2022

The Role of PI4KB in Cellular Localization of Small GTPases

Parisa Sadrpour
Wright State University

THE ROLE OF PI4KB IN CELLULAR LOCALIZATION OF SMALL GTPASES

A thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

by

PARISA SADRPOUR

M.S., Azad University, Iran, 2011

B.S., Azad University, Iran, 2006

2022

Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

July 28, 2022

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Parisa Sadrpour ENTITLED The role of PI4KB in cellular localization of small GTPases BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

_____

Kwang-Jin Cho
Thesis Director

_____

John Paietta, Ph.D.
Chair, Biochemistry and Molecular Biology Department
College of Science and Mathematics

Committee on Final Examination:

_____

Kwang-Jin Cho, Ph.D.

_____

Michael Leffak, Ph.D.

_____

Hongmei Ren, Ph.D.

_____

Barry Milligan, Ph.D.
Dean of the Graduate School

ABSTRACT

Sadrpour, Parisa. M.S., Department of Biochemistry and Molecular Biology, Wright State University, 2022. The role of PI4KB in cellular localization of small GTPases

Constitutively active oncogenic mutant K-Ras is one the principal contributors to human cancers including 90% of pancreatic, 50% of colorectal and 32% of non-small cell lung cancers. However, except for K-Ras G12C oncogenic mutant, which only presents in about 13% of non-small cell lung cancer patients, there is no anti-K-Ras therapy for a considerable subset of K-Ras mutations in human tumors, reflecting challenges for targeting oncogenic K-Ras activity.

K-Ras is a membrane-bound small GTPase; when active, it triggers multiple signaling pathways regulating a variety of key cellular functions such as cell growth, proliferation and survival. To initiate these signaling cascades, K-Ras must localize to the plasma membrane (PM), where gets activated by guanine-nucleotide exchange factors and interact with its downstream effectors. Based on this dependency of K-Ras localization to the PM for its biological activity, removing K-Ras from the PM has been suggested as an anti-K-Ras approach. The exact mechanism of K-Ras interaction with the PM is not fully elucidated. Extensive studies have shown a specific preference of K-Ras for interacting with phosphatidylserine (PS), an acidic phospholipid in the inner PM leaflet, and decrease in the PS contents dissociates K-Ras from the PM and blocks K-Ras signaling.

Towards our goal of targeting the PM/K-Ras interaction, our recent study has indicated that phosphatidylinositol 4-kinase IIIβ (PI4KB), which converts PI to PI 4-phosphate at the Golgi complex, is involved in the PM enrichment of PS and K-Ras. We found that upon PI4KB inhibition, K-Ras and PS redistribute from the PM to mitochondria and other endomembranes, respectively. The aims of this dissertation are to 1) characterize PI4KB as an anti-K-Ras target in human pancreatic cancer cell lines expressing oncogenic mutant K-Ras, and 2) assess the mechanism of K-Ras translocation to mitochondria upon PI4KB inhibition. Our proliferation assay data demonstrate that chemical inhibitors for PI4KB reduced the growth of human pancreatic

cancer cells harboring oncogenic mutant K-Ras, but not wild-type K-Ras. These data suggest that PI4KB could be a target for treating human pancreatic cancers.

In the second aim of the study, we sought to elucidate whether the polybasic domain (PBD) of K-Ras is involved in mitochondrial translocation of K-Ras in PI4KB inhibited cells. K-Ras is distinct from the other Ras isoforms, H-Ras and N-Ras, for its PM targeting signal. While H- and N-Ras bind the PM via the C-terminal prenyl and palmitoyl lipid moieties, K-Ras binds the PM through the C-terminal prenyl lipid moiety and PBD made of hexa-Lys residues. Also, the PBD electrostatically interacts with the PM PS, and depleting PM PS content dissociates K-Ras from the PM. Our data show that PI4KB inhibition translocates K-Ras, but not H -Ras, to mitochondria. Thus, we propose that K-Ras PBD is involved in translocation to mitochondria upon PI4KB inhibition. To test this, we examined the mitochondrial localization of four small GTPases containing PBD in PI4KB-inhibited cells: Rac1, RalA, Arl4a and Arl4c. Our data demonstrate that PI4KB inhibition promotes mitochondrial translocation of RalA, which has a closer sequence and structure to K-Ras compared to the other three small GTPase, even though K-Ras and RalA use different prenyl groups for the PM anchoring.

Taken together, here we identified PI4KB as a possible therapeutic candidate for treating human pancreatic cancers harboring oncogenic mutant K-Ras. Moreover, different observations made for the effect of PI4KB inhibition on the localization of four small GTPases containing PBD indicate that PBD-mediated PM anchoring is not a deceptively simple electrostatic interaction, which only is based on positive charges provided by PBD. Although further studies are needed to explore the exact mechanism of this interaction, we propose that in addition to positive charges, primary sequences of PBD, and intramolecular interactions (non-covalent bonds) also play roles. Moreover, it is feasible to consider the possibility that the membrane localization of small GTPase is also regulated by their effector proteins, where they recruit to and/or stabilize the membrane localization of small GTPases.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Acknowledgements

I would like to express my deepest appreciation to my thesis advisor, **Dr. Kwang-Jin Cho**, for allowing me to join his lab and work on such a wonderful project in the cancer biology field. Through his mentorship and guidance, not only I was able to further develop my lab skill sets needed to conduct my thesis project, but also provide me the opportunity to explore greater levels of my passions and potential by challenging myself. Moreover, I appreciate all his perceptive feedback for my future profession as a research scientist. I also must acknowledge all his support throughout my study, especially whenever I ran into tough times and he reminded me that I am not alone in this path.

Besides my advisor, I was delighted to have **Dr. Michael Leffak**, and **Dr. Hongmei Ren**, on my thesis committee. I appreciate all their insightful comments and feedback which helped me to have a better understanding of different aspects of my project and as a result adding more depth to my thesis.

This thesis would not have been possible without my lab mates: **Karen M. Henkels**, **Kristen M. Rehl**, **Taylor Miller**, **Sarah E. Kovar**, **Christian M. Garrido**, and **Mackenzie Duff**. Special thanks to **Karen M. Henkels**, and **Kristen M. Rehl**, who both have been a great help for my project by teaching me numerous lab techniques. Along with all training and supports, they also have been good friends for me since the initial days I joined the lab.

My sincere thanks also goes to **Dr. David Ladle**, director of Microscopy Core Facility at Wright State University, for his enormous assistance with confocal microscopy imaging, which constitutes a significant part of my study. I appreciate all his time and efforts for providing training and technical supports which allow me continues access to the instrument for generating data.

Furthermore, I could not have undertaken this journey without having supports and assistance of all my colleagues in Biochemistry and Molecular Biology (BMB) department at Wright State University. I am truly grateful to be a part of the BMB department and appreciate everyone for providing such a friendly environment. It was a great pleasure meeting a group of nice people and thanks to all of you for making an extraordinary work experience at Diggs Laboratory.

I would like to take this opportunity to express my appreciation to a number of people who inspired me including my previous PIs and mentors: **Dr. Reza A. Ghiladi**, **Dr. Frank Scholle**, **Dr. Mirsasan Mirpour**, **Dr. Leili Chamani-Tabriz**, **Dr. Abbas Bahador**, and **Dr. Mehdi Assmar**.

And lastly, I am extremely grateful to my dear husband and family for their unconditional love and support which keep me inspired, motivated and confident. I believe a big part of my passion for doing research is coming from the way that my parents nurtured my curiosity, since my childhood, by encouraging me to question “why” and figure out “how” things work.

I. Introduction

1.1 Ras superfamily of small GTPases

The Ras superfamily of small GTPases including over 150 family members, which are divided into **5 subfamilies: Ras, Rho, Rab, Arf, and Ran**. This classification is based on their sequences, structural similarities and cellular functions (Gerwert, Mann, & Kotting, 2017; Gray, von Delft, & Brennan, 2020; Matos, 2021).

1.2 Small GTPases structure and function

Small GTPases regulate a wide range of cellular processes such as gene expression, cell proliferation, differentiation, actin cytoskeleton remodeling, vesicular trafficking, and nuclear transport (Arrazola Sastre et al., 2020; Gray et al., 2020; Qu et al., 2019; Takai, Sasaki, & Matozaki, 2001). Small GTPases, as defined by their name, can bind to a guanosine triphosphate (GTP) and intrinsically hydrolyze it to guanosine diphosphate (GDP) (Qu et al., 2019; Rojas, Fuentes, Rausell, & Valencia, 2012) through the N-terminal G-domain made of about 170 amino acid residues (Reiner & Lundquist, 2018). In the G-domain, as shown in Figure 1, there are five conserved sequences of polypeptide loops (G-Box; G1-G5) responsible for GTP binding, and the major conformational changes occur in Switch I and Switch II regions required for the effector binding and GTP hydrolysis (Cherfils & Zeghouf, 2013; Toma-Fukai & Shimizu, 2019).

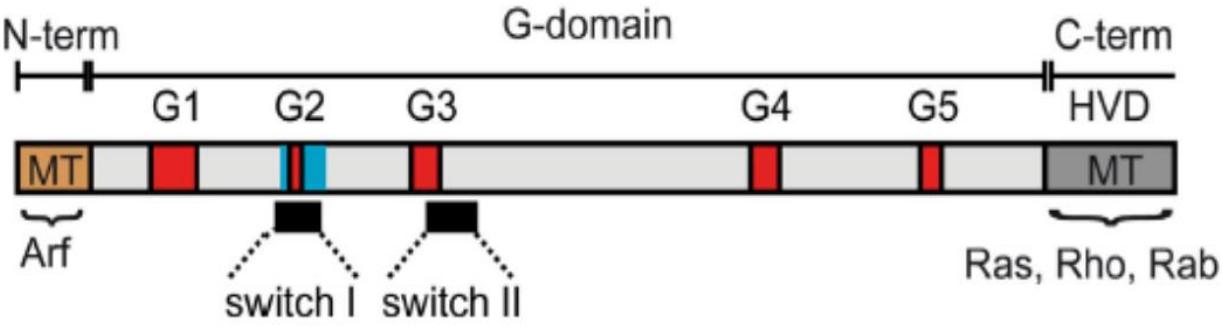

Figure 1- Schematic representation of the general structure of Ras superfamily of small GTPases.

All GTPases share highly conserved regions in their G-domain which contains multiple nucleotide binding motifs (G1-G5). In contrast, they are distinguished by variable region in membrane targeting motif (MT) located at the C-terminal of the protein except for Arf subfamily that their N-terminus is also involved in membrane targeting. Figure adapted from (Neely & Hidalgo, 2014).

Small GTPases switch between active GTP-bound and inactive GDP-bound states. For this nucleotide exchange and cycling between active/inactive forms, small GTPases need to directly interact with three group of molecules: first, guanine nucleotide exchange factors (GEFs), which induce conformational changes of guanine nucleotide-bound small GTPases, resulting in dissociating the guanine nucleotide and stimulating GTP binding. Second, GTPase-activating proteins (GAPs), which catalyze the intrinsic GTP hydrolysis activity of small GTPases, instantly converting to the GDP-bound inactive state. Third, GDP-dissociation inhibitors (GDIs), which keep GDP-bound small GTPases in inactive form in cytosol and away from the plasma membrane for their activation (Arrazola Sastre et al., 2020; Cherfils & Zeghouf, 2013; Phuyal & Farhan, 2019; Toma-Fukai & Shimizu, 2019).

1.3 Ras small GTPases

Ras proteins belong to Ras subfamily of small GTPases; in mammalian, there are three Ras isoforms: K-Ras (**K**irsten **ra**t **s**arcoma), H-Ras (**H**arvey **ra**t **s**arcoma), N-Ras (**N**euroblastoma **RAS** viral oncogene homology); where for K-Ras there are two splicing variants arise from alternative RNA splicing at the fourth exon (K-RAS4A and K-RAS4B) (K. Chen, Zhang, Qian, & Wang, 2021; Hobbs, Der, & Rossman, 2016). Since K-Ras4B is the splice variant predominantly expressed in many tissues (Hancock, 2003; Hobbs et al., 2016; Spencer-Smith & O'Bryan, 2019), K-Ras4B, referred to K-Ras hereafter, has been studied extensively.

H-Ras was the first human oncogene discovered in 1982 (Cox & Der, 2010); soon after, other Ras isoforms (N-Ras and K-Ras), were identified. To date, Ras proteins are considered as one of major cancer drivers with the rate of about 19% for all human cancers. Among Ras proteins, K-Ras is the most frequently mutated isoforms (K. Chen et al., 2021), found in almost 90% of pancreatic ductal adenocarcinoma (PDAC), 32% non-small cell lung adenocarcinoma (NSCLC) and 50% of colorectal adenocarcinoma (CRC) (Prior, Hood, & Hartley, 2020).

1.4 Ras signaling

Under basal physiological conditions, cell surface receptors that are stimulated by extracellular signaling activates Ras at the PM, resulting in stimulation if a wide range of Ras signaling pathways (Figure 2). The Ras-RAF-MEK-ERK is the first recognized Ras signaling pathway and it is a central regulator of cell growth, differentiation and apoptosis (Castellano &

Downward, 2011); activation of RAF (a protein serin/threonine kinase) by activated Ras results in activation of MEK 1 and 2, which are mitogen-activated protein kinase (MAPK) kinases. MEK, in turn activates MAPKs, such as extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) (Bhattacharjee & Gurung, 2015). Another well-studied Ras signaling pathway is PI3K/AKT, which has a role in cell cycle progression and survival (Castellano & Downward, 2011). Another Ras downstream effector is RAL guanine nucleotide dissociation stimulator (RALGDS), which is implicated in cell differentiation, apoptosis(Moghadam et al., 2017) and endosomal trafficking(J. K. M. Lim & Leprivier, 2019). Lastly, phospholipase Cε (PLCε) is involved in protein kinase C (PKC) activation which has a role in cell motility. The other effect of PLCε is stimulation of Ca2+ signaling (J. K. M. Lim & Leprivier, 2019).

