed. Dimerization surfaces have been identified by

molecular dynamics (MD) simulations that allow the building of membrane-bound KRAS oligomers up to and including pentamers [109, 110] To some extent these surfaces have been validated by mutating interacting residues, which leads to reduced nanoclustering in intact cells and altered biology in more complex analyses [109, 110]. What is clear is that dimers involve low affinity protein interactions and only exist in the constrained two diffusional space of a membrane surface. However, the lipid composition of that membrane is also critical; reconstitution experiments with simple bilayers have failed to visualize any evidence of RAS dimerization or oligomerization [111], whereas more complex supported bilayers that emulate the lipid composition of plasma membrane faithfully recapitulate KRAS nanoclustering behavior [112]. Similarly, isolated KRAS anchors also display lipid-dependent nanoclustering behavior. In sum, anchor-lipid interactions are likely the driving force for KRAS nanoclustering on the PM with additional regulation, or modulation, provided through G-domain-lipid and G-domain-G-domain interactions. A thorough review of RAS PM targeting, spatial organization and anchor-induced lipid sorting can be found in [105].

KRAS signaling through the MAPK pathway occurs primarily on the PM, but has also been reported to occur from intracellular membranes such as the Golgi [27, 37], ER, endosomes [113, 114] and mitochondria [115, 116]. For example, EGFR activation leads to initial RAS activation on the PM and subsequently on Golgi membranes. RAS activation on the PM is rapid and transient (1–10 minutes) while activation on the Golgi is delayed and sustained (>20 minutes) [117]. The nature of MAPK signaling from these different platforms also varies. GTP-KRAS nanoclusters on the PM operate as digital switches which translate a graded/analog input (RAF kinase activities) into amplified fixed/digital output (pp-ERK) [103, 118, 119]. In contrast, RAS signaling on the Golgi generates a graded pp-ERK output, indicating the requirement of a higher activation threshold for the MAPK module in the Golgi compared to at the PM [120]. Interestingly, high-threshold graded Golgi MAPK signaling has clear biological relevance in the context of thymic deletion of highly autoreactive T-cells during fetal development [121]. The fact that one signaling module can generate different signaling dynamics and produce fundamentally different outputs points towards the importance of the nanoscale heterogeneity present within membrane environments.

4. KRAS Inhibitors

The quest to identify KRAS inhibitors has taken over 30 years, a journey that earned KRAS an “undruggable” status [122]. The first crystal structure of RAS solved in 1989 showed that, other than the nucleotide binding site, KRAS lacks any deep pockets that can be susceptible to small molecule binding [99, 123, 124]. Nevertheless, many combination therapies and new approaches to inhibiting KRAS signaling are emerging which can broadly be categorized into three strategies: (1) Direct inhibition of KRAS by targeting the nucleotide binding pocket, or allosterically modulating KRAS activity by binding newly identified surface pockets, (2) inhibition of downstream effectors of KRAS, and (3) inhibition of KRAS-PM interactions.

4.1 Direct KRAS Targeting:

4.1.1: Non-nucleotide Site Binding—RAS proteins have a picomolar affinity for GTP, which when combined with the cellular concentration of GTP in the millimolar range, essentially eliminates the feasibility of developing of GTP-competitive inhibitors for KRAS [69, 125]. This contrasts with the relative ease of designing ATP-competitive inhibitors against receptor tyrosine kinases (RTKs) that have binding affinities for ATP in the low micromolar to millimolar range [126]. Many attempts have been made to engineer proteins that specifically bind KRAS, including whole antibodies [127], single chain variable fragments [128], affibodies [129], monobodies [130, 131], DARPins [132], anticalins and other biologics that bind to allosteric sites on RAS (reviewed in [133]). Allosteric inhibitors targeting RAS dimerization and protein-protein interactions, rather than nucleotide analogs, seem to offer a feasible approach to inhibit RAS function [130, 131, 134, 135]. Moving forward, some degree of isoform and mutant specificity may be required to limit potential toxicity that may arise from inhibiting all RAS proteins [136]. For example, some pan-RAS inhibitors disrupt RAS-RAF interaction by binding to sites adjacent to the effector binding site and have shown promising results *in vitro* and *in vivo*; however, concern with toxicity in normal cells arise when targeting all isoforms [137].

KRAS allosteric sites were discovered by Gorfe *et al.* and colleagues through ensemble docking, MD simulations, bioinformatics, and *in vitro* validation of potential inhibitors. They showed that helix 5 and loop 7 are involved in allosteric regulation of the nucleotide-binding switch, and identified three adjacent transient binding pockets, termed p1, p2 and p3 (Figure 2). Virtual screening and ensemble docking were then used to identify novel small molecule binders to these pockets that could not be captured by traditional crystallography [138, 139]. Their efforts led to the identification of a novel pyrazolopyrimidine-based allosteric KRAS inhibitor, compound 11 (Figure 3A), that binds GTP-KRAS at the “p1” pocket with an IC50 of ~1–5 μM, disrupting RAS-RAF binding, inhibiting KRAS signaling and cancer cell proliferation [140].

Macromolecule inhibitors bind their targets with high specificity and affinity but their large size hinders uptake by cells. While small molecule drugs overcome this issue, their small size means a smaller surface area to bind targets and lower target affinity [141–143]. To circumvent these issues, Quevedo *et al.* used intracellular antibody capturing technology [144, 145] to develop an antibody fragment that binds to all RAS isoforms in the GTP-bound conformation. They showed that their product bound to RAS with low K_d, high K_{on} and low K_{off}, and inhibited tumor growth *in vivo* [146]. Since it bound to RAS with such desirable properties, they used it as a starting point to screen for other compounds that bound RAS at the same location and discovered a compound, Abd-7, that bound RAS adjacent to the effector binding region. Treatment with Abd-7 reduced signaling through the PI3K and MAPK pathways as measured by decreased levels of p-AKT and pp-ERK in colorectal and non-small cell lung cancer cell lines. Their work showed that using intracellular antibody fragments as starting points for drug development represents a viable option for the generation of small high-affinity compounds [147].

Another intriguing mechanism visualizes targeting RAS-membrane interaction sites to lock KRAS in an orientation with respect to the PM that occludes access to the effector binding site. This was demonstrated by the compound, Cmpd2, discovered using nanodiscs coupled with NMR spectroscopy to generate and characterize atomic-scale structures of KRAS on a 20% PtdSer lipid bilayer [148].

4.1.2: KRAS G21C Inhibitors—Mutant G12C KRAS is found in approximately 13% of non-small cell lung cancer (NSCLC), 3% of colorectal cancer and 2% of other solid tumors [149]. Many compounds have been developed to target KRAS G12C following the pioneering work of Shokat and his group [150] who developed a thiol-reactive compound which binds to a shallow pocket below switch II and forms a disulfide bond with the mutant cysteine to lock KRAS in the GDP-bound state [151–155]. One such KRAS G12C inhibitor, ARS-853, was among the first compounds to show drug-like qualities in potency and selectivity [154]. KRAS G12C has a much faster nucleotide cycling time ($t_{1/2}$ of nucleotide release = 9.9 minutes, $t_{1/2}$ hydrolysis = 27 minutes) compared to G12D and G12V mutants (>3 hours) [156]. Because of this fast cycling time, such mutants remain sensitive to upstream signals rendering ARS-853 more effective when coupled with EGFR inhibitors like erlotinib that increase the cellular fraction of GDP-KRAS. In contrast, combination treatment with MEK1/2 inhibitors renders ARS-853 treatment less effective due to loss of negative feedback inhibition of ERK1/2 on SOS1 (a RAS GEF), increasing the amount of GTP-KRAS that cannot be bound by ARS-853 [154]. These *in vitro* studies led to the development of ARS-1620 by structure-based design, a highly potent and selective KRAS G12C inhibitor that induced tumor regression *in vivo* [151].