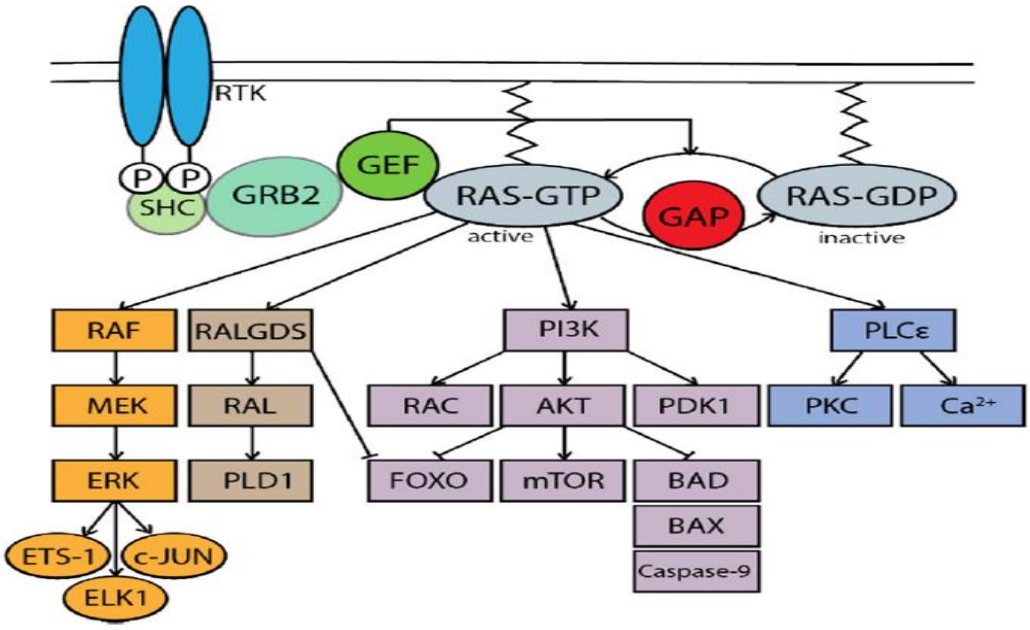

Figure 2- Activation of RAS at the plasma membrane leads to trigger multiple signaling pathways, through interactions with different downstream effectors.

Upon stimulation of receptors tyrosine kinase (RTK), Src homology 2 domain containing transforming protein (SHC) and growth factor receptor-bound protein 2 (GRB2) recruit GEF to the PM where facilitate nucleotide exchanges and activation of Ras. This leads to initiation of signaling cascades through activation of downstream effectors. Figure adapted from (J. K. M. Lim & Leprivier, 2019).

1.5 Activating point mutation of Ras and hotspots in human cancers

Point mutations at amino acid residues G12, G13, and Q61 have been shown as well-characterized hotspots for Ras in cancers. These residues are located near the nucleotide-binding site and switch regions involved in conformational changes upon GTP binding, and thereby selective interactions with effectors (Figure 1) (Neely & Hidalgo, 2014). The amino acid substitution at aforementioned residues interferes the stable interaction of Ras with GAPs, which causes prolonged Ras GTP binding, resulting in enhanced Ras/effector interaction and ultimately upregulation of Ras downstream signaling pathways and oncogenic transformations in cells (Eser, Schnieke, Schneider, & Saur, 2014; Munoz-Maldonado, Zimmer, & Medova, 2019).

1.6 Ras isoform structures and trafficking

Similar to other small GTPases, all three Ras isoforms (H-, N- and K-Ras) are almost identical in their G-domain, which is responsible for the catalytic activity of the protein. In contrast, these isoforms differ in the C-terminal hypervariable region (HVR), which plays a role in membrane targeting (Figure 3). In HVR, there is a tetrapeptide motif called CAAX (C, cysteine; A, aliphatic amino acid; X, any amino acid). The CAAX motif undergoes a number of post-translational modifications including prenylation, proteolysis and methylation(Cox et al., 2015). After the synthesis in the cytosol, the Cys residue of CAAX motif undergoes an obligatory and irreversible step of farnesylation, an attachment of a 15C farnesyl lipid moiety by farnesyltransferase (FTase). This event allows the association of Ras proteins with endoplasmic reticulum (ER), where the next step of modification occurs; in the ER, RAS-converting enzyme 1

(RCE1), a farnesylcysteine-directed endoprotease, cleaves the AAX motif. Lastly, the farnesylated Cys gets methylesterified by isoprenylcysteine carboxyl methyltransferase (ICMT) (Cox et al., 2015; Prior & Hancock, 2012). After the post-translational modifications, H-Ras, N-Ras, and K-Ras4A traffic from ER to Golgi and then to the PM. However, the mechanism through which K-Ras4B translocates from the ER to the PM is not fully understood (Apolloni, Prior, Lindsay, Parton, & Hancock, 2000; Magee & Marshall, 1999).

Since farnesylation was identified as the first required step for Ras PM localization and activity, FTase inhibitors were developed as anti-Ras drugs. However, despite promising results in pre-clinical study phases of H-Ras-driven cancers, these inhibitors failed to inhibit K-Ras activity in cancer patients in clinical trials. This unexpected failure was led to discovering another feature of K-Ras; it turned out that upon FTase inhibition, N-Ras and K-Ras4B (in contrast to H-Ras and K-Ras4A) alternatively get geranylgeranylated by geranylgeranyltransferase I (GGTase I) and the 20C geranylgeranyl lipid moiety is sufficient for the PM localization of K-Ras4B in the absence of FTase (Cox et al., 2015).

Ras protein contains two PM targeting signals: the first signal, which is common in all Ras isoforms is the farnesyl lipid moiety at the Cys in CAAX motif. The second signal, which is distinct for each Ras isoform, involves the palmitoylated Cys 181 and 184 in H-Ras and Cys 181 for N-Ras, while K-Ras4B contains polybasic domain (PBD) made of a series of Lys residue as the second signal (Kattan et al., 2019; Zhou & Hancock, 2018) .

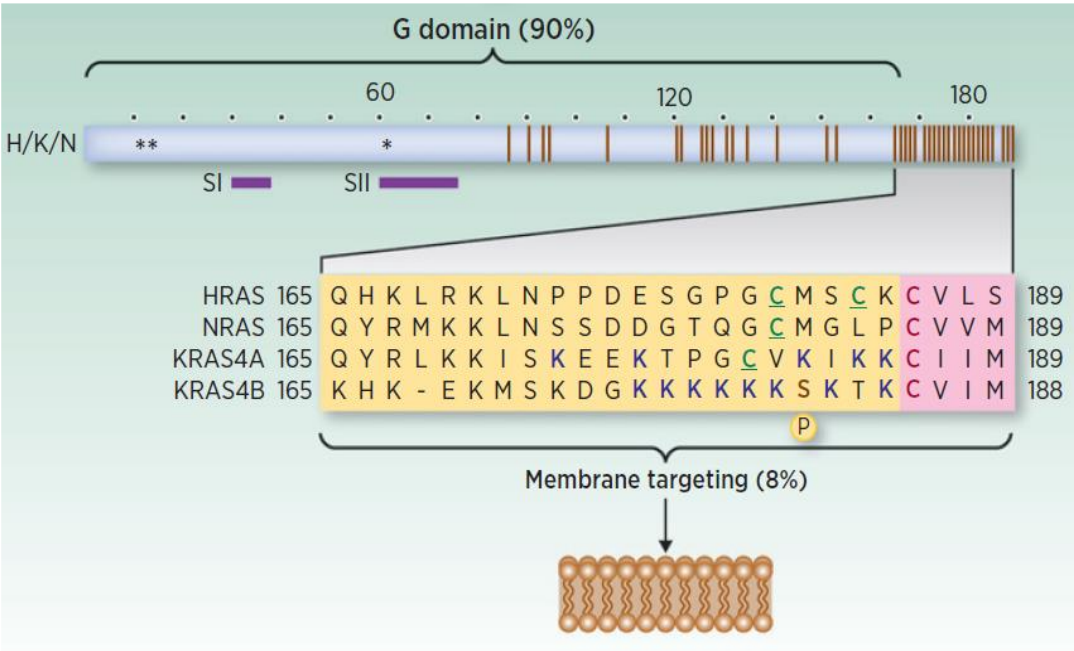

Figure 3- Hypervariable region of Ras isoforms.

Unlike high levels of similarities in G-domain, Ras isoforms are highly divergent in their membrane targeting motif, known as hypervariable region or HVR, in the C-termina. In HVR, CAAX motif (shown in pink box) undergoes different post-translational modifications, including prenylation of Cys residues which provides the first signal for the membrane targeting; the second signal is isoform-specific for H-/N-Ras and K-Ras4A splice variant includes additional palmitoylation of Cys residues (shown in green). However, for K-Ras unlike other isoforms, there is no palmitoylation in this region, instead PBD of K-Ras4B composed of six Lys residue, account as second signal. Figure adapted from (Cox, Der, & Philips, 2015).

K-RAS4B is distinguished from other isoforms, due to the presence of PBD, which confers an additional affinity towards negatively charged plasma membrane (PM) via electrostatic interactions.

Among all Ras isoforms, the PM anchoring of K-Ras4B is a matter of utmost importance in different aspects; first, it is the most frequently mutated cancer-related Ras isoform, and secondly, K-Ras4B as the only non-palmitoylated PBD-containing isoform uses a complex combinational membrane anchor compromised of PBD and prenyl group (can be either farnesyl or geranylgeranyl) (Abdelkarim et al., 2019; Prior & Hancock, 2001; Zhou & Hancock, 2018).

1.7 Perturbing K-Ras localization to the PM as an approach for blocking K-Ras activity

There are different strategies reported to prevent K-Ras activity; one of them is to prevent Ras-GTP formation by inhibiting Ras interaction with GEFs. Another approach is to inhibit Ras downstream effectors; for example, inhibiting the MAPK and PI3K/AKT signaling pathways. Another approach is to lock K-Ras in the GDP-bound inactive conformation. Lumakras (sotorasib), which recently approved by FDA, directly binds to the GDP-bound inactive K-Ras G12C mutant and form a covalent bond to the mutant Cys, resulting in blocking its oncogenic signaling (Canon et al., 2019; Ostrem, Peters, Sos, Wells, & Shokat, 2013). However, only about 13% of NSCLC patients harboring oncogenic mutant K-Ras have the G12C point mutation and still a vast majority of K-Ras mutations are remained undruggable (K. Chen et al., 2021). Lastly, since Ras signaling is highly dependent to the PM localization, impairment of Ras localization to the PM disrupts K-Ras signal transduction (Cox et al., 2015; Hobbs et al., 2016; Kattan et al., 2019; Spiegel, Cromm, Zimmermann, Grossmann, & Waldmann, 2014).

1.8 K-Ras/PM interaction

Extensive studies have reported that Ras localization to the PM is a critical requirement for Ras activation and its interaction with downstream effectors for conducting signal transduction (Abankwa, Gorfe, & Hancock, 2008; Abdelkarim et al., 2019; Adhikari et al., 2021; Goswami et al., 2020; Spiegel et al., 2014). Moreover, studies have shown that about 5 to 6 Ras molecules are laterally segregated into distinct nanoscale domains in the PM, termed nanoclusters, which are pivotal platform for Ras signal transduction by providing proper orientation for effectors interactions (Abankwa & Gorfe, 2020; Abankwa et al., 2008; Barcelo et al., 2013; Parker & Mattos, 2015; Zhou et al., 2017). These nanoclusters are isoform-specific and nucleotide-dependent, where active GTP-bound nanoclusters of an isoform is distinct and nonoverlapping not only with another isoform clusters, but also with nanoclusters of the same isoform in the inactive GDP-bound state (Zhou & Hancock, 2018).

Altogether, with respect to high flexibility of the isoform-specific HVR, in contrast to highly conserved sequences of G-domain, the differences in HVR, in addition to playing roles in membrane targeting, may explain the distinct isoform-specific functionality of K-Ras as the only non-palmitoylated containing PBD Ras isoform. For instance, palmitoylation of Ras isoforms in the Golgi facilitates their vesicular trafficking to the PM, while K-Ras by deviation from the ordinary ER-Golgi-PM trafficking rout, proceeds from the ER to PM via unknown mechanism (Apolloni et al., 2000). Moreover, K-Ras and H-Ras have shown different preferences for interaction with specific lipid species at the PM (Abdelkarim et al., 2019; Prior & Hancock, 2012; Zhou, Gorfe, & Hancock, 2021).

1.9 Phosphatidylserine (PS)

PS is a negatively charged phospholipid and asymmetrically concentrated in the inner leaflet of the PM (Yang, Lee, & Fairn, 2018). K-Ras selectively interacts with the PM PS over other PM phospholipids via the farnesyl moiety and PBD (Zhou & Hancock, 2020; Zhou et al., 2017; Zhou, Prakash, Liang, Gorfe, & Hancock, 2021). Reducing the PM PS content has shown to dissociate K-Ras from the PM, and due to the PM localization dependency for signal transduction of K-Ras, reducing the PM PS enrichment can block K-Ras signaling (Kattan et al., 2019; van der Hoeven et al., 2013; Zhou et al., 2017; Zhou et al., 2015).

PS is synthesized in mitochondria-associated membranes (MAMs) of the ER (Kay & Fairn, 2019), and lipid transfer proteins (LTPs), ORP5 and 8, exchange the newly synthesized PS from the ER with phosphatidylinositol 4-phosphate (PI4P) from the PM at ER-PM membrane-contacting site (MCS). PI4P, another negatively charged phospholipid in the PM, is synthesized by phosphatidylinositol 4-kinase IIIα (PI4KIIIA) (Chung et al., 2015). At the ER, Sac1 phosphatase, converts PI4P to PI to maintain the PI4P gradient between the PM and ER (Figure 4) (Chung et al., 2015; Moser von Filseck, Vanni, Mesmin, Antonny, & Drin, 2015).

It is worth emphasizing that the PS trafficking mediated by OR5/8 occurs at the expense of constant degradation of PI4P at the ER by Sac1 and concurrent PI4P production at the PM by PI4KA. This results in stablishing high and low concentrations of PI4P at the PM and ER, respectively, which is required for continuous transport of PS from the ER to the PM at the ER-PM MCS.

Perturbation of any components in this process fails to transport PS from the ER to PM. This in turn, can result in K-Ras dissociation from the PM and consequently inhibition of K-Ras signaling (Adhikari et al., 2021; Kattan et al., 2019).

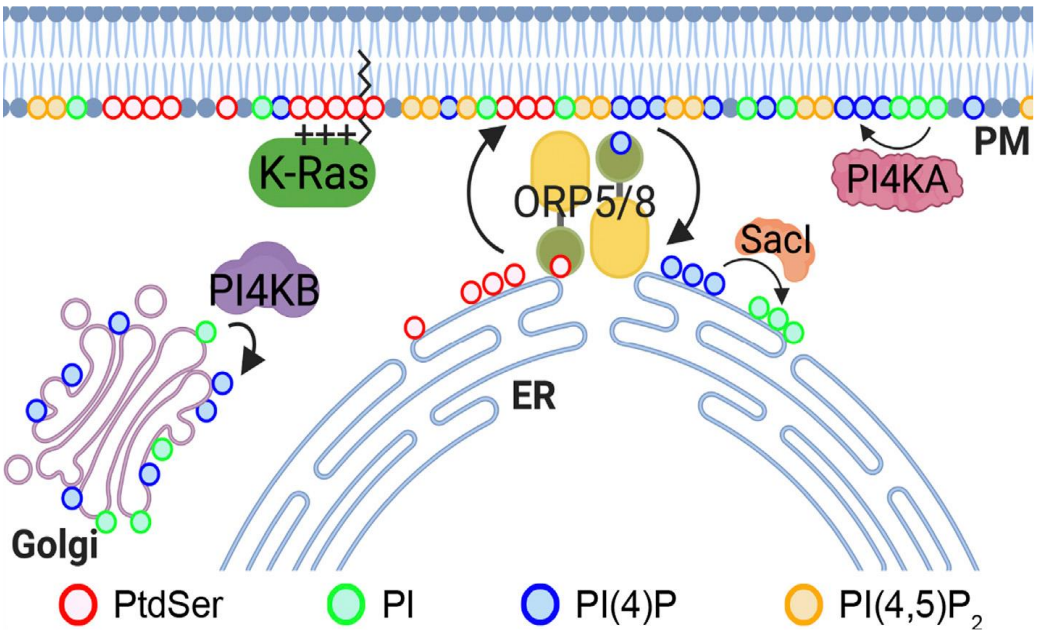

Figure 4- The PM and Golgi PI4P contents are involved in the PM enrichment of phosphatidylserine and subsequently K-Ras.

phosphatidylserine after being synthesized in the ER, exchanges with PI4P from the PM, by lipid transfer proteins ORP5/8. The required PI4P gradient between the PM and the ER, for PS transport to the PM, is provided by together activity of two enzymes at the ER and PM; Sac1 phosphatase by using up PI4P as substrate, prevent the increasing of PI4P in the ER, whereas PI4KA, on the other side, at the PM produce PI4P from PI. The Golgi PI4P, synthesized by PI4KB, also contributes in the PM PS. Interruption in this process lead to decrease in the level of the PM PS and therefore dissociation of K-Ras from the PM. Figure adapted from (Henkels, Rehl, & Cho, 2021).

1.10 PI4P at the Golgi complex regulates the PM localization of PS and K-Ras

While the PM PI4P is a primary precursor for PM PI (4,5) P_2, an important signaling lipid in the PM, PI4P is also synthesized at the Golgi complex by PI-4K IIIβ (PI4KB). Golgi PI4P content is involved in the membrane biogenesis and cellular vesicular trafficking at the *trans*-Golgi network (Graham & Burd, 2011). The synthesis of Golgi PI4P is regulated by PI4KB recruitment to the Golgi, where it is further phosphorylated by protein kinase D as an additional regulatory layer (Boura & Nencka, 2015). Although some studies have suggested that active GTP-bound Arf1 small GTPase recruits PI4KB to the Golgi membranes (D'Angelo, Vicinanza, Di Campli, & De Matteis, 2008), other studies have shown that this PI4KB requitement by Arf1 might be through an indirect mechanism and it has been suggested that ACBD3 (acyl-CoA binding domain containing 3), also called PAP7, GCP60, GOCAP1 or GOLPH1, is more likely to be the major PI4KB recruiter to the Golgi (Boura & Nencka, 2015). It has been further shown that glucose starvation dissociates PI4KB from the Golgi and promotes its nuclear accumulation (Graham & Burd, 2011; Venditti, Masone, Wilson, & De Matteis, 2016). However, the exact mechanism of this translocation is not fully understood.

In our recent study, we found that chemical inhibition or genetic depletion of PI4KB reduces the PM enrichment of both PS and K-Ras. We showed that upon PI4KB inhibition, LactC2, a well-characterized PS marker, redistributes from the PM to endomembrane and mitochondria. This reduction in the PM PS content results in K-Ras dissociation from the PM. We observed an enhanced colocalization of K-Ras with mitochondrial marker in cells after PI4KB inhibition, indicative of K-Ras translocation to mitochondria.

Studies have provided evidence that Golgi PI4P content supports a fraction of PM PI4P pool (Dickson, Jensen, & Hille, 2014) and depletion of Golgi PI4P content by blocking PI4KB activity dissociates PS and K-Ras from the PM (Miller et al., 2019). However, there is not much known about the details of the mechanism, by which the Golgi PI4P transport to the PM is regulated and how this Golgi-to-PM PI4P cargo plays a role in the PM PS content alterations, and as a consequence the PM K-Ras localization. To the best of our knowledge, the simplest explanation is that the Golgi PI4P depletion by PI4KB inhibition causes interference in PI4P transport from the Golgi to the PM, resulting in reduced PM PI4P pool. This in turn, perturbs the high concentration of the PM PI4P content required for the ORP5/8-mediated PM-PI4P/ER-PS exchange, and ultimately a decline in the PM enrichment of PS and K-Ras.

1.11 Lipid trafficking in the PM-Golgi- ER circuit

Lipid transport across cellular membranes occurs by vesicular secretory and non-vesicular trafficking pathways (Figure 5). Vesicular secretory pathways involve the intra/extra-cellular transport of lipids and proteins, which often contributes to the bulk lipid transport in a non-selective manner with access to both leaflet of bilayer membranes. In contrast, highly selective non-vesicular lipid trafficking, which is mediated by LTPs at MCSs, often distributes lipid species asymmetrically in one leaflet of the double-layer membrane of a cellular compartment at the MCS, using a steep gradient provided by a second lipid species (Agliarulo & Parashuraman, 2022; Lenoir, D'Ambrosio, Dieudonne, & Copic, 2021; Yang et al., 2018).

PI (phosphatidylinositol) is synthesized in the ER, and is transported to the PM and Golgi, where it is further phosphorylated by PI4 kinases to PI4P. While the PM, Golgi and secretory vesicles are three main components with high PI4P contents, the Golgi is known as a canonical source of PI4P in cell. A recent study has shown that disruption of Golgi trafficking, similar to Golgi PI4P depletion by translocating of Sac1 to the Golgi, leads to reduction in the PM $PI(4,5)P_2$ levels (Dickson et al., 2014). Although, PM PI4P depletion showed a higher degree of reduction in $PI(4,5)P_2$, this reduction further increased when Golgi PI4P was also simultaneously depleted. These data suggest that vesicular transport of the Golgi PI4P to the PM partially contributes to the PM PI4P contents needed for maintaining $PI(4,5)P_2$ levels at the PM (Dickson et al., 2014). Golgi PI4P plays a major role in Golgi vesicular trafficking by recruitment of PI4P-binding proteins required vesicle formation and exit from the *trans*-Golgi network (TGN). A number of effectors involved in transport of Golgi-membrane derived vesicles destinated to the PM has been identified: four-phosphate-adaptor protein (FAPP) (De Matteis, Di Campli, & Godi, 2005) and Golgi

phosphoprotein 3 (GOLPH3) (De Tito, Hervas, van Vliet, & Tooze, 2020). GOLPH3 is identified as a membrane curving protein which needs to interact with PI4P and MYO18A to form a complex along with F-actin for sufficient Golgi-to-PM trafficking (Rahajeng et al., 2019) .

The intertwined PI4P and PS metabolisms have been shown in yeast and mammalian cells, where they are regulated by PI4-kinases and PI4P-phosphatase (Lenoir et al., 2021). As described previously, PI4P/PS counter-lipid transport at the ER-PM MCS is mediated by ORP5/8, while this transport highly relies on PI4P gradient provided by ER-localized PI4P phosphatase, Sac1, and PI4KA at the PM.

A similar LTP-mediated lipid transport mechanism has been suggested for exchanges of Golgi PI4P with the ER sterol by another LTP, oxysterol-binding protein (OSBP). This lipid transport mechanism also relies on the PI4P gradient across the Golgi-EM MSC, where provided by phosphatase activity of the ER-localized Sac1, and Golgi PI-4 kinases (Balla, 2020; David, Castro, & Schuldiner, 2021). In addition to ceramide and cholesterol, LTPs mediated trafficking has been shown for PS transport from the ER to the Golgi (Agliarulo & Parashuraman, 2022). Particularly, ORP10 and ORP11 transport PS from the ER to the Golgi (Lenoir et al., 2021), where consequently upon reaching to the TGN, PS traffics to the PM via vesicular secretory pathway (Leventis & Grinstein, 2010).

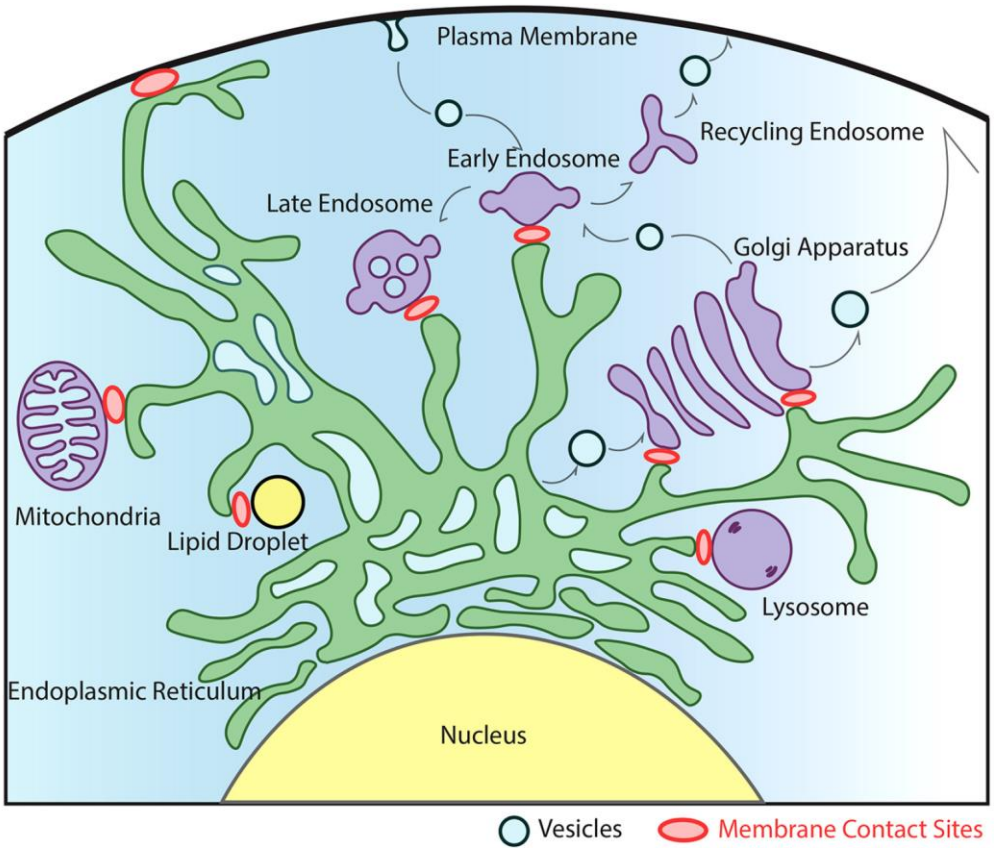

Figure 5- lipid traffics intracellularly through vesicular and non-vesicular pathways.

Vesicular transport pathway, in addition to lipid transport, is also involved in endocytosis (by vesicle formation at the PM), and protein trafficking (mainly from the ER and Golgi) to the PM or endomembranes. Alternatively, LTPs selectively shuttle lipid species between organelles at membrane contact sites. Figure adapted from (Yang, Lee, & Fairn, 2018).

1.12 Hypothesis and Specific Aims

Our previous study has shown that Golgi PI4P synthesized by PI4KB is involved with PM enrichment of PS and K-Ras (Miller et al., 2019). We found that upon PI4KB inhibition, PS redistribute to endomembrane and mitochondria while K-Ras is mainly translocated to mitochondria. However, since a similar observation was not made for intracellular localization changes of H-Ras (Ras isoform lacks PBD) in PI4KB-inhibited cells, we assumed that this different result is due to the involvement of PBD of K-Ras in this interaction.

Studies have demonstrated that the PM mislocalization of K-Ras blocks its signal transduction (Adhikari et al., 2021; Kattan et al., 2019). Also, it has been shown that K-Ras signaling is required for the growth of K-Ras-driven pancreatic cancer cells (Hayes et al., 2016). In addition, the observation of mitochondrial localization of K-Ras, but not H-Ras, in PI4KB-inhibited cells suggests a possible role of K-Ras PBD in this mitochondrial translocation. Based on these findings, we hypothesize that K-Ras redistribution from the PM to mitochondria, upon PI4KB inhibition is in a PBD-dependent manner, and the PM mislocalization can inhibit the growth of pancreatic cancer lines as a consequence of blocking K-Ras signaling. Therefore, we will test our hypothesis through two specific aims:

1) Identifying the effect of PI4KB inhibition on the growth of pancreatic cancer cell lines harboring oncogenic mutant K-Ras.