The first-in-human clinical trials with Amgen's G12C inhibitor AMG 510 are underway with promising preliminary results in lung cancer patients (clinicaltrials.gov identifier NCT03600883, phase1/2), but not colorectal cancer patients. Although 47% of lung cancer patients (11 out of 23) experienced tumor shrinkage, this occurred in only 3% (1 out of 29) of colorectal cancer patients [149, 157, 158]. Clinical trials studying the effects of combining AMG 510 with MEK or PD1 inhibitors in solid KRAS G12C tumors (clinicaltrials.gov identifier NCT04185883, phase 1b) and trials comparing AMG510 treatment versus Docetaxel in NSCLC (clinicaltrials.gov identifier NCT04303780, phase 3) are underway. Oher pharmaceutical companies have also manufactured KRAS G12C inhibitors that are currently in clinical trials such as Mirati Therapeutics MRTX894 (clinicaltrials.gov identifier NCT03785249, phase1/2) [159].

Recent work elucidated potential resistance mechanisms to AMG 510 in three models of KRASG12C lung cancer [160]. Single-cell RNAseq revealed that inhibitor treatment increased p21 and p27 expression levels indicative of a quiescent (G0) state [161] in 80% of the cell population while the remaining 20% were termed "adapting" cells. Adapting cells initially undergo growth inhibition upon treatment but this is followed by accumulation of GTP-KRAS and finally MAPK reactivation, a pattern seen in many cases of acquired drug resistance [162–164]. A genome-wide knock-out screen revealed that heparin-binding epidermal growth factor (HBEGF), aurora kinase A (AURKA) and KRAS were essential for the AMG 510-adapting cell population. Increased HBEGF in adapting cells led to activated EGFR signaling, which proved essential for escaping the G0 state. The role of AURKA in

mitogenic signaling [165–167] and acquired resistance to PI3K or EGFR inhibitors has been well established [168, 169]. In this setting, AURKA stabilized the interaction between GTP-KRAS and CRAF; and co-treatment with AMG 510 and an AURKA inhibitor inhibited CRAF and ERK activation more extensively than treatment with either inhibitor alone. Cells expressing higher KRAS levels quickly convert GDP-KRAS to GTP-KRAS by increased upstream EGFR signaling and maintain the GTP-KRAS-CRAF interaction downstream by AURKA signaling, ultimately leading to cell cycle progression and escape from G0 [160]. These results likely explain why the majority of patients in the AMG 510 clinical trial have only had partial responses [157].

BI-2852 (available at https://opnme.com/molecules/kras-bi-2852) targets KRASG12C via a fundamentally different mechanism from other G12C inhibitors. BI-2852 binds to a pocket between switch I and switch II (SI/II-pocket), rather than above switch II (SII-pocket), which is unaffected by KRAS activation status [170]. Therefore, BI-2852 blocks interaction of both GDP- and GTP-KRAS with SOS1 [171] as well as GTP-KRAS with GAPs [172], CRAF and PI3Kα, inhibiting signaling and proliferation of *KRAS*-mutant cells in the low micromolar range [173, 174]. An obvious limitation of these inhibitors is that they are mutation specific, and most *KRAS*-mutant tumors have G12V or G12D mutations that have yet to be successfully targeted. However, this is a very active area of research [147, 175–177], with the SI/II pocket garnering most attention.

4.2 Downstream Effector Targeting

Identifying a suitable therapeutic window for agents that target KRAS or the MAPK pathway has been difficult as these pathways are essential for regulating cell growth and survival in normal cells. Efficacy has also been a recurring issue when trying to target the MAPK pathway, as doses required for efficient inhibition can be toxic [178–180]. Therefore, acquired drug resistance in tumor cells treated with these drugs is inevitable due to the strong selective pressure on cancer cells, which are inherently genetically unstable and able to adapt quite quickly. Downstream effector inhibitors have been extensively reviewed elsewhere [1, 3, 69, 99, 122, 181, 182] and so will only be considered briefly here to illustrate some mechanisms of resistance.

In the case of BRAF inhibitors for example, resistance is acquired through increased HGF secretion by stromal cells which activates both the MAPK and PI3K pathways via MET activation. Concordantly, *BRAF*-mutant melanoma patient data showed that those with stromal HGF had a significantly worse treatment response [183]. MET activation may also have a role in mitigating the action of gefitinib, an EGFR inhibitor, in non-small cell lung cancer [184–188]. HGF-induced resistance is immediate and innate as opposed to acquired resistance to drugs developed over time, but could be reduced by a combination of MEK and AKT inhibition [183]. BRAFV600E colorectal cancer cell lines treated with RAF inhibitors also develop resistance through EGFR activation [162, 164]. One recurring theme of resistance seen with many BRAF inhibitors is paradoxical activation of ERK due to alleviation of negative feedback mechanisms imposed by ERK on upstream components of the pathway [189–192], which is also seen with MEK1/2 inhibitors [99].

Rigosertib was developed as a RAS-mimetic that binds the RAS binding domain (RBD) of RAF [193]. This molecule inhibits RAS signaling but the mechanism of action remains unclear since Rigosertib binds the RAF-RBD with low affinity and cannot dissociate RAF from GTP-bound RAS. Rigosertib may induce mitotic stress and the generation of reactive oxygen species leading to JNK-mediated inhibition of SOS and RAF, to suppress the MAPK pathway in a KRAS non-specific manner [194].

SHP2 is a tyrosine phosphatase that acts upstream of KRAS to promote MAPK signaling and also mediates resistance to BRAF or MEK inhibitors in specific ERK-dependent tumors [195]. In addition, combination treatment with SHP2 inhibitors also abrogated the RTK reactivation observed with KRASG12C inhibitors, leading to sustained MAPK inhibition with better outcomes *in vitro* and *in vivo*. Several SHP2 inhibitors are currently under clinical evaluation since SHP2 inhibition has shown efficacy in RAS-mutant glioblastoma, pancreatic and lung cancers and in gastroesophageal cancers with WT KRAS amplification (ClinicalTrials.gov Identifiers: NCT03114319; NCT03565003; NCT03634982) [196–200]. Full details on all clinical trials involving RAS effector therapies can be found in a recent review [16].

4.3 Synthetic Lethality

Much work has been directed to identifying tumor-specific vulnerabilities that may be targeted to cause tumor cell death, a phenomenon termed synthetic lethality [201]. Such vulnerabilities may include non-oncogenic metabolic or genetic programs on which cancer cells have become dependent, so called non-oncogene addiction [202, 203]. Normal cellular processes that become critical for the maintenance of *KRAS*-mutant tumors include regulation of oxidative, genotoxic and ER stress, autophagy, macropinocytosis, glycolysis, and enhanced glutamine uptake in PDAC. Inhibitors of these pathways are currently in different stages of preclinical and clinical evaluation (reviewed in [204]).

For example, inhibiting KRAS function in PDAC cell lines leads to increased autophagy as a mechanism of cell survival [205–207]. Targeting both the MAPK and autophagy pathways has synergistic effects *in vitro* and *in vivo* in *KRAS*-mutant PDAC, *NRAS*-mutant melanoma and *BRAF*-mutant colorectal cancer. Moreover, coinhibition of MEK and autophagy using trametinib and hydroxychloroquine, respectively, in a single PDAC patient led to significant reduction in levels of CA19–9 tumor marker, overall tumor burden and cancer-associated pain [206].

KRAS G12R tumors exhibit a selective vulnerability to inhibitors of PI3K and macropinocytosis. Though quite rare in other cancers (~1% in NSCLC and CRC), G12R mutations comprise up to ~20% of KRAS mutations in PDAC. KRAS G12V and KRAS G12D expression stimulate macropinocytosis while KRAS G12R lacks this ability. The G12R substitution causes a structural change in switch II that impairs binding to the PI3K p110α subunit and hence activation of PI3K/AKT signaling, which in turn is essential for macropinocytosis. KRAS-independent upregulation of PI3Kγ signaling is therefore required to support macropinocytosis in KRAS G12R-PDAC. In consequence KRAS G12R-PDAC is more sensitive than G12D-PDAC and G12V-PDAC to ERK/MAPK and autophagy

inhibition. Interestingly, simultaneously targeting both pathways was synergistic in G12D- and G12V-PDAC but additive in G12R-PDAC [208].