2) Elucidate the role of PBD of K-Ras as a factor involved in mitochondrial translocation in PI4KB-inhibited cells.

II. Materials and Methods

2.1 Maintaining mammalian cell lines

Human pancreatic ductal adenocarcinoma cells (PDACs) including BxPC-3, AsPC-1 and Panc 10.05 were grown in RPMI-1640 (Cat# 302001; ATCC), MIA PaCa-2 and PANC-1 were grown in DMEM (Cat# 10569010; Gibco) and HPAF-II were maintained in EMEM (Cat#20-200; ATCC). All cancer cell lines were grown in media supplemented with 10% fetal bovine serum (FBS) (Cat#: 16000-044; Gibco), and 2 mM L-glutamine (Cat# CA009-010; GenDEPOT). Madin-Darby canine kidney (MDCK) were grown in DMEM, supplemented with 10% FBS and 2mM L-glutamine. MDCK cells stably expressing GFP-tagged small GTPases (Rac1, RalA, Arl4 and Arl7) were grown in complete DMEM (supplemented with 10% FBS and 2 mM L-glutamine) containing blasticidin (4 μg/mL) as a selective marker. Cells were routinely tested for mycoplasma using MycoAlert PLUS Mycoplasma Detection Kit (Cat# LT07-710; Lonza). All cell lines were grown in 37°C incubator with 5% CO_2 injection.

2.2 Antibodies

Primary antibody: TOM20, mouse monoclonal antibody (Cat# SC-136211 SANTA CRUZ BIOTECHNOLOGY, INC). Secondary antibody: Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 555 (Invitrogen, Cat# A-21422), were used for IF staining.

2.3 Expression of GFP-tagged small GTPases in MDCK cells

2.3.1 GFP-small GTPases constructs

The full-length cDNA of RalA was cloned into cloning sites of pEGFP-C1 vectors. As the first step, cleavage sites of restriction enzymes (complementary to the vectors' RE sites at the multiple cloning site) were created by forward (F-SalI-RalA: AAGTCGACGCTGCAAACAAGC) and reverse (R-KpnI-RalA: CCGGTACCTTATAAAATGCAGCATCT) primers in PCR reaction using Phusion DNA polymerase and Phusion high-fidelity master mix (Thermo Scientifics, Cat# F530L).

After agarose gel electrophoresis of PCR products, the amplicons were extracted from agarose gel using GeneJET PCR Gel Extraction Kit (Thermo Scientific, Cat# K0692). The amplicon and pEGFP-C1 were then incubated with SalI and KpnI in Fast Digest Green (10 X) Buffer (Thermo Scientific, Cat# B72). The digested amplicon and vector were gel extracted followed by ligation using Quick Ligation Kit (New England Biolabs, Cat# M2200). Thereafter, ligated constructs were transformed into *E. coli* strain DH5α competent bacterial cells using heat shock method, followed by plating bacteria on LB-agar plates containing selective marker (kanamycin). After overnight incubation at 37 °C, colonies were picked up and after confirming by colony PCR (amfiSure PCR ONE PCR Master Mix (2X), Cat# P7000-005), selected corresponded bacterial suspensions were inoculated into 5 mL LB broth for growing for an overnight at 37 °C, in a shaker incubator. The day after, after preparation glycerol stocks, recombinant plasmids, GFP-RalA, were extracted from bacteria using QIAprep Spin miniprep kit (Qiagen, Cat# 27106).

2.3.2 Mammalian expression plasmid constructs

In order to generate MDCK cells stably express GFP-tagged proteins, the constructs were cloned into a vector with pEF6 promoter; this vector contains blasticidin and ampicillin resistance gene markers for mammalian and bacterial expression, respectively. As previously discussed, restriction enzyme cleavage sites (BamHI, EcoRI) were added into the constructs using primer listed in Table 1 in PCR (Phusion DNA pol. and 5X Phusion buffer).

Table 1- Sequence of primers used in Phusion PCR.

Construct	Forward primer	Revers Primer
GFP-RalA	5'CGGTCTCGACGAATTCCACCATGG TGAGCAAG G 3'	5'GGAGAGGGGCGGATCCTTATAAAA TGCAGCATCT TTC TCTG 3'
Arl4-GFP	5'CGGTCTCGACGAATTCCACCATGG GGAATGGGCTGT 3'	5'GGAGAGGGGCGGATCCTTACTTGT ACAGCTC GTC CAT G 3'
Arl7-GFP	5'CGGTCTCGACGAATTCCACCATGG GCAACATCTCCT 3'	5'GGAGAGGGGCGGATCCTTACTTGT ACAGCTCG TCCATG 3'

The cloning process and transformation was performed as described above and extracted plasmid was prepared for transfection into MDCK cells.

2.3.3 Transfection

MDCK cells were seeded on a 6-well plate containing 2 mL complete DMEM, on Day 2, when the cell confluency reached about 90%, cells were transfected using Lipofectamine 3000 transfection kit (Invitrogen, Cat# L300015). In two separate tubes we prepared diluted lipofectamine and DNA plasmid master mix, respectively. In the first tube, 5 μL of Lipofectamine 3000 were diluted with 125 μL Opti-MEM (Gibco, Cat# 31985070), and mixed by gentle finger flick. In the second tube, DNA master mix was made in by adding 4.0 μg DNA into pre-mixed solution of 125 μL Opti-MEM and 5 μL P3000 reagent. Then 130 μL of tube 1 solution (diluted lipofectamine) were added into tube 2 (containing DNA master mix), mixed well by gentle finger flick 5-6 times, incubated for 10-15 min at room temperature.

Cells were washed with PBS, 1850 μL of complete DMEM was added cell. Then the mixture of DNA-lipofectamine was added dropwise onto the cells; followed by incubation at 37 °C, for 24 hr. Then a day after, the blasticidin treatment (10 μg/mL) started and continued for 7 days. Thereafter, 4 μg/mL blasticidin in DMEM was used for maintaining cells.

2.4 Study subcellular localization of small GTPases using confocal microscopy

In order to quantitively measure the mitochondrial translocation of small GTPase upon PI4KB inhibition, we used Manders' coefficient as a tool to quantify colocalization of immunofluorescent stained mitochondrial marker and GFP-tagged small GTPase.

The principal of Mander's coefficient is based on the assessment of overlapping fractions of one protein colocalized with the second protein in the same subcellular site in a dual-channel confocal microscopy image (Dunn, Kamocka, & McDonald, 2011; MANDERS, VERBEEK, & ATEN, 1993). Each of these two proteins are fluorescently labeled with different fluorophores. Here, we have MDCK cells expressing GFP- fused with either Rac1, RalA, Arl4 and Arl7, also to visualized mitochondria, we did immunofluorescence staining for the mitochondrial marker, TOM20 (Galmes et al., 2016) which is a mitochondrial outer membrane protein.

After collecting images, we used Manders' coefficient plugin in ImageJ software to calculated the number of red pixels (from mitochondrial marker) colocalized with green pixels (from GFP-tagged proteins) divided by total number of red pixels. The higher values of Manders coefficient shows the higher fraction of mitochondrial marker are colocalized with GFP-tagged proteins, or in other words, higher percentage of GFP-tagged localized to mitochondria (Miller et al., 2019). Therefore, any increases in Manders coefficient numbers after each PI4KB inhibition treatment, comparing to control, demonstrate the mitochondrial translocation of the small GTPase in PI4KB inhibited cells.

2.4.1 Seeding cells and drug treatment

In order to evaluate mitochondrial translocation of small GTPases upon PI4KB inhibition, MDCK cells stably expressing GFP-tagged small GTPases were seeded on a glass coverslip with a density of 2X 10^5 for GFP-Rac1, 1.5X10^5 for GFP-RalA, 1x10^5 for GFP-Arl4, and 2x10^5 for GFP-Arl7 per well in a 12-well plate. 24h later, we started a 48h drug treatment; aliquot of each

PI4KB inhibitors in 2 mL of complete DMEM was prepared with the concentration of 1 μM for BF738735 or BQR-695 and 0.1 μM for IN-10 compound. As control, we used 1:1000 dilution of DMSO in 2 mL complete DMEM.

2.4.2 Fixing cells and Immunofluorescence staining

On Day 4, after 48h drug treatment cells were fixed followed by immunofluorescence staining against TOM20 for visualizing mitochondria. Cells were washed twice with ice-cold 1X phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) in PBS for 30 min in the dark. Then cells were permeabilize with 0.2% (W/V) Tx-100 (freshly made in PBS) for 10 min, in dark, followed by quenching with 50mM NH_4Cl for 10min at RT, dark. Cells were blocked with blocking buffer containing 2.5% BSA (10%) and 2.5% Fish gelatin (10%) in PBS for 30min at RT, in dark. After blocking, cells were incubated with TOM20 primary antibody diluted (1:100) in blocking buffer for 1 to 1.5 hr. Cells were washed with PBS (4 times) and incubated in dark for 30 min with secondary antibody (Goat anti-Mouse IgG, Alexa Fluor™ 555), diluted 1:500 ratio in blocking buffer. At the end, coverslips were washed with PBS, and then MilliQ water, and mounted in mowoil as a mounting medium on slides. Slides kept an overnight in dark at 4 °C until imaging.

2.4.3 Confocal imaging and analysis

Images were collected using an Olympus Fluoview FV1000 confocal laser scanning biological microscope. Images were analyzed for co-localization quantifications of mitochondrial marker with GFP-proteins using Manders' coefficient plugin in ImageJ software. For each reported Manders' coefficient values, at least three representative images from each coverslip were collected. From three independently experiment for each treatment, the mean value and standard error of the mean was calculated for Manders' coefficient value (Mean ± SEM). Then to evaluate mitochondrial translocation, statistical significance of differences between Manders' coefficient values of PI4KB inhibitors and DMSO treatments were analyzed by One-way ANOVA (p-value < 0.05) using Prism statistical software.

2.5 Cell Proliferation assay

2.5.1 Cell seeding and drug treatment

Human pancreatic cancer cell lines were seeded in 100 μL complete growth medium on a 96-well plate; a day after, when the confluency was about 40%-50%, drug treatment was started for 72h with various serially diluted drug concentrations in 100 μL complete growth medium:

BF738735: 1 nM, 50 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM, 50 μM.

BQR-695: 50 nm, 100 nm, 500 nM, 1 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM.

IN-10: 1 nM, 50 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM, 50 μM.

In each experiment DMSO (as control), and drug concentrations were applied onto 3 wells (triplicate). Every 24 h, 100 μL complete growth medium containing corresponded drug concentration was replaced for each treatment.

2.5.2 Measurement of Cell proliferation rate

On day five, media was aspirated and after twice washing with cold PBS, 100μL of 1X dye solution CyQUANT NF dye reagent in 1X HBSS buffer (CyQUANT NF Cell Proliferation Assay Kit; Cat#C35006; Invitrogen) were applied into each well.

Then, after dark incubation at 37°C, for 30 min and 1h, fluorescence intensity was measured using BioTek Synergy H1 microplate reader with excitation λ: 480 nm and, emission λ: 530 nm.

III. Results

3.1 Specific Aim 1. Identifying the effect of PI4KB inhibition on the growth of pancreatic cancer cell lines harboring oncogenic mutant K-Ras.

3.1.1 PI4KB inhibition redistributes K-Ras and PS from the PM to mitochondria and endomembranes, respectively.

In our preliminary data, we found that PI4KB inhibition translocates K-Ras from the PM to mitochondria. To look at K-Ras mislocalization, we co-expressed GFP-K-Ras and RFP-PDHA1 (as a mitochondrial marker) in Madin-Darby canine kidney (MDCK) cells. Then we knocked out PI4KB using CRISPR/Cas9 system (Figure 6A). As it is shown in Figure 6C, in the control MDCK cells which were transfected with an empty vector, GFP-K-Ras is mainly localized to the PM; Manders' coefficient, which calculates the fraction of GFP-K-Ras co-localized with RFP-PDHA1, an indication of K-Ras localization to mitochondria, shows that only about 20% of PDHA1 is colocalized with K-Ras. In other words, a small fraction of K-Ras is localized to mitochondria. In *PI4KB* knockout (KO) cells, however, GFP-K-Ras noticeably co-localizes with RFP-PDHA1, and Manders' coefficient suggests that about 50% of mitochondrial marker is colocalize with K-Ras, suggesting an enhanced K-Ras translocation to mitochondria.

Furthermore, we looked at PS distribution in *PI4KB* KO MDCK cells co-expressing GFP-LactC2, a well-studied PS marker, and RFP-PDHA1 (Figure 6B and D). In control cells, LactC2 was localized to the PM, while In *PI4KB* KO cells, LactC2 is extensively redistributed. The increased Manders' coefficient also suggest that PS is translocated to mitochondria. Interestingly, LactC2 was found at regions where there is no PDHA1. This suggests that PS is redistributed to mitochondria and endomembranes after *PI4KB* KO.

To further examine the PM localization of K-Ras and PS after *PI4KB* KO, we performed quantitative electron microscopy (EM) (unpublished data, Cho's lab). Briefly, we prepared intact basal PM sheets from *PI4KB* KO MDCK cells expressing GFP-K-RasG12V or -LactC2 and labeled with anti-GFP antibody conjugated with 4.5 nm gold particles. We imaged them by EM and counted the gold particles, which represent the level of K-Ras or PS at the inner PM leaflet (Figure 6E and F). Our data showed that in *PI4KB* KO cells, there were about a 30%-reduction in the level of GFP-K-Ras while the content of LactC2 which represents PS showed almost 40% decrease in the inner PM leaflet. Together with confocal microscopy, our data suggest that *PI4KB* knockout translocates K-Ras and PS from the PM to mitochondria and endomembrane, respectively.

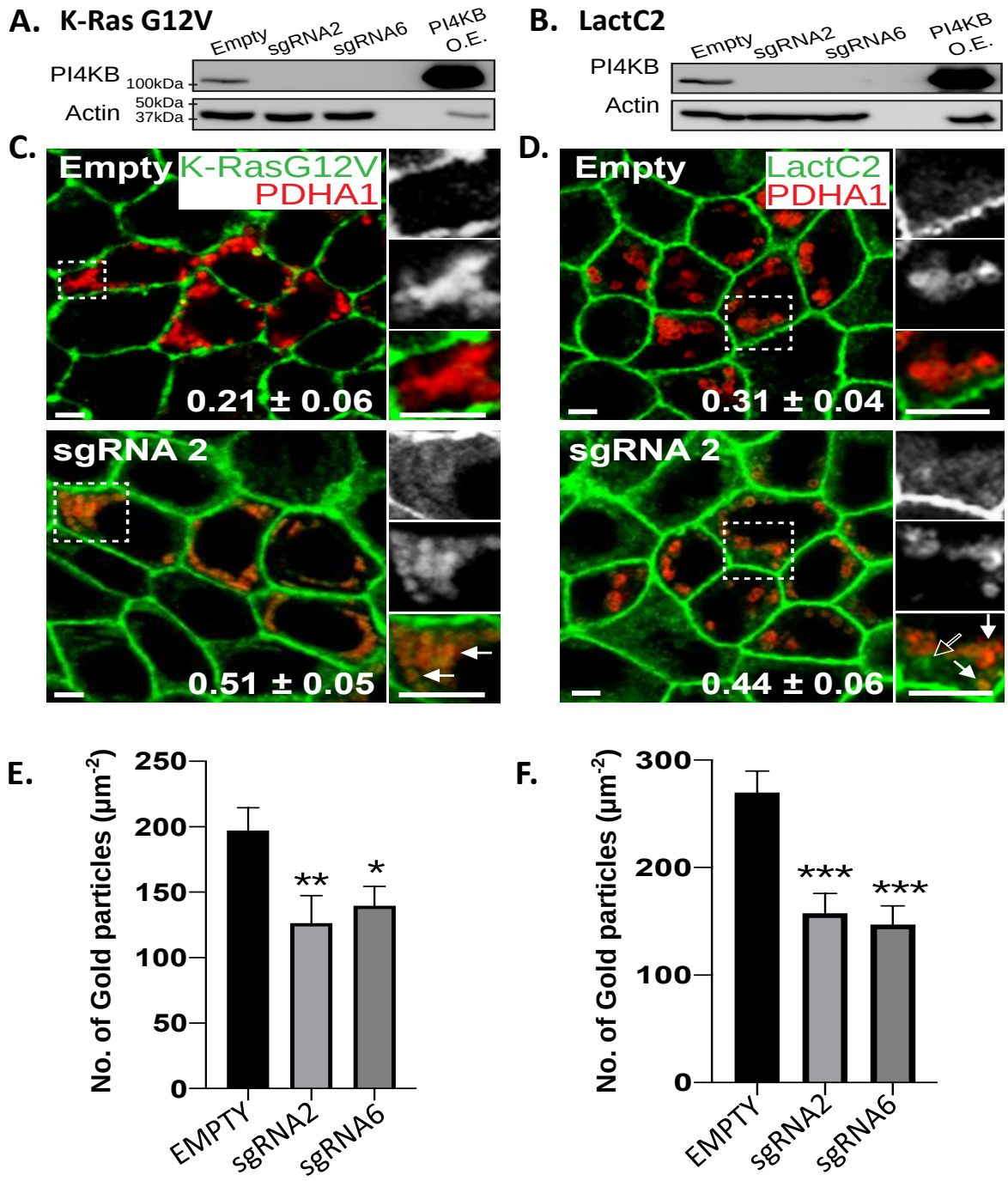

Figure 6- PI4KB knocked out redistribute K-Ras and PS from the PM.

Immunoblot data representing PI4KB knocked out in cells expressing oncogenic GFP-K-Ras (A.) and GFP-LactC2 (B.). RFP-PDHA1 was expressed in the MDCK cells expressing GFP-K-Ras (panel C.) or GFP-LactC2 (panel D.) and these cells were imaged by confocal microscopy. The increased Manders coefficient (mean values ± SEM, from 3 independent experiments) after *PI4KB* KO indicates an increase in the colocalization of mitochondrial marker with GFP-K-Ras or GFP-LactC2. Quantification of the PM mislocalization of GFP-K-Ras (E.), and GFP-LactC2 (F.) after *PI4KB* KO in aforementioned cells.

3.1.2 PI4KB mRNA expression is higher in human pancreatic adenocarcinoma.

As the first step of conceptual framework, we assessed the PI4KB mRNA expression level in human PDAC samples from The Cancer Genome Atlas (TCGA) database, since ~90% of PDAC are driven by oncogenic K-Ras. We took 354 pancreatic RNA-sequencing data from TCGA TARGET GTEx study containing expression profiles of TCGA solid-PDAC samples and GTEx samples from normal pancreatic tissues of healthy individuals. First, we compared the PI4KB mRNA level in the normal pancreatic tissue versus primary PDAC. As illustrated in Figure 7A, the PI4KB mRNA level was significantly higher in primary PDAC tissues than normal tissues. Furthermore, we sought to identify whether there is a correlation between the mRNA expression levels of PI4KB and K-Ras in these data set. For this reason, we plotted the PI4KB mRNA level against that of K-Ras for normal and PDAC tissues. As demonstrated in Figure 7B, Pearson's correlation shows a strong positive correlation, suggesting that samples with high PI4KB mRNA levels are likely to have high K-Ras mRNA levels and that PDAC tissues express higher mRNA levels of PI4KB and K-Ras than normal pancreatic tissues. These data suggest that mRNA levels of PI4KB and K-Ras in both normal and PDAC tissues can be targeted for oncogenic mutant K-Ras activity.

We further took more tissue sample data to explore whether the PI4KB expression levels is associated with prognosis of survival rate in cancer patients harboring wild-type (WT) or oncogenic mutant K-Ras. As it is shown in the graph Figure 7C, which is for cancer patients harboring WT K-Ras, there was no difference of survival rate between samples with low and high PI4KB mRNA levels. However, the data in the graph for cancer patients harboring oncogenic mutant K-Ras demonstrated that patients with low PI4KB level survived significantly longer than

patients with high PI4KB level (Figure 7D). Altogether, these data suggest that PI4KB may be a target for oncogenic mutant K-Ras signaling implicated in pancreatic cancer cells.

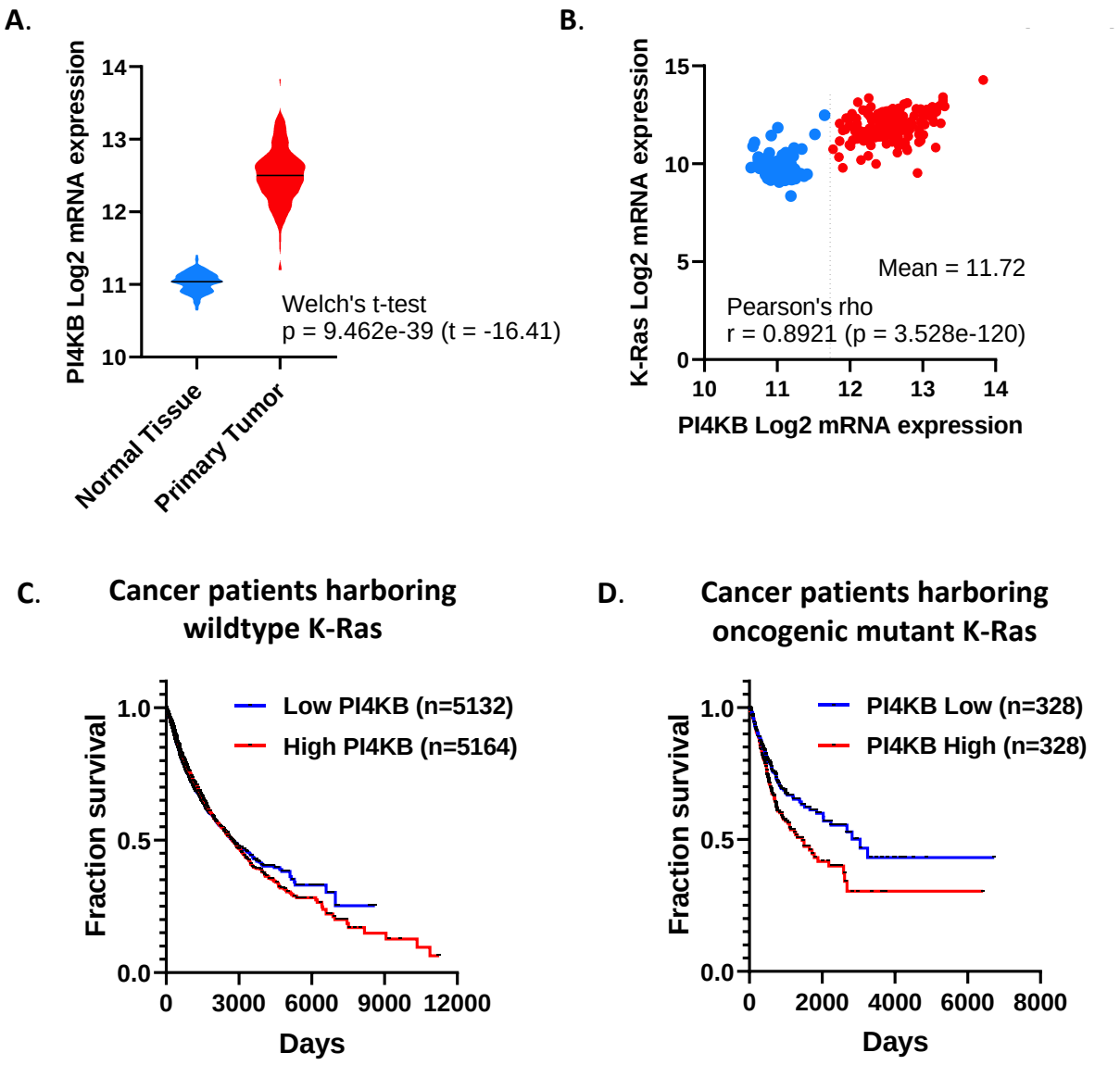

Figure 7- PI4KB expression levels in cancer patient samples.

Violon plot representing the level of PI4KB mRNA expression levels in normal pancreatic tissues versus primary pancreatic tumor samples from TCGA TARGET GTEx-PANCAN database (panel A). The correlation of K-Ras mRNA expression level in samples with low and high PI4KB mRNA expression level; based on the mean value (11.72) samples were divided into two groups: blue and red dots represent samples with low and high level of PI4KB mRNA expression levels, respectively (panel B). Overall survival rate analysis of cancer patients harboring WT K-Ras (panel C), and oncogenic mutant K-Ras (panel D) based on the PI4KB mRNA expression

3.1.3 The effect of PI4KB inhibition on K-Ras signaling in pancreatic cell lines

Since the PM localization of K-Ras is essential for the biological activity and signal transduction (Chavan et al., 2015; Henkels, Rehl, & Cho, 2021; Schmick et al., 2014), dissociation of K-Ras from the PM can block its signaling. Moreover, it has been shown that knockdown of endogenous K-Ras in pancreatic cancer cell lines harboring oncogenic mutant K-Ras, but not WT K-Ras reduce their viability and clonogenic growth (Hayes et al., 2016). Taken together, since PI4KB inhibition dissociates K-Ras from the PM, we hypothesize that PI4KB inhibition may block the growth of human pancreatic cancer cell lines expressing oncogenic mutant K-Ras. To address our hypothesis, we chose a panel of pancreatic cancer cell lines; including BxPC-3, a control cell line that harbors WT K-Ras, and cell lines (AsPC-1, Panc 10.05, HPAF-II, HPAC, MIA PaCa-2, and Panc-1) that express oncogenic mutant K-Ras with different point activating mutations. These cells were treated with three different PI4KB inhibitors for 72h, and cell proliferation was measured by using a fluorescent dye that stains cellular DNA contents.

As it is shown in Figure 8, BF738735 compound significantly inhibited all cell growth, except MIA PaCa-2, compared to BxPC-3 cell line. For IN-10, all cell lines showed significantly lower cell growth compared to BxPC-3 cell line. BQR-695 compound significantly reduced the growth of Panc10.05 and HPAC cells, whereas other cells were not affected by this PI4KB inhibitor. These data show that PI4KB inhibition tends to show greater inhibition in the growth of pancreatic cancer cell lines harboring oncogenic mutant K-Ras over that of WT K-Ras, suggesting that PI4KB could be a drug target for treating pancreatic cancer cells harboring different oncogenic mutant K-Ras.

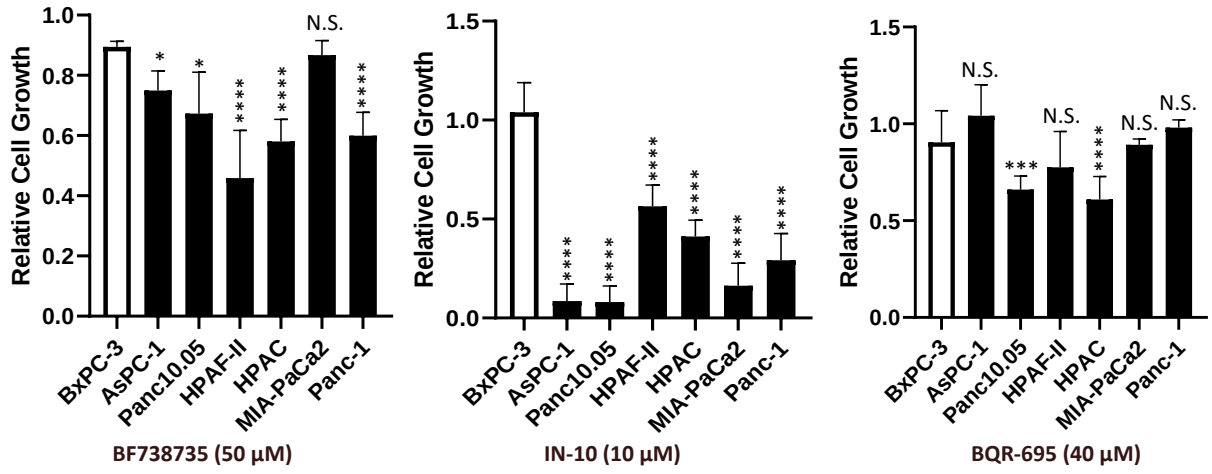

Figure 8- Proliferation assay study for PI4KB inhibitors.