A genome-wide synthetic lethal RNAi screen in *RAS*-mutant tumors yielded multiple hits in the mitotic stress response pathway, including subunits of the APC/C complex and the mitotic kinase PLK1 [209]. Concordantly, mutant *RAS* positive lung cancer patients had increased overall survival if APC/C levels were low, whereas APC/C activity level had no prognostic bearing in mutant *RAS* negative patients. PLK1 is activated at the G2/M transition by Aurora A Kinase leading to mitotic progression. Activated KRAS may lead to oncogene-induced senescence and so *KRAS*-mutant cells seem to have a higher dependence on PLK1 to progress through mitosis, as evidenced by increased levels of total and activated (phospho-)PLK1 [209]. This compensatory mechanism was also seen with KRAS G12C inhibitor-resistant cells, which had increased Aurora A Kinase levels [160]. In this context the PLK1 inhibitor, BI-2536 significantly inhibited the growth of KRAS transformed HCT116 and DLD-1 colorectal cancer cell xenografts. Other pathways essential for *RAS*-mutant cell growth include ribosomal biogenesis, RNA splicing and mRNA translation, and posttranslational modifications such as neddylation and sumoylation [209]. An extensive list of RAS synthetic lethal functional genetic screens can be found in [204]. Unfortunately many of the early hits from these studies could not be validated or recapitulated pharmacologically [209–211], and to date none have advanced to the clinic for the treatment of mutant RAS cancers [212].

4.4 Inhibiting KRAS Posttranslational Modification and Plasma Membrane Recycling

A valid mechanism to inhibit KRAS function is to block PM localization [213]. As discussed previously, KRAS requires the posttranslational attachment of a membrane anchor to localize to the PM. The first step in this process can be blocked by Farnesyl Transferase inhibitors (FTIs). Highly potent and relatively non-toxic FTIs were developed in the early 1990s that blocked the growth of HRAS mutant tumors in mouse models [214–217]. Concordantly, an ongoing phase 2 clinical trial conducted by Kura Oncology for the FTI tipifarnib in patients with *HRAS*-mutant relapsed or refractory urothelial carcinomas has seen promising results: 5 of the 13 patients treated achieved objective responses and 4 experienced progression-free survival greater than 6 months (clinicaltrials.gov identifier NCT02535650) [218]. Kura oncology is conducting another phase 2 tipifarnib clinical trial in *HRAS*-mutant Head and Neck Squamous Cell Carcinoma (clinicaltrials.gov identifier NCT02383927) and have reported a 56% overall response rate with progression free survivals of 6.1 months compared to 2.1 months on previous therapies [219]. However, FTIs did not provide such promising results in earlier trials with *KRAS*-mutant tumors because in FTI-treated cells, KRAS and NRAS are alternatively prenylated by geranylgeranyltransferase I (GGTaseI) allowing normal PM localization [3, 220–224]. Attempts to circumvent this escape mechanism to date have been unsuccessful. A GTTase1 inhibitor (GGT1–2418) that was well tolerated in phase 1 studies had limited efficacy (https://drugs.ncats.io/drug/M67G28K74K) [16], and combining FTIs with GTTase1 inhibitors (GGTIs), whilst showing promising efficacy in inhibiting prenylation of all RAS isoforms in preclinical experiments, proved to be too cytotoxic, with a therapeutic index too low to be implemented as a treatment option [225, 226]. Probably because together, FTase

and GGTase1 prenylate a large number of proteins important for normal cellular growth and function [227]. One dual prenyltransferase inhibitor, “L-778,123” that advanced to two different phase 1 clinical trials failed to inhibit KRAS prenylation [228, 229] despite some apparent efficacy *in vitro* [230].

A second approach to prevent PM KRAS localization involves blocking the function of PDEδ, which as reviewed above promotes recycling of cytosolic KRAS to maintain the fidelity of PM targeting [47, 49]. Inhibiting PDEδ leads to the accumulation of KRAS on endomembranes, whence it cannot signal. An early small molecule PDEδ inhibitor, Deltarasin, bound to the prenyl-binding pocket of PDEδ with nanomolar affinity and inhibited KRAS signaling in *in vitro* PDAC models but its effective *in vivo* dose was in the micromolar range and caused off-target cytotoxicity that limited efficacy [231]. A second generation PDEδ inhibitor, Deltazinone 1, to some extent circumvented these problems and was efficacious against PDAC [232]. Other compounds, such as Deltasonamide, 1 and 2 also inhibit *KRAS*-mutant colorectal cancer cell proliferation but suffer from low membrane permeability [233, 234].

4.5 Inhibiting KRAS Plasma Membrane Localization

An unbiased high content screen with a library of 1,120 FDA-approved small molecules was used to identify inhibitors of KRAS PM localization [235]. Some hits, also identified previously, included weak amphiphilic bases which only caused moderate dissociation of KRAS from the PM [236], but the top hit, fendiline, significantly mislocalized KRAS (IC_{50} =3.14 μM) as well as the PtdSer (IC_{50} =3.16 μM) from the PM in a dose-dependent manner. Importantly, fendiline had no effect on NRAS or HRAS localization or on CAAX processing [235, 237]. Fendiline is an L-type voltage-gated Ca^{2+} channel blocker originally used to treat angina [238]. Other Ca^{2+} channel blockers had no effect on KRAS, indicating a new function unrelated to Ca^{2+} channel activity and unrelated to changes in intracellular Ca^{2+} levels [235, 239]. Fendiline is a racemic compound; however, only the R-fendiline stereoisomer (hereafter fendiline, Figure 3B) significantly mislocalized KRAS. Fendiline treatment abolished KRAS-dependent MAPK and PI3K signaling at concentrations similar to those required for KRAS mislocalization, and more potently inhibited proliferation of *KRAS*-mutant cells in a panel of 21 pancreatic, lung, colorectal and endometrial cancer cell lines [235]. This selectivity was also observed *in vivo* in xenograft mouse models of *KRAS*-mutant or *KRAS*-WT pancreatic tumors with no observed organismal toxicity [240]. Because fendiline reduced KRAS PM levels and inhibited both the MAPK and PI3K/AKT pathways, it may have potential use as an adjuvant therapy with kinase inhibitors that lead to paradoxical re-activation of these pathways; such as mTOR and BRAF inhibitors that activate MAPK signaling [241, 242]. Fendiline derivatives have been synthesized that show increased potency in terms of KRAS mislocalization, inhibition of cell proliferation *in vitro* and tumor growth *in vivo* [243].

Fendiline operates as an indirect inhibitor of lysosomal acid sphingomyelinase (ASM) which converts sphingomyelin (SM) to ceramide (Cer), leading to SM accumulation in the lysosome [240, 244, 245]. Fendiline treatment reduces PM cholesterol levels, increases PM SM levels, and decreases PM PtdSer and cellular ceramide levels. Concordantly, since

KRAS PM localization and nanoclustering depends on PM PtdSer levels, supplementation with exogenous PtdSer rescued fendiline-induced KRAS mislocalization and MAPK signaling [237]. ASM facilitates NPC2-mdiated cholesterol efflux from the lysosome in part explaining the decreased cholesterol content seen in the PM of fendiline-treated cells [240]. More broadly, SM accumulation destabilizes lysosomal membranes [246] and impedes membrane fusion events such as those occurring during autophagy [247].

Fendiline and other ASM inhibitors, such as siramesine and cationic amphiphilic drugs (CADs), are concentrated in the lysosome where they interfere with the binding of ASM to bis-monoacyl-glycerophosphate (BMP) on the inner lysosomal membrane [248]. Displacement of ASM from BMP results in relocation to the lysosomal lumen where it undergoes degradation [249]. Siramesine and other CADs such as desipramine and amlodipine selectively kill *KRAS*-mutant HCT116 colon cancer cells but not the *KRAS*-null isogenic line [248]. Cancer cells generally have destabilized lysosomes due to elevated proteolytic activity and dependency on autophagy [250] and siramesine has been shown to effectively inhibit autophagic flux [251, 252]; other clinically relevant CADs probably have the same effect. The additional effect of fendiline on KRAS PM localization would add loss of KRAS signaling to these detrimental shared effects on lysosomal function, perhaps accounting for the increased sensitivity of *KRAS*-mutant cancer cells to fendiline treatment [253, 254].