Pancreatic cancer cells expressing oncogenic mutant K-Ras (black bars) or Wild type K-Ras (white bars) were seeded on a 96-well plate, and after 72h treatment with PI4KB inhibitors (BF738735, IN-10 and BQR-695), the cell proliferation rates was measured. Each cell line was tested in three independent experiments and the mean values of drug treated cells were compared to control (DMSO) by unpaired t test. Then the data sets of each cell line compared to BxPC-3 using ordinary one-way ANOVA test. (N.S.-not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

3.2 Specific Aim 2. Determine the effect of PI4KB inhibition on mitochondrial translocation of small GTPases containing PBD.

Membrane-bound Small GTPase containing PBD

Our previous published and unpublished confocal and EM data show that upon PI4KB inhibition, K-Ras dissociated from the PM and translocated to mitochondria (Miller et al., 2019). Moreover, PI4KB inhibition redistributes PS from the PM to mitochondria and other endomembrane. However, PI4KB inhibition did not alter cellular localization of H-Ras, a Ras isoform does not contain PBD (Miller et al., 2019). It has been extensively reported that K-Ras interacts with the PM PS via its C-terminal farnesyl lipid chain and PBD, and reducing the PM PS content dissociates K-Ras from the PM (Cho et al., 2016; Kattan et al., 2019; Zhou & Hancock, 2020). H-Ras however, binds the PM via a farnesyl chain and two palmitoyl lipid moieties, and does not require PM PS content for the PM binding (Chavan et al., 2015; Zhou & Hancock, 2015). Based on these observations, we hypothesize that K-Ras translocation to mitochondria upon PI4KB inhibition is through the PBD. To test this hypothesis, we examined the cellular localization of four PM small GTPases containing a PBD after PI4KB inhibition by confocal microscopy (Table 2).

As listed in Table 2, these small GTPases localize to the PM and have different types of PBD and lipid moieties; K-Ras contains a series of Lys residues and the C-terminal 15C farnesyl lipid tail, whereas Rac1 and RalA have a PBD made of Lys and Arg residues with the C-terminal 20C geranylgeranyl lipid tail. For Arl4 and Arl7, they have a PBD made of Lys and Arg residues with the N-terminal 14C myristoyl lipid chain.

Table 2 - Small GTPases containing PBD tested in the study. Amino acid residues in red represent a polybasic domain. G (shown in green) and **C** represent a myristoylated Gly and a prenylated Cys, respectively.

Small GTPase	C-terminal polybasic domain sequence	Lipid moiety
K-Ras	--KMSKDGKKKKKKSKTK**C**VIM-C'	C-terminal farnesyl
Rac1	--IRAVLCPPPVKKRKRK**C**LLL-C'	C-terminal geranylgeranyl
RalA	--NGKKKRKSLAKRIRER**C**CIL-C'	C-terminal geranylgeranyl
Arl4	N'-MGNGLSDQTS--KLHDMIIKRRKMLRQQKKKR-C'	N-terminal myristoyl
Arl7	N'-MGNISSNISA--DKLYEMILKRRKSLKQKKKR-C'	N-terminal myristoyl

3.2.1 PI4KB inhibition does not promote the mitochondrial localization of Ras-related C3 botulinum toxin substrate 1 (Rac1).

Rac1 is a member of Rho subfamily of small GTPases, cycling between GTP-bound active and GDP-bound inactive forms (Jou & Nelson, 1998). As an RhoGTPase, its signaling pathways is a master regulator for cell polarization and migration (Remorino et al., 2017), cytoskeleton remodeling, cellular adhesion, proliferation, apoptosis, and reactive oxygen species production (Marei & Malliri, 2017). Deregulated Rac1 at the protein level and activity have shown in different cancers including breast cancer, melanoma, non-small cell lung cancer (NSCLC), and colorectal cancer (CRC), suggesting the role of Rac1 in different stages of tumor initiation and progression (Marei & Malliri, 2017).

To examine whether PI4KB inhibition promotes translocation of Rac1 to mitochondria, we generated MDCK cells stably expressing GFP-Rac1 and treated these cells with DMSO or different PI4KB inhibitors; BF738735 and IN-10. After 48h treatment, cells were fixed with 4% paraformaldehyde (PFA) followed by immunofluorescence staining for Translocase of the outer mitochondrial membrane complex subunit 20 (TOM20) as a mitochondrial marker. Then, cells were imaged using confocal microscopy and Manders' coefficient was calculated for each treatment to quantitate the fraction of TOM20 co-localized with Rac1. Our data show that Rac1 is extensively distributed throughout cells presumably to the PM, nucleus and endomembranes (Phuyal & Farhan, 2019) in control cells. When cells were treated with PI4KB inhibitors, Manders' coefficient compared to DMSO-treated cells was not changed, suggesting that, unlike K-Ras, PI4KB inhibition does not promote the mitochondrial localization of Rac1.

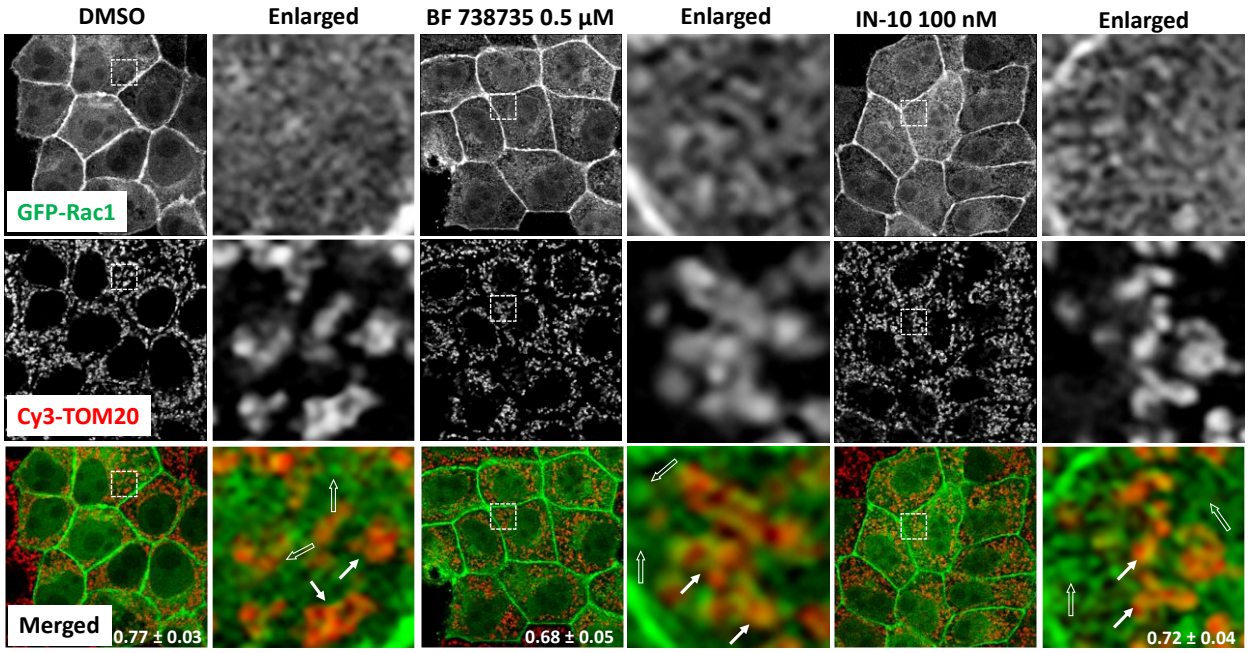

Figure 9- PI4KB inhibition does not enhance mitochondrial localization of Rac1.

MDCK cells stably expressing GFP-Rac1 were treated with DMSO (control) or PI4KB inhibitors for 48h. Cells were fixed with 4% PFA and after immunofluorescence staining for TOM20, were imaged using a confocal microscope. Manders coefficient from three independent studies was calculated using ImageJ software.

3.2.2 PI4KB inhibition promotes the mitochondrial localization of Ras-like (Ral) A.

Ral GTPases belong to the Ras subfamily and as defined by their name, they share a lot of structural similarities (about 50%) with Ras isoforms (Yan, Jones, & Theodorescu, 2015). Ral GTPase is known as a key effector for promoting Ras-dependent cancer cell growth (Gentry et al., 2015; K. H. Lim et al., 2005; Neel et al., 2011), where its signal transduction is mainly involved with vesicle trafficking, migration, autophagy (Zago, Biondini, Camonis, & Parrini, 2019), and regulation of mitochondrial functions (Gentry et al., 2015). Of the two Ral isoforms (RalA and RalB), RalA signaling has shown to play a critical role in the anchorage-independent growth of

pancreatic cancer (Martin & Der, 2012) and CRC (Gentry et al., 2015). It has been shown that activated Ras recruits RalGDS (a GEF for Ral) from the cytosol to PM, which consequently leads to nucleotide exchanges and Ral activation at the PM. In other words, the PM localization of Ras dictates the stimulatory effect of Ral by recruiting RalGDS to the PM (Matsubara et al., 1999). Targeting RalA in cancer therapy has attracted more attention in recent years (Yan et al., 2015).

To examine whether PI4KB inhibition promotes the mitochondrial localization of RalA upon PI4KB inhibition, MDCK cells stably expressing GFP-RalA were treated with PI4KB inhibitors and prepared for confocal microscopy as described in Section 3.2.1. In DMSO-treated control cells, RalA is localized to the PM and other endomembranes likely to be recycling endosomes, mitochondria, exocyst complex and perinuclear compartment (Gentry et al., 2015; K. H. Lim et al., 2005; Pollock, Schinlever, Rohani, Kashatus, & Kashatus, 2019; Shipitsin & Feig, 2004). Manders' coefficient shows that about 50% of TOM20 is co-localized with RalA. After treatment with PI4KB inhibitors, Manders' coefficient is increased indicating that a higher fraction of RalA is translocated to mitochondria when PI4KB was inhibited. Therefore, our data suggest that PI4KB inhibition promotes RalA translocation to mitochondria.

We further evaluated the PM localization of RalA upon PI4KB inhibition using quantitative EM. We seeded MDCK cell expressing GFP-RalA on gold EM grid and after drug treatment for 48h with PI4KB inhibitors, we prepared intact basal PM sheets. As it was described in Section 3.1.1, we immunolabeled basal PM sheets with anti-GFP antibody conjugated with 4.5 nm gold particles and imaged them by EM. As it is indicated in Figure 11, we found that in PI4KB inhibited cells, there was about 30% reduction in the level of GFP-RalA in the inner leaflet PM.

Together with confocal microscopy, our data suggest that PI4KB inhibition translocates RalA from the PM to mitochondria.

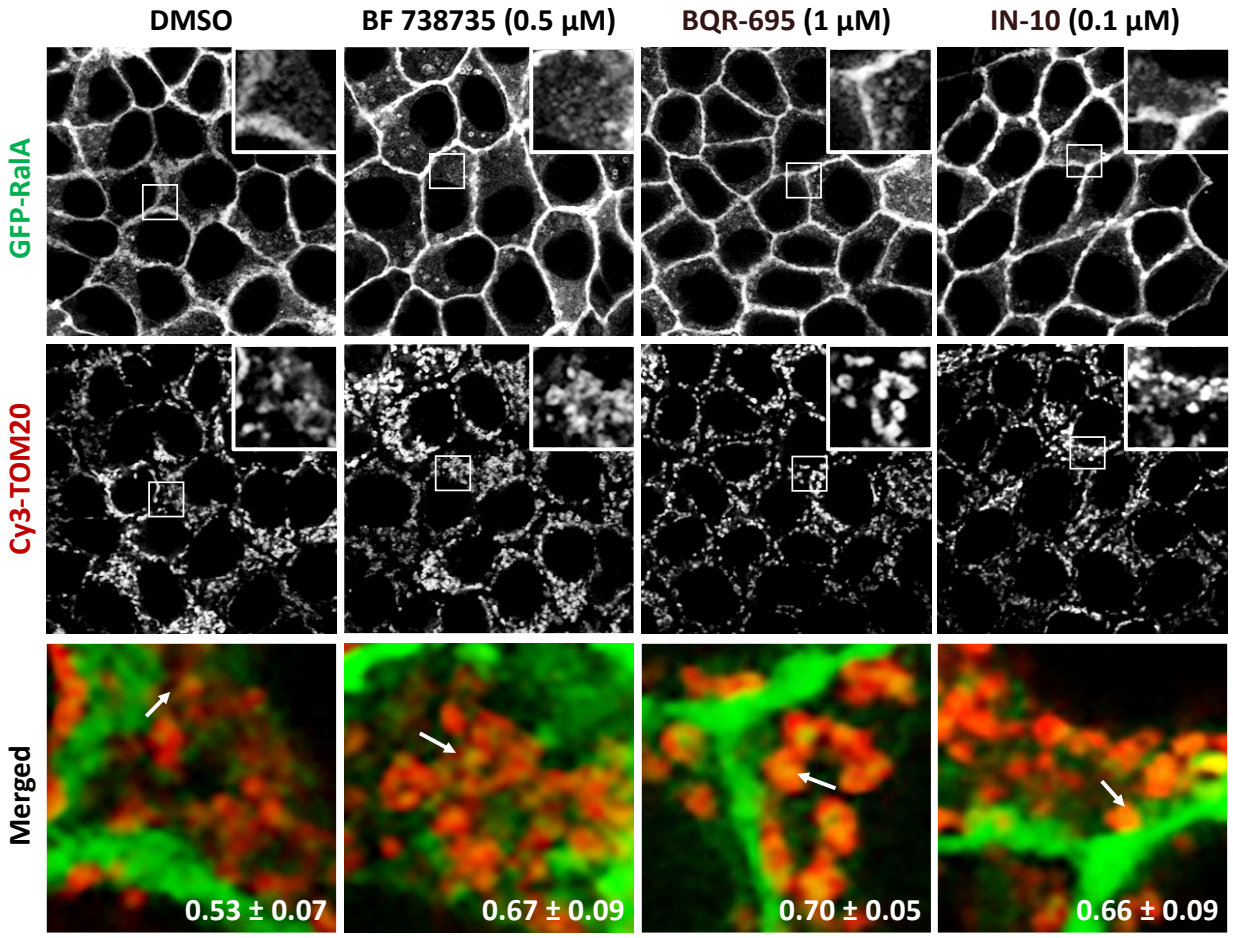

Figure 10- PI4KB inhibition translocates RalA to mitochondria.