Three FDA-approved tricyclic antidepressants, desipramine, imipramine and amitriptyline, which also inhibit ASM, [244, 255] mislocalize KRAS and PtdSer at similar IC_{50} values in a dose-dependent manner and significantly decrease KRAS nanoclustering on the PM. These effects were translated to decreased MAPK signaling and preferential inhibition of *KRAS*-mutant pancreatic, colon, lung and endometrial cancer cells over *KRAS*-WT cells. ASM inhibitors also block mutant *let-60* (K*RAS* homolog) signaling in *C. elegans* [240].

Sphingomyelin is generated by *de novo* synthesis from serine and palmitate by serine palmitoyl transferase (SPT) [256, 257], or by addition of a choline head group from phosphocholine to ceramide by sphingomyelin synthases (SMases) [258, 259]. Several targetable components of SM metabolism that decrease KRAS function were identified by an RNA interference screen against 18 enzymes of the Cer-SM biosynthetic pathway in the *C. elegans* system. Among the identified genes whose inhibition most potently suppressed *let-60* signaling (~70%) were *sphk-1* (sphingosine kinase 1), *hyl-2* (ceramide synthase) and, unsurprisingly, *asm-1* which encodes acid sphingomyelinase) [240]. SPHK1 is a kinase that phosphorylates sphingosine to sphingosine-1-phosphate (S1P), an extensively-studied active oncometabolite that promotes cell proliferation and survival [260, 261].

Validation of hits with pharmacological inhibitors in mammalian cells revealed that inhibition of ceramide synthase with fumonisin B1, dihydroceramide desaturase with GT11, or ceramide kinase with K1 potently misloclaized PM KRAS and PtdSer. These enzymes function in the Cer-SM *de novo* biosynthetic pathway and their inhibition *in vitro* significantly perturbed cellular SM levels or SM distribution and depleted PM PtdSer. Fumonisin B1, GT11 and K1 concordantly dose-dependently mislocalized KRAS from the PM at concentrations similar to those needed to inhibit their enzyme targets [240].

Additionally, inhibiting ORMDL3, a negative regulator of SPT, by very low concentrations of staurosporine (STS) [237] increased SM content by *de novo* synthesis [262].

G01 (3-O-methyl oxanthroquinone ethyl ester) is a derivative of oxanthroquinone, a polyketide structure discovered from a high content screen for inhibitors of KRAS PM binding (Figure 3C) [263]. G01 is an inhibitor of acylpeptide hydrolase (APEH), a prolyl-oligopeptidase that removes Nα-acylated amino acids from peptides and functions in protein degradation of oxidized and glycated proteins [264–270]. G01 treatment significantly inhibited the PM localization and nanoclustering of HRAS, KRAS4B (KRAS), KRAS4A and PtdSer with an IC_{50} of ~ 1μM [264, 271]. Further analysis suggests that this effect of G01 on multiple RAS isoforms [271] reflected a disruption of RE function which is crucial for maintaining RAS PM localization [49, 272]. The importance of the RE in maintaining RAS on the PM is echoed by studies showing that thioesterase inhibitors which prevent HRAS and NRAS depalmitoylation [273, 274], PDEδ inhibitors or RAB11 knockdown which prevent KRAS loading on to the RE [49, 231, 275] all lead to their redistribution to endomembranes. Similarly, a commercially available APEH inhibitor called ebelactone A inhibited APEH function at concentrations similar to that required to mislocalize KRAS from the PM [264, 269, 276]. Interestingly, in a cohort of patients encompassing 33 different types of cancer, APEH was expressed at significantly higher levels in *KRAS*-mutant as opposed to *KRAS*-WT tumors [264]. Precisely how APEH inhibition impairs RE function remains unclear, but may be related to aberrant proteasomal regulation [264].

G01 blocks MAPK signaling in cells expressing constitutively active KRAS or HRAS at the same concentrations that cause mislocalization from the PM. This translated to decreased proliferation of a panel of 14 cell lines comprising pancreatic, colon, endometrial and lung cancer cell lines treated with G01. Interestingly, cell lines harboring mutant *KRAS* were more sensitive to treatment than cell lines expressing WT *KRAS* in all but the lung cancer cell lines [271]. G01 also inhibited ASM and NSM, leading to elevated SM and cholesterol content [264] as seen with fendiline and fumonisin B1 [237, 240].

One unifying feature of these different mechanisms of lipid homeostasis is that they indirectly regulate PM PtdSer levels. Thus, their disruption decreases PM PtdSer content, and hence the capacity of KRAS to stably localize to and nanocluster on the PM. These observations in turn suggest that other approaches to disrupt PtdSer PM localization will also abrogate KRAS function.

4.5.1 Phosphatidylserine and KRAS Function—PtdSer accounts for 4–6% of total cellular lipids [277–279] but is the major anionic lipid species of the inner leaflet of the PM, accounting for ~25mol% [4, 280]. PtdSer is synthesized on the cytosolic leaflet of the ER by PtdSer synthase-1 (PSS1) and PtdSer synthase-2 (PSS2) [281] which, respectively, replace the choline or ethanolamine headgroups of phosphatidylcholine (PC) or phosphatidylethanolamine (PE) with serine [282–284]. Deletion of both PSS isoforms is lethal [285]; whereas cells with a non-functional PSS1 are viable [286–288]. From the ER, PtdSer is transported to mitochondria, PM, endosomes, lysosomes, and the trans-Golgi network by lipid transport proteins (LTPs) or vesicular transport [289–292]. Among intracellular organelles, PtdSer is most enriched in recycling endosomes (REs) [293]. PtdSer

binding proteins such as evectin-2 mediate retrograde trafficking between the RE and Golgi and likely contribute to the maintenance of high PtdSer levels on the PM [282, 293, 294].

PtdSer is mainly transported from the ER to the PM by Oxysterol Related Binding Protein 5 and 8 (ORP5 and ORP8) encoded by *OSBPL5* and *OSBPL8*, respectively [295]. ORP5 and ORP8 share 80% sequence identity [296] and are ER-resident proteins that function as lipid exchangers at membrane contact sites (MCSs) between the ER and PM, or mitochondrial outer membrane [297, 298]. MCSs are microdomains where two organelle membranes are arrayed within 10–30 nm of each other to facilitate molecule exchange [299]. At ER-PM MCSs, ORP5 and ORP8 couple PtdSer transport against its concentration gradient with PI4P transport down its concentration gradient. Dedicated modes of lipid transport between membranes generate membrane asymmetry which is then maintained by a variety of "trapping" mechanisms including asymmetric breakdown and synthesis. In the case of PI4P, it is synthesized on the PM by PM-localized PI4KIIIα and degraded immediately after transport to the ER by the ER-resident phosphatase SAC1P (Figure 4) [298–301].

PI4-kinases function mainly at the PM and Golgi to phosphorylate PI at position D4 of the inositol head group to synthesize PI4P, the precursor of $PI(4,5)P_2$ (PIP_2) and $PI(3,4,5)P_3$ (PIP_3) which are involved in phospholipase C (PLC) and PI3K/AKT signaling, respectively [302, 303]. There are three classes of PI4-kinases (PI4KI, PI4KII, PI4KIII) defined by sequence similarity and biochemical properties, each of which contain an α and a β isoform [302, 304]. The key isoform essential for PtdSer transport to the PM is PI4KIIIα which is recruited to the PM by a multiprotein complex containing the TTC7 scaffold protein and EFR3A, a palmitoylated protein that tethers the complex to the PM [304, 305].

Once at the PM, PtdSer is actively concentrated in the inner leaflet by ATP-dependent aminophospholipid flippases of the P4 subfamily of P-type ATPases [306–310]. In the PM, PtdSer has a multitude of functions involved in coagulation, signaling cascades, protein recruitment, phagocytosis and the apoptotic response [283, 311–314]. There are two pools of PtdSer, a reactive mobile pool and an immobile pool constrained by interactions with cortical actin [108, 279].

4.5.2 The ORP5/8 PtdSer Transport System as a Novel Targetable Pathway for KRAS Inhibition—The human ORP family contains 12 members in 6 subfamilies categorized by protein homology and gene organization [315, 316]. All members contain a highly conserved lipid binding domain that can transport cholesterol or different phospholipids [295, 317, 318]. The lipid transport domain in ORP5/8 is called the Oxysterol-binding-protein-Related-Domain (ORD) and binds PI4P or PtdSer; binding of these lipids is mutually exclusively because the hydrophobic pocket can only accommodate a single lipid molecule [299]. Both ORP5 and ORP8 contain an N-terminal Pleckstrin-Homology (PH) domain that binds to PI4P or PIP_2 on the PM [297, 319–322] and a C-terminal hydrophobic transmembrane domain that tethers them to the ER [323–325]. Two splice variants of ORP8 exist: ORP8S and ORP8L which has an additional 42 amino acid acidic/negative stretch at the N-terminus. ORP5 is predominantly located at ER-PM MCSs, while the majority of ORP8L is ER localized, and ORP8S is intermediate between the two. The decreased levels of ORP8L at ER-PM contacts compared to ORP8S may be due to the

ORP8L-specific negative N-terminus [297]. Additionally, ORP5 contains a cluster of basic/positive arginine residues that makes this protein especially favorable among the three for interaction with the negatively charged inner leaflet of the PM [326].

There is some debate about how ORP5 and ORP8 mechanistically interact. Some studies suggest that ORP5 helps recruit ORP8 to the PM when PIP_2 levels rise due to elevated PI4P content that is subsequently phosphorylated to produce PIP_2. Under these conditions ORP5 and ORP8 colocalize and directly interact with each other [297], consistent with studies showing an increased PI4P/PtdSer exchange rate upon dimerization [296]; by contrast other work suggests that ORP8 binding inhibits ORP5 recruitment to the PM [326]. There is also debate on which phosphoinositide ORP8 or ORP5 bind, with some studies accounting the difference to minor distinction in amino acid sequence and charge of each ORP protein [297]. More specifically, Sohn *et al.* showed that ORP8, and not ORP5, may preferentially bind PIP_2 because it forms only 12 H-bonds with PI4P but 16 H-bonds with PIP_2 and has additional electrostatic interactions with the two additional phosphates of PIP_2 compared to PI4P. Therefore, while ORP5 activity at the PM depends on PI4P levels, ORP8 activity and recruitment depends on both PI4P and PIP_2 levels [326]. It is yet to be determined if PI4P and PIP_2 carry the same weight in terms of importance for PtdSer PM transport; however, it is clear that PIP_2 levels can regulate PtdSer exchange by recruiting more ORP8 to the ER-PM MCSs, or by OPR8 using PIP_2 for exchange, or a combination of both mechanisms [297, 327, 328].

Knockdown of ORP5 or ORP8 in *C. elegans* completely reverted the activated *let-60* multi-vulva phenotype (MUV), indicative of pathway inhibition. In addition, and concordant with the concept that disrupting ER/PM PI4P concentration gradient will affect KRAS signaling, SAC1P knockdown also suppressed the MUV phenotype without affecting organismal viability. Similarly, knockdown of either ORP5 or ORP8 in human colorectal, breast and pancreatic cancer cell lines resulted in significant mislocalization of PtdSer and KRAS from the PM, along with decreased nanoclustering of any KRAS remaining on the PM and concomitant abrogation of KRAS-dependent MAPK activation [4]. Importantly, double knockdown of both homologs was not additive or synergistic, indicating that knockdown of either ORP protein alone is sufficient to mislocalize KRAS from the PM [4].

Knockdown of either ORP protein also decreased proliferation and anchorage-independent growth of *KRAS*-dependent but not *KRAS*-independent pancreatic cancer cells. More specifically, the *KRAS*-independent cell line PANC-1 was sensitive to ORP5 but not ORP8 knockdown, while *KRAS*-dependent lines were sensitive to either knockdown [4]. It has been reported that *KRAS*-independent cell lines depend on oxidative phosphorylation rather than glycolysis for metabolism, and hence have a higher amount of reactive oxygen species [329]. This observation is reconciled with studies showing ORP8 levels are higher at ER-mitochondria MCSs but ORP5 is the predominant homolog that interacts with mitochondrial outer membrane proteins [330]. Studies have shown that cells lacking either ORP have altered mitochondria morphology (reduced cristae) and reduced oxygen consumption. ORP5/8 interact with the VAPB-PTPIP51 protein complex on mitochondrial associated ER membranes (MAMs) and exchange mitochondrial PI4P with ER PtdSer for mitochondrial PE synthesis [330]. Interestingly, PANC-1 cells have a higher basal expression of ORP5

compared to other PDAC cell lines such as MiaPaCa-2 [331], which may indicate a more important role for this homolog in this cell line.

Corroborating evidence for a role of ORP5/8 in KRAS cancers comes from data showing that PDAC patients with increased ORP5 mRNA expression have poorer overall survival, with 1-year survival rates of high versus low ORP5 expression being 36.4% and 73.9%, respectively. The effect of high ORP5 levels on overall survival (OS) is seen across all stages of PDAC but is most pronounced for patients with stage I to stage III tumors with high ORP5 who have an OS of 6.7 months compared to 22.4 months for those with low ORP5 levels. High levels of ORP5 is also associated with increased invasion of pancreatic cancer cells *in vitro* and is manifested in patients as early relapse [332]. The consequences of increased ORP5 levels are not restricted to pancreatic cancer, as it is also highly expressed in metastasis-positive lung tumor samples [333]. In some pancreatic cancers, ORP5 expression correlates with upregulation of the cholesterol synthesis pathway and, although the mechanism is unclear, some have speculated that the effects of high ORP5 in these cancers can be tied back to reports that statins reduce invasion and metastasis in PDAC cells [331, 334, 335]. Ectopic expression of ORP5 also induced the expression of the efflux protein pump ABCB1 known as Multidrug Resistance gene MDR1 [331]. Others have shown that the lipid transferring function of ORP5 is absolutely required for cancer cell proliferation, invasion and migration *in vitro* and that when overexpressed, ORP5 interacts with mTORC1 by virtue of its ORD. Overexpression or down-regulation of ORP5 induced and repressed, respectively, mTORC1 activity *in vitro*. This may in part be due to the mislocalization of mTOR from lysosomes in ORP5 knockdown cells [336]. The most recent work; however, directly links both ORP5 and ORP8 to KRAS function and provides a straightforward explanation for the correlation of ORP5 levels with tumor progression. As such, in the context of what is now known, earlier models and speculations regarding ORP5 may not be accurate, moreover the link to KRAS function can also rationalize why high levels of ORP8 correlate negatively with patient survival in a variety of cancers including PDAC and NSCLC [4].