MDCK cells stably expressing GFP-RalA were treated with DMSO (control) or PI4KB inhibitors for 48h. Cells were fixed with 4% PFA and after immunofluorescence staining for TOM20, were imaged using a confocal microscope. Manders coefficient from three independent studies was calculated using ImageJ software.

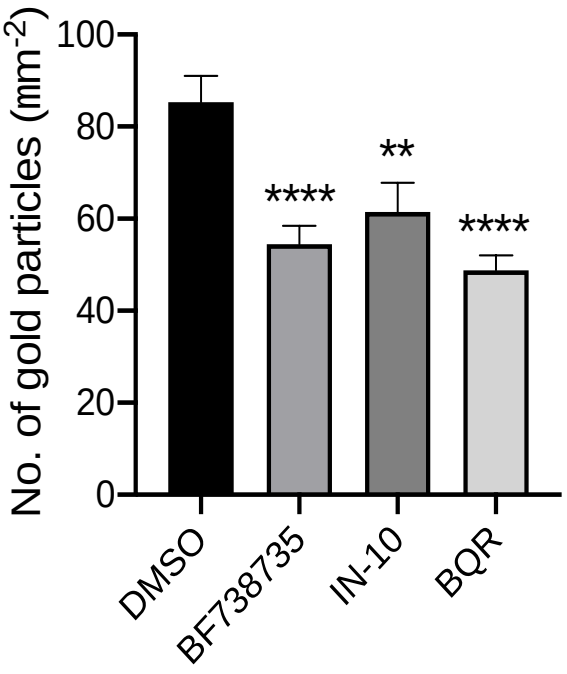

Figure 11- PI4KB inhibition reduced the level of RalA localized to the inner leaflet of the PM.

MDCK cells stably express GFP-RalA were seeded on EM grid, after 48 h treatment with BF738735 (1 μM), IN-10 (0.1 μM), and BQR-695 (1 μM), basal plasma membrane was prepared. Cells were labeled with GFP antibody conjugated to nanogold particles, and imaged by EM. Significant differences between GFP-RalA localized to the PM in drug-treated and DMSO controls were evaluated by one-way ANOVA (**p<0.01, **** p<0.0001).

3.2.3 PI4KB inhibition does not promote the mitochondrial localization of ADP-ribosylation factor (Arf) 4a and c.

Arf-like (Arl) GTPases belong to a subgroup of Arf subfamily, based on structural similarities (Hofmann, Thompson, Sanderson, & Munro, 2007; Jacobs et al., 1999). Arls are the only members of Arf subfamily distinguished by the PBD in their C-terminus, which has been suggested for the distinct contribution in the PM interaction of the protein with negatively charged membrane phospholipids, specifically PtdIns(4,5)P_2 (PIP_2) for Arl4C (Hofmann et al., 2007) . Arl4 subfamily includes three isoforms: Arl4a (called Arl4), Arl4c (also called Arl7), and Arl4d (Jacobs et al., 1999). In addition to the PM anchoring, studies have suggested that the PBD act as nuclear localization signal (Hofmann et al., 2007; Jacobs et al., 1999). Moreover, the PBD has a role in recruitment of cytohesin (act as a GEF for Arl) through interaction with their PH domains, even though cytohesin potentially might not able to interact with the PM with high affinity (Hofmann et al., 2007). For instance, recruitment of cytohesin-2 (act as a GEF for Arf6) to the membrane by the three Arl4 isoforms (Hofmann et al., 2007) leads to further recruitment of Arf6 to the PM and initiation of Arf6-Rac-Rho signaling pathway involved in actin remodeling (Casalou, Faustino, & Barral, 2016; Donaldson & Jackson, 2011; Harada et al., 2021; Hofmann et al., 2007).

Arl4c, through Wnt-β-catenin and EGFR-Ras signaling pathways, contributes in epithelial morphogenesis. Upregulated expression of Arl4c has shown in different cancers such as colon, lung, liver, and in particular invasion of pancreatic cancer samples (Harada et al., 2021). In addition to structural similarities, Arl isoforms also share different common functionalities with Arf subfamily; for example, Arl4a and Arl4c are involved with vesicle biogenesis and cellular trafficking (Pasqualato, Renault, & Cherfils, 2002). Specifically, Arl4a has a role in the Golgi

integrity and endosome-to-Golgi trafficking, regulating actin cytoskeleton rearrangement (Sztul et al., 2019; Zhang et al., 2020), and cell motility (Casalou et al., 2016). Arl4c, has also shown its implication in actin remodeling, cell migration (Sztul et al., 2019), proliferation, and cell cycle progression (Zhang et al., 2020); Arl4c inhibition has shown to block Rac1 activity, resulting in lower cell migration, proliferation and invasion in colorectal and lung cancer cells (Casalou et al., 2016).

3.2.3.1 PI4KB inhibition does not promote the mitochondrial localization of Arl4a.

We generated MDCK cells stably expressing GFP-Arl4a and treated with PI4KB inhibitors. Cells were prepared for confocal microscopy as described in Section 3.2.1. In DMSO-treated control cells, GFP-Arl4a localized to the PM and endomembranes likely to be the Golgi complex and endosomes (K. J. Chen, Chiang, Yu, & Lee, 2020; Lin et al., 2011). Manders' coefficient suggests that GFP-Arl4a localized to mitochondria.

In PI4KB-inhibited cells, Manders' coefficient was not changed, suggesting that PI4KB inhibition doesn't the mitochondrial localization of Arl4a.

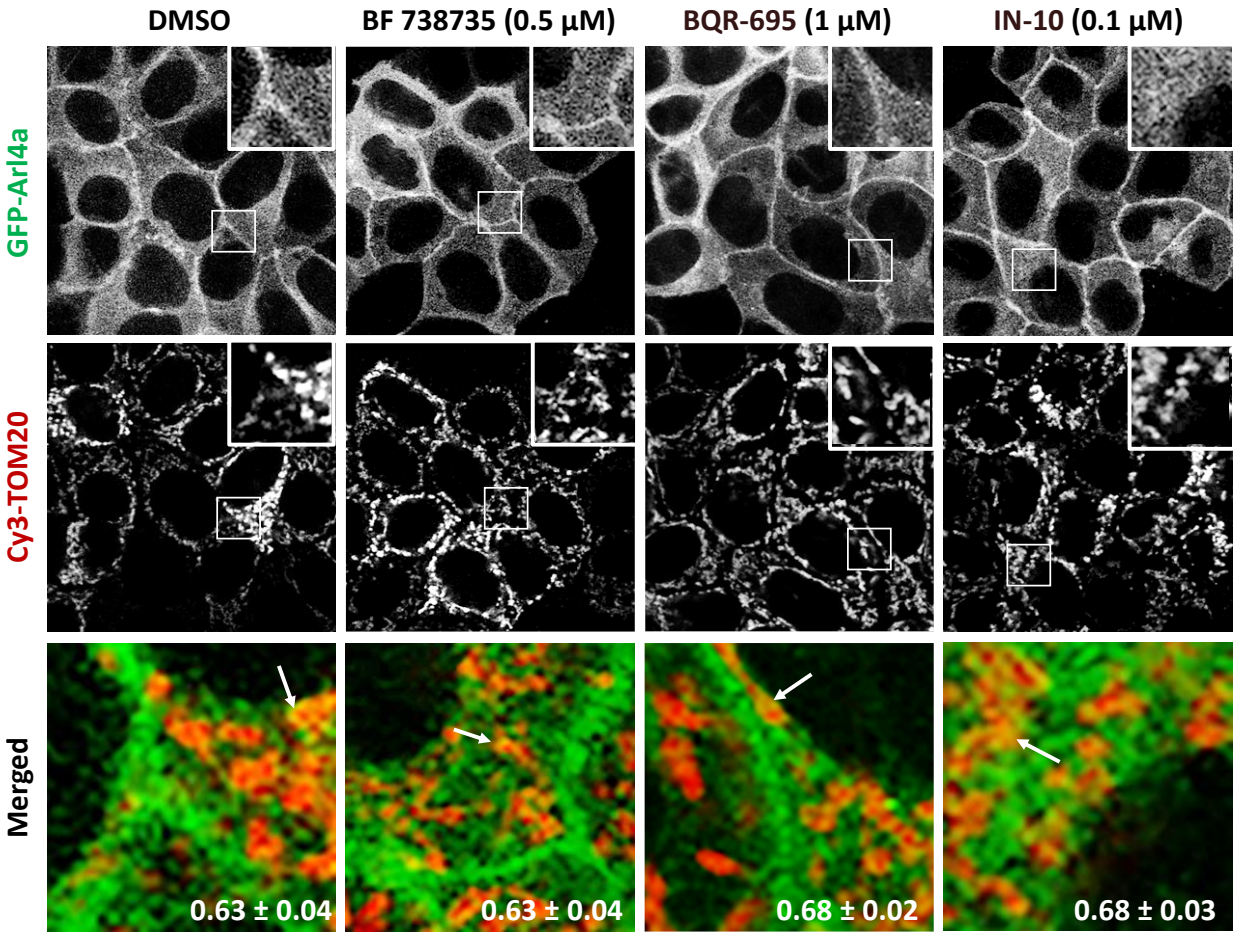

Figure 12- PI4KB inhibition does not enhance mitochondrial localization of Arl4a.

MDCK cells stably expressing GFP-Arl4a were treated with DMSO (control) or PI4KB inhibitors for 48h. Cells were fixed with 4% PFA and after immunofluorescence staining for TOM20, were imaged using a confocal microscope. Manders coefficient from three independent studies was calculated using ImageJ software.

3.2.3.2 PI4KB inhibition does not promote the mitochondrial localization of Arl4c.

In control cells, GFP-Arl4c was localized to the PM as well as endomembranes including mitochondria. However, when cells were treated with PI4KB inhibitors, we did not see an obvious changes in Arl4c localization. Moreover, Manders' coefficient was not changed significantly compare to control cells. Therefore, our confocal microscopy data suggest that PI4KB inhibition does not promote mitochondrial of Arl4c in cells.

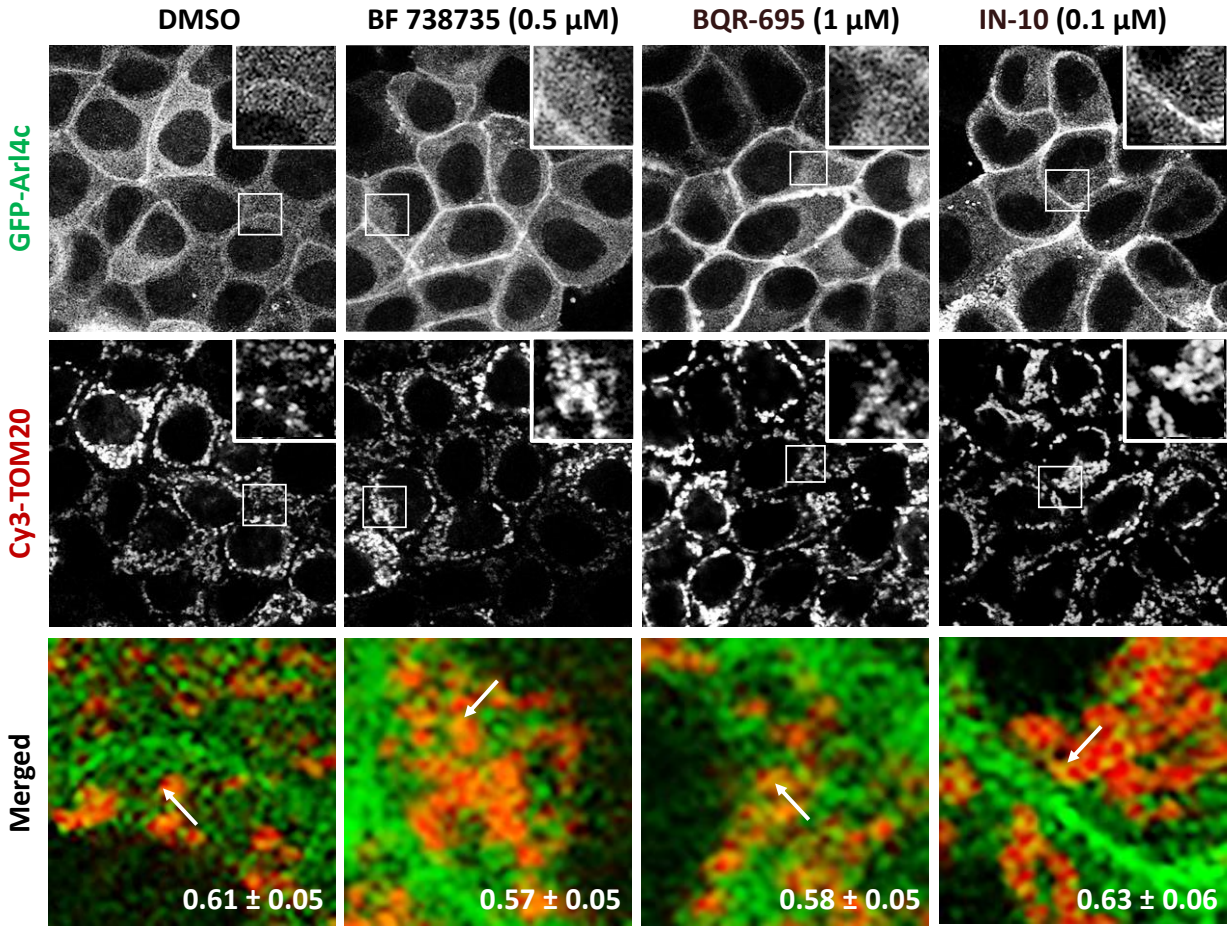

Figure 13- PI4KB inhibition does not enhance mitochondrial localization of Arl4c.