No inhibitors of ORP5 or ORP8 have been described but PtdSer-PI4P exchange can be inhibited by targeting PI4KIIIα upstream of this pathway. Overexpression of PI4KIIIα results in increased localization of ORP5/8 to PM-ER MCSs; conversely, inhibition of PI4KIIIα using the small molecule inhibitor A1 [337] leads to the dissociation of these complexes. In support of the rationale of targeting this protein in cancer, PI4KIIIα has been shown to contribute to the invasion and metastasis of pancreatic cancer in a difference analysis screen [338], and to the acquired resistance against gemcitabine in pancreatic cancer [339]. No activating mutations in PI4-kinases or inactivating deletions of their phosphatases have been identified in cancer [302]. Several pan and selective PI4K inhibitors have been synthesized (reviewed in [303]). The tool compound demonstrating the most selectivity towards PI4KIIIα is the small molecule C7 (IC_{50} against PI4KIIIα, PI4KIIIβ, PI3K: 6.3 nM, 1,250 nM, inactive, respectively) (Figure 3D). Treatment with C7 depletes PI4P from the PM [340, 341], which should lead to elimination of the PI4P concentration gradient required for ORP function, effectively phenocopying ORP5/8 knockdown. Concordantly, C7 treatment significantly mislocalized both PtdSer and KRAS from the PM in a dose-dependent manner, with the highest mislocalization occurring at concentrations

previously reported to decrease PIP_2 and PIP_3 (generated from PI4P) levels [4, 341]. This translated to selective inhibition by C7 of the proliferation of *KRAS*-dependent PDAC cells, with minimal effects on *KRAS*-WT PDAC cells and no effect on non-transformed immortalized pancreatic cells. These results tie in nicely with the observations that ORP5 levels are specifically elevated in *KRAS*-mutant tumors across multiple cancer types, indicating the dependence of KRAS function, and hence *KRAS*-addicted cells, on ORP5 [4]. In addition to reducing the PI4P PM-to-ER gradient, PI4KIIIα inhibition may have other relevant mechanistic actions. For example, levels of PM-localized ORP5 and ORP8 are decreased upon PI4KIIIα inhibition [326]. Recent work has shown that knockdown of EFR3A, part of a protein complex that recruits PI4KIIIα to the PM, also leads to decreased PtdSer and KRAS PM content and reduced PDAC cell growth *in vitro* [342]. Others have shown that depleting PM PI4P by PI4KIIIα inhibition or knockout not only decreases PM PtdSer content, but also lowers overall PtdSer levels by 50% [297, 301, 326]. The overall reduction of PtdSer is due to its accumulation in the ER leading to product inhibition of PSSI and PSSII.

Prolonged overexpression of PIP5K, which converts PI4P to PIP_2, also depletes PM PI4P, in turn decreasing PM PtdSer. Under physiological conditions, increased PIP5K activity increases PM PIP_2 levels which leads to ORP5/8 redirecting more PI4P to the ER. Therefore, the ORP5/8 system also functions to regulate PIP_2 levels on the PM. PIP_2 dysregulation can be seen upon ORP5 knockdown, where PI4P accumulates on the PM leading to more PIP_2 generation and consequent recruitment/activation of ORP8 to exchange PIP_2 for PtdSer [4, 326]. In this context, some experiments suggest that ORP8 actually exhibits dual activity as a PtdSer/PI4P and PtdSer/PIP_2 exchanger, as it has been reported that ORP5 knockdown leads to increased ORP8 protein expression which partially restores PtdSer PM levels, but that knocking down ORP8 did not lead to increased ORP5 expression [4]. This compensatory mechanism does not seem to operate or be relevant in *KRAS*-dependent cancer cells where loss of ORP5 or ORP8 is sufficient to significantly reduce cell proliferation [4]. Although PtdSer transport depends predominantly on ORP5 and ORP8 exchange with PI4P, ORP10 can also transport PtdSer from the ER to the trans-Golgi (TGN) from where it can be transported to the PM; however, knockdown of ORP10 did not alter PM PtdSer levels suggesting that this pathway is not a major contributor to PM PtdSer [343].

A critical question when considering PI4KIIIα as a drug target is toxicity. Indeed, one study claimed that pharmacological targeting of PI4KIIIα is not feasible since oral administration of PI4KIIIα inhibitors to mice induced gastric mucosal epithelial degeneration at high doses. Mice treated at lower doses remained alive but still exhibited differing degrees of GI toxicity probably due to the oral route of drug administration [337]. However, all of the PI4K inhibitors synthesized and tested on the mice variably inhibited multiple isoforms including PI4KIIIα, PI4KIIIβ, PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ [337, 344]. To date, the compound C7 seems to be the most selective PI4KIIIα inhibitor [303, 340] and at least in tissue culture experiments shows a high degree of selective toxicity for *KRAS*-mutant cancer cell lines with minimal effects on normal pancreatic cells [4]. PI4KIIIα inhibition also increases the radiosensitivity of diverse cancer cell lines *in vitro* as well as in immune-competent and

nude mouse models of breast and brain cancer [345]. Thus, selective targeting of PI4KIIIα seems well tolerated in these animals at concentrations that result in anti-tumor effects.

Inhibition of MAPK signaling can lead to upregulation of PI3K-AKT-mTOR signaling as a compensatory mechanism and *vice versa* [346–348]. However, inhibiting PI4KIIIα can act in *KRAS*-mutant cells to simultaneously inhibit both the MAPK and PI3K pathways, which is an attractive notion given that 93% of *PIK3CA* mutations in PDAC co-occur with *KRAS* mutations [69, 349–353]. First, PI4KIIIα inhibition has the additional effect of decreasing pAKT activation by decreasing PIP_3 PM levels (Figure 4) in breast cancer cells [345]. Secondly, PtdSer has been shown to be critical for the activation of PI3K/AKT signaling as it facilitates AKT-PIP_3 binding and AKT conformational changes required for phosphorylation and downstream signaling [354]. Thirdly, one study showed that suppressing ORP5 increased levels of PTEN, a negative regulator of AKT/mTOR signaling [331].

Finally, PI4KIIIα as a target to inhibit KRAS function may circumvent the limitations seen with other inhibitors that show mutation-specific activity. For example, the EGFR inhibitor cetuximab, shows efficacy with KRAS G13 but not G12 mutants in advanced colorectal cancer [355]. In studies evaluating PtdSer mislocalization as anti-KRAS treatment, cell lines of different cancer types harboring different KRAS mutations were similarly affected [4, 235, 240, 271]. Additionally, PtdSer mislocalization most potently affected *KRAS*-dependent transformed cells, followed by *KRAS*-independent transformed cells and finally *KRAS*-WT cells. This demonstrates that levels of PM PtdSer are a selective vulnerability that may be exploited in *KRAS*-driven cancers.

5. Conclusion

RAS genes are the most frequently mutated oncogenes and drive of some of the deadliest human cancers, hence much effort has been expended to find anti-RAS therapies. The major clinical problem is KRAS. In addition to targeting classical downstream effectors, recent KRAS targeting strategies have expanded to include direct inhibition of KRAS, combination therapies exploiting KRAS-specific vulnerabilities, inhibiting KRAS post-translational processing and recycling, and preventing KRAS PM localization. The major benefit of targeting KRAS PM interactions has been the observed selectivity of these methods against oncogenic *KRAS* over WT *KRAS* irrespective of the mutation, and the ability to simultaneously downregulate two major KRAS signaling pathways without observable toxicities on organismal viability. One intriguing drug target is PI4KIIIα which has merit as a novel treatment target for *KRAS*-dependent tumors and warrants further research as inhibitors suppress the MAPK pathway by mislocalizing KRAS from the PM, and the PI3K/AKT/mTOR pathway by depleting both PM PtdSer and PIP_3 required for the activation of this pathway.

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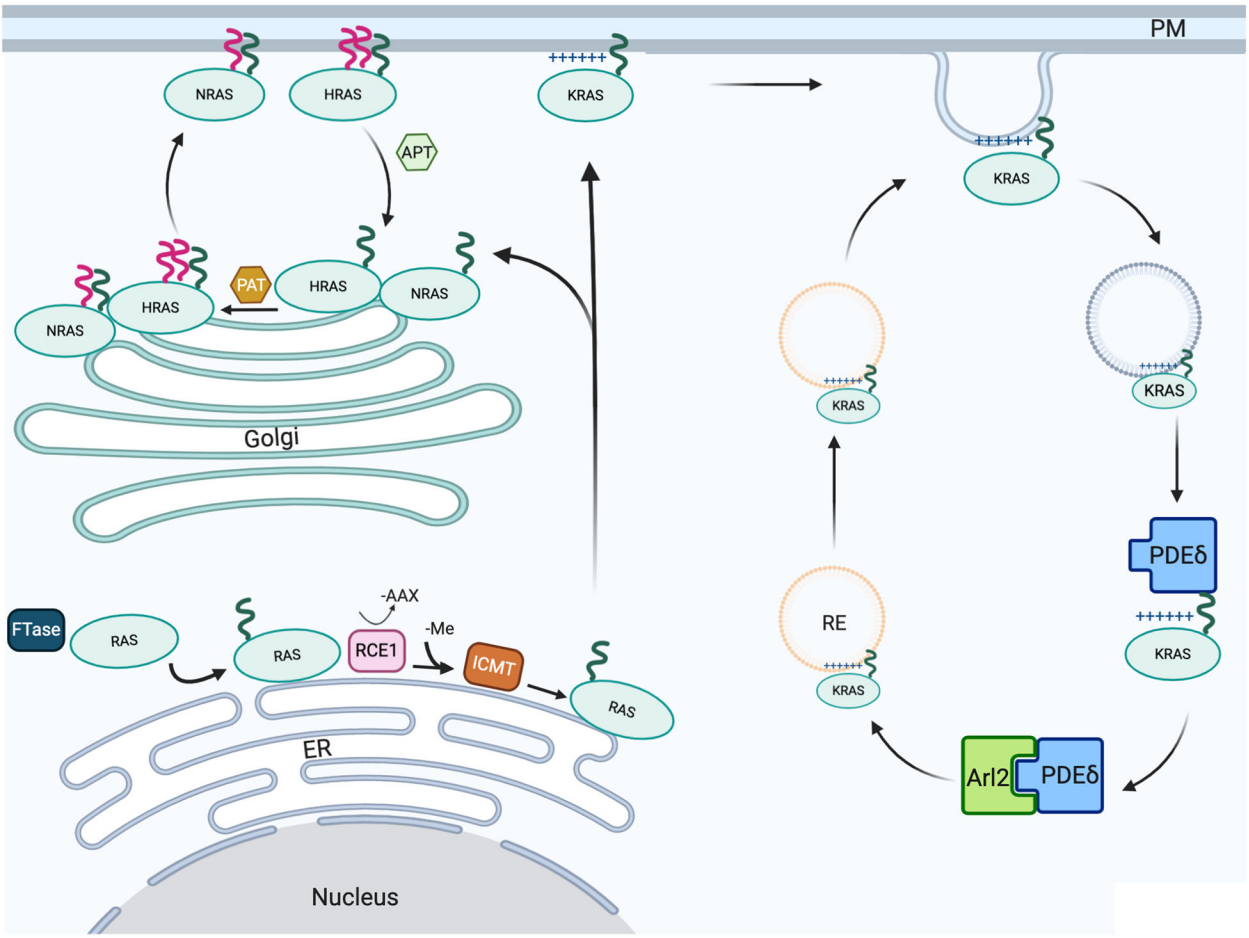

Figure 1. Schematic of RAS posttranslational processing, plasma membrane targeting and recycling.

Following mRNA translation in the cytosol, the three RAS isoforms (HRAS, NRAS and KRAS) are trafficked to the PM in a series of steps in specific subcellular localizations. HRAS and NRAS are recycled through palmitoylation-depalmitoylation cycles; KRAS is recycled via the recycling endosome which is enriched with PtdSer. Green and red lines indicate farnesyl and palmitate, respectively. The (+) symbol denotes polybasic residues of the KRAS hypervariable region. FTase, farnesyl transferase; RCE, Ras-converting enzyme 1 protease; ICMT, isoprenylcysteine carboxylmethyltransferase; PAT, palmitoyl acyl transferase; APT, acyl-protein thioesterase; PDEδ, phosphodiesterase delta; Arl2, ADP-ribosylation factor-like protein 2; ER, endoplasmic reticulum; Golgi, Golgi apparatus; RE, recycling endosome; PM, plasma membrane; -Me, methyl group.

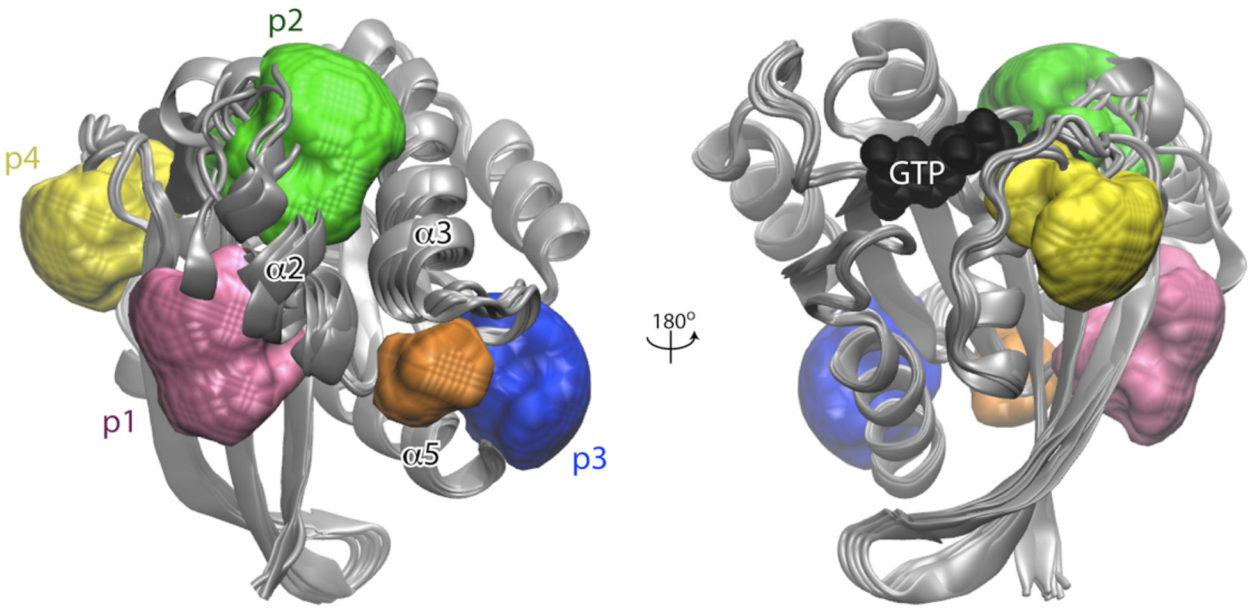

Figure 2. Druggable allosteric pockets on KRAS.

Ensemble fragment mapping analysis identified four allosteric binding pockets, p1, p2, p3 and p4 (pink, green, blue and yellow, respectively), on the catalytic domain of KRAS. P1 is located near the core-beta sheet while p2 is between helix 3 switch 2. P3 and p4 are near the C-terminus and behind switch 1, respectively. Image reproduced from Grant *et al.* with permission [356].

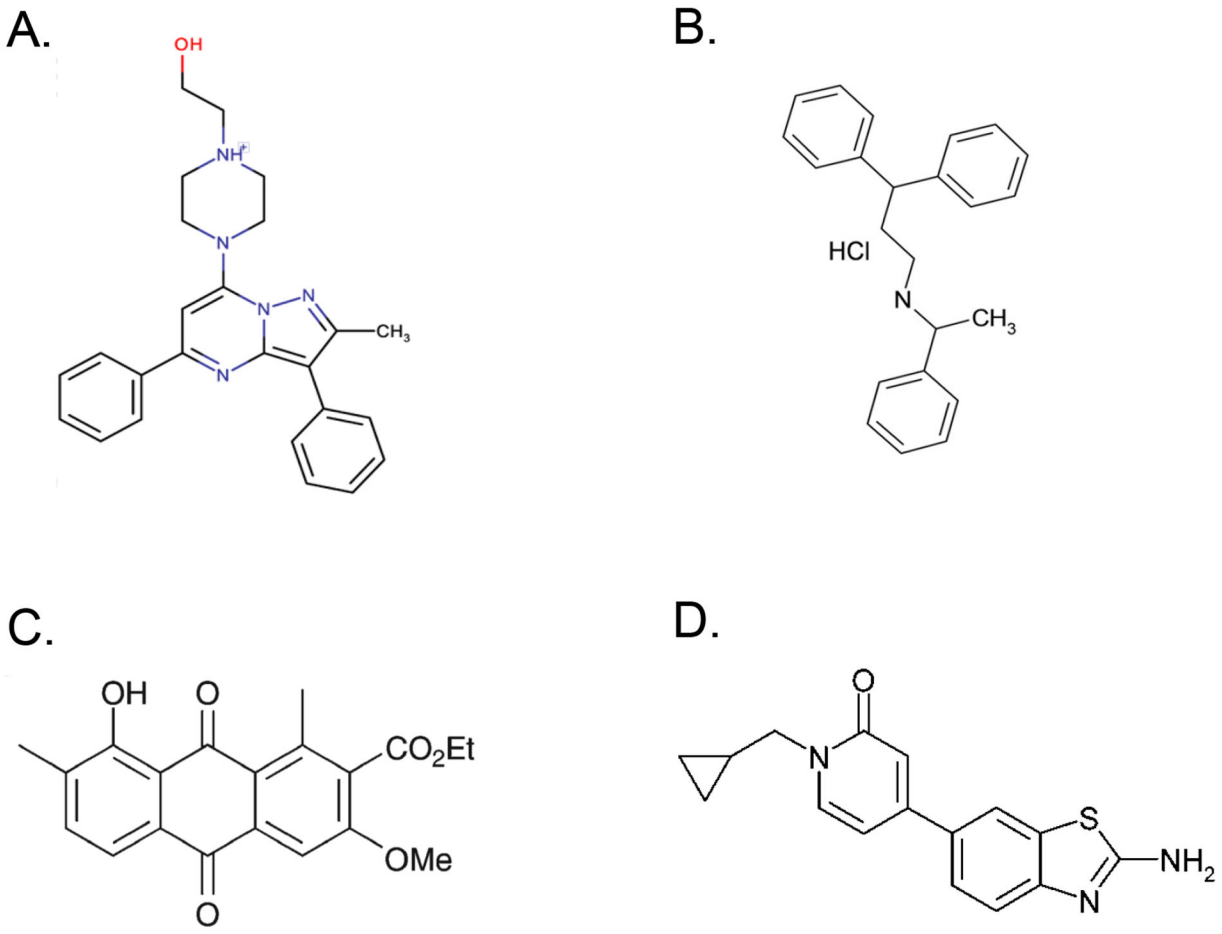

Figure 3. Chemical structures of several KRAS signaling inhibitors.

(A) Compound 11 binds to the p1 allosteric pocket on KRAS to inhibit its function; image reused with permission from McCarthy *et al.* [140] (https://pubs.acs.org/doi/10.1021/acsomega.8b03308). **(B)** Fendiline inhibits acid sphingomyelinase in the lysosome, resulting in PtdSer and KRAS mislocalization from the PM. **(C)** G01 inhibits acid sphingomyelinase as well but also perturbs RAS recycling to the PM through the recycling endosome pathway by inhibiting APEH; republished with the permission of ASBMB, from "An oxanthroquinone derivative that disrupts RAS plasma membrane localization inhibits cancer cell growth", Tan *et al.*, vol. 293, issue 35, 2018; permission conveyed through Copyright Clearance Center, Inc. [271]. **(D)** C7 selectively inhibits PI4KIIIα leading to depletion of PI4P from the PM and concomitant mislocalizaion of PtdSer and KRAS; image reused from Raubo *et al.* with permission from Royal Society of Chemistry [340].

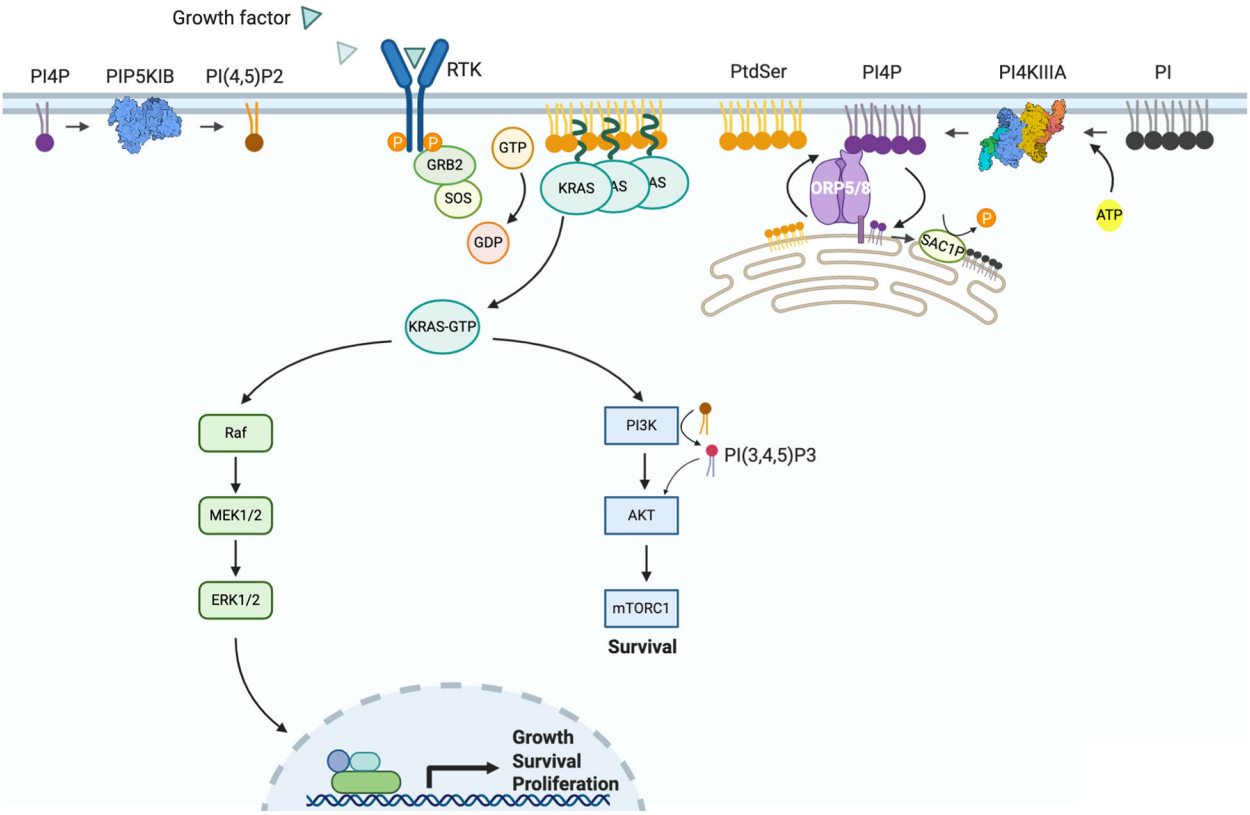

Figure 4. ORP5 and ORP8 transport PtdSer to the plasma membrane in exchange for PI4P.

(A) ORP5 and ORP8 are lipid exchangers that transport PtdSer from the ER to the PM where they then bind to and transport PI4P to the ER. PI4P is immediately hydrolyzed to phosphoinositide (PI) by ER-resident SAC1P creating a PI4P concentration gradient that drives the counter transport process. PM PtdSer molecules are critical structural elements of KRAS nanoclusters that act as signaling platforms to activate many pathways, most importantly the RAF-MEK-ERK and PI3K-AKT-mTORC1 pathway which promote cell proliferation and survival. On the PM, PIP5K phosphorylates PI4P to PI(4,5)P2 which can subsequently be phosphorylated to PI(3,4,5)P3 by PI3K leading to the activation of the AKT pathway. ORP5/8: Oxysterol Related Binding Protein 5 and 8; SAC1P: SAC1 Like Phosphatidylinositide Phosphatase; PM, plasma membrane; ER, endoplasmic reticulum; PtdSer, phosphatidylserine; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P2, phosphatidylinositol-4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; PI4KIIIA, Phosphatidylinositol 4-kinase III alpha; PIP5K1B, phosphatidylinositol-4-phosphate 5-kinase type 1 Beta; RAF, Rapidly Accelerated Fibrosarcoma; MEK, mitogen-activated protein kinase kinase; ERK, extracellular-signal-regulated kinase; PI3K, phosphoinositide 3-kinase; AKT, also known as protein kinase B (PKB); mTORC1, mammalian target of rapamycin complex 1.