MDCK cells stably expressing GFP-Arl4c were treated with DMSO (control) or PI4KB inhibitors for 48h. Cells were fixed with 4% PFA and after immunofluorescence staining for TOM20, were imaged using a confocal microscope. Manders coefficient from three independent studies was calculated using ImageJ software.

IV. Discussion

Although the recent FDA-approved drug against K-Ras G12C-driven cancers has been considered as a breakthrough, after all unsuccessful efforts for finding anti-Ras therapy, only about 13% of NSCLC patients can benefit from this treatment. Since this selective inhibitor only targets K-Ras G12C, the majority subsets of K-Ras mutations in PDAC, CRC, and NSCLC patients are still needed to be addressed. One strategy for blocking activity of K-Ras with any activating point mutations is to dissociate K-Ras from the PM, since K-Ras activity heavily relies on the PM binding. Studies have suggested that PS content in the PM plays a critical role for the interaction of K-Ras with the PM, and thereby K-Ras activity (Cho et al., 2016; Zhou, Gorfe, et al., 2021; Zhou et al., 2014). To this end, our lab has recently identified PI4KB, the Golgi-resident PI kinase, as a potential target for regulating hyperactivity of oncogenic mutant K-Ras (Miller et al., 2019).

We have shown that inhibition of PI4KB led to redistribution of K-Ras and PS from the PM to mitochondria and other endomembranes, respectively (Miller et al., 2019). Although the mechanism of the PS and subsequent K-Ras redistribution from the PM in PI4KB-inhibited cells is still under investigation, we have explored the capability of PI4KB as an anti-K-Ras target for practical applications. First, using the TCGA database, we found that the mRNA expression level of PI4KB in the samples from pancreatic cancer is significantly higher than normal pancreatic tissue from healthy individuals (Figure 7A). Moreover, cancer patients with oncogenic mutant K-Ras showed a poor prognosis when the PI4KB mRNA expression level is high (Figure 7C).

Implementation of Golgi apparatus and PI4KB has been shown across multiple cancer types (Tan et al., 2020; Tokuda et al., 2014; M. G. Waugh, 2014; Mark G. Waugh, 2019); a

comprehensive study has shown that the upregulation of PI4KB is correlated with accelerated progression of lung adenocarcinoma harboring mutant K-Ras (Tan et al., 2020). Here, we further showed that PI4KB inhibitors tend to reduce the growth of pancreatic cancer cell lines expressing oncogenic mutant K-Ras, which is, in part, by K-Ras dissociation from the PM, and thereby blocking K-Ras signaling transduction.

Using three chemical PI4KB inhibitors, we found a level of inconsistency in terms of cell lines response to drug treatments (Figure 8). These observed differences perhaps are due to the non-specific activity of these PI4KB inhibitors towards other PI4-kinase isoforms or even other kinases. Since this is an inherent problem for all chemical inhibitors, to mitigate this issue, we further consider to evaluate and confirm our proliferation assay data for the same cell lines after genetically silencing *PI4KB* by shRNA-induced knockdown or CRISPR/Cas9-induced knockout. Altogether, our data suggest that PI4KB could be a target for treating pancreatic cancers harboring oncogenic mutant K-Ras.

Upon PI4KB inhibition, K-Ras translocates from the PM to mitochondria while the cellular localization of H-Ras is not altered. Thus, we presume that the presence of PBD in K-Ras is implicated in different observations were made for K-Ras and H-Ras. To explore whether the PBD-mediated interactions of K-Ras drives the mitochondrial translocation, we investigated the effect of PI4KB on mitochondrial translocation of other small GTPases containing PBD including Rac1, RalA, Arl4a and Arl7c. Among the four small GTPases tested, only RalA showed an increased level of mitochondrial localization in presence of PI4KB inhibitors. Compared to other small GTPases tested, Ral GTPases have shown a higher level of sequence and structural similarities with Ras (Guin & Theodorescu, 2015; Yan & Theodorescu, 2018), suggesting that

these similarities provide a PM anchor closer to that of K-Ras, which results in the same observations for these two proteins. Moreover, perhaps this PBD-dependent anchoring is more complex rather than presence/absence of a PBD in HVR.

As previously described, all GTPases share certain levels of conservative regions in their G-domain. For instance, for K-Ras and Rho (Rac1 belongs to Rho family), there are four conserved regions scattered across the G-domain which made up a 30% similarity between these two families (Chardin & Tavitian, 1986). However, Ral and K-Ras, in addition to sharing these four aforementioned regions, have five additional highly conserved regions corresponding to the K-Ras amino acid residues at 28-49, 64-73, 75-84, 97-104 and 152-164. Among them, residues between positions 28 and 49 of K-Ras are involved in the interaction with its downstream effectors for the transformation activity (Chardin & Tavitian, 1986).

Rac1, which does not translocate to mitochondria upon PI4KB inhibition (Figure 9), interacts with the PM through the C-terminal anchor which comprised of PBD, geranylgeranyl lipid tail as well as palmitoyl chain on Cys178 (Marei & Malliri, 2017; Michaelson et al., 2008; Navarro-Lerida et al., 2012). Although the PM is known for Rac1 activation and signaling initiation, different studies have reported that nuclear localization of Rac1 is also in a PBD-dependent manner, where PBD functions as an NLS (nuclear localization signal) (Phuyal & Farhan, 2019); such a nuclear translocation promotes Rac1 nuclear signaling involved in cell cycle progression, actin dynamic in the nucleus (Marei & Malliri, 2017). In aggressive tumors, higher levels of nuclear Rac1 has shown to be implicated in cancer cell invasion (De, Rozeboom, Aske, & Dey, 2020; Navarro-Lerida et al., 2015; Phuyal & Farhan, 2019) . Moreover, unlike K-Ras, the PM interaction of Rac1 is identified to be highly dependent on PIP_3 and PIP_2 (Remorino et al.,

2017), but not PS (Das et al., 2015; Maxwell, Zhou, & Hancock, 2018). Also, Rac1 palmitoylation at the Cys in immediate adjacent to the PBD is reversable and regulates its localization and lipid sorting (Maxwell et al., 2018). Taken together, it is plausible that the PBD and lipid anchors of Rac1 provide a unique Rac1/membrane binding characteristic that is different from K-Ras. This in turn, provides a stable membrane binding of Rac1 after PI4KB inhibition.

We further showed that the mitochondrial localizations of Arl4a and Arl4c were not enhanced upon PI4KB inhibition. For all three Arl4 isoforms, both N-terminal myristoyl lipid moiety and PBD are required for the PM localization, and deletion of the last eight or nine residues that include PBD of Arl4c leads to dissociation from the PM (Hofmann et al., 2007). Hofmann *et al.* showed that PM PIP_2 depletion can dissociate Arl4c from the PM (Hofmann et al., 2007) while in another study by Heo *et al.*, it was found that PM PIP_2 depletion alone had a minimal effect on Arl4c PM localization, but concurrent PIP_2 and PIP_3 depletion resulted in remarkably reduction in the PM enrichment of Arl4c (Heo et al., 2006). Similar observations were made for Rit, another PM small GTPase containing a PBD with prenyl lipid moiety, while there weren't any changes for H-Ras cellular localization (Heo et al., 2006). Taken together, our data suggest that the PM localization of Arl4a and 4c is dependent on PIP_2 and PIP_3, but not PS, in the PM. Consequently, PM PS depletion by PI4KB inhibition does not alter the PM localization of Arl4a and 4c.

It is important to identify that there are levels of uncertainty about metabolism of lipid species, including those which are involved in the membrane interactions of small GTPases; here, we point out some of them:

1- PI4P and PS turnovers across cell membranes, although they are important membrane lipid species due to their (direct/indirect) role in mediating signal transduction;
2- The mechanism by which their levels are strictly maintained at certain levels; this includes recognizing involved enzymes at each cellular compartment.
3- Functional purposes of the tightly regulated levels of PI derivatives and PS, including their regulatory effects on the other lipid species trafficking to/from different subcellular parts.

In this regard, we need to have more in depth understanding about how different PI4 kinase isoforms in different subcellular sites work together and along with phosphatases to stablish equilibrium needed for PS transport to the PM (from the ER or (if there is) possibly other subcellular sites). Furthermore, despite the proven central role of Golgi in processing and transporting cargoes, as well as endocytosis and exocytosis, there are not much known in details about effectors implicated in direction and promotion of Golgi vesicular trafficking particularly, cargo molecules destinated for the PM.

Indeed, having better understanding of spatial distribution of PS and PI derivatives along with mechanisms and enzymes by which their subcellular levels and trafficking are regulated will shed light on different cellular functions driven by these lipid species across cells which in turn lead us to find accurate therapeutic targets for uncontrolled activity of membrane bound small GTPases in future investigations.

Therefore, considering current gaps of knowledge, any future study which evolve our understanding regarding any of these described aspects will remarkably improve insights into the findings in our current study. This will get us closer to explore the answer of our fundamental question which is “how the Golgi PI4P content regulates the PS content at the PM”.

To further elucidate the role of PI4KB in the crosstalk between Golgi, ER, mitochondria and the PM circuit implicated in lipid trafficking across endomembranes and the PM, and subsequently localization of small GTPases, we have multiple unanswered questions needed to be addressed:

What are the direct Golgi-related effects of *PI4KB* KO on:

- Golgi PI4P levels; whether other PI4 kinases (IIα, β) at Golgi restore a fraction of PI4P depletion resulted in PI4KB KO?
- Golgi morphology?
- Forward vesicular trafficking pathway from Golgi to the PM, since PI4P is required in vesicular formation of PI4P-GOLPH3-MAYO18A and F-actin complex (Rahajeng et al., 2019)?
- Lipid transport between Golgi and ER?

We further would like to identify changes in the PS/PI4P counter-transport between the ER and PM affected by PI4KB inhibition at Golgi; since the Golgi PI4P partially contributes to the PM PI4P pool (Dickson et al., 2014) required for stablishing the PI4P gradient within the PM-ER

MCS by continually PI4P transport to the ER and subsequent degradation by Sac1. The resultant PI4P gradient, then allows the PS transport against its gradient from the ER to the PM.

It has been shown that PI4P at the PM is increased following inhibition of Sac1 at the ER (Dickson et al., 2014). Moreover, while it is controversial, some studies have suggested the possibility of Sac1 translocation to the Golgi under certain conditions (such as glucose deprivation) (Dickson et al., 2014). Since we have observed an increased glucose consumption in PI4KB inhibited cells (Miller et al., 2019), we presume the elevated mitochondrial glucose consumption may possibly mimics a (false) glucose starvation condition for the rest of the cell which may lead to Sac1 translocation from the ER to the Golgi. However, along with this escalated level of glucose consumption, we did not find any changes in the amount of ATP synthesis or cellular respiration (Miller et al., 2019). Therefore, morphological and other functional criteria in mitochondria worth investigating which may further provide us more accurate explanations and reasons for the observed elevated glucose consumption and unchanged ATP production.

In sum, evaluating 5 small GTPases containing a lipid anchor and PBD for the PM interaction, we found that membrane interactions of small GTPases includes a complex of different factors work in concert with each other to define a complicated combinational PM anchor. Although the presence of PBD may play a role, there are also many other determinants involved in this interaction; for instance, as it is shown, prenyl lipid moieties including number of carbons and saturation state of the lipid chain. Moreover, as it is shown for K-Ras, the G-domain interaction with the PM PS and HVR can also add even more complexity to the membrane anchors. For PBD, itself, it seems different PBDs (primary sequences including the type and number of the basic residues) confer various characteristics for the interaction; for example, it has been shown that

when K-Ras hexa-Lys PBD substitutes with hexa-Arg, even though with equal positive charges, this affects the lipid sorting for membrane interaction. Also, preference of PBD in K-Ras for interaction with PS rather than PIP_2 (with higher negative charges) suggests that the PBD/PM anchoring is beyond a simple electrostatic interaction (Abdelkarim et al., 2019; Kattan et al., 2019; Zhou & Hancock, 2018; Zhou et al., 2017).

Conclusion and Future directions

This project set out to investigate the effect of PI4KB inhibition in the PM mislocalization of oncogenic mutant K-Ras and subsequently blocking K-Ras signaling transduction, implicated in K-Ras driven cancers. Based on the TCGA samples data analysis, we found the higher expression levels of PI4KB mRNA in primary pancreatic tumor tissues, and strong correlation between elevated mRNA levels of PI4KB K-Ras. Moreover, this higher PI4KB mRNA level seems to be a poor prognostic marker for the survival rate in patients harboring oncogenic mutant K-Ras. In addition, our data suggest that PI4KB inhibitors tend to block the growth of pancreatic cancer cell lines harboring oncogenic mutant K-Ras, but not WT K-Ras. Taken together, our data suggest that PI4KB may be a target for treating pancreatic cancer patients harboring oncogenic mutant K-Ras. To further evaluate our hypothesis, we will perform anchorage-dependent cell growth assay (soft-agar assay) on pancreatic cancer cell lines in the presence of PI4KB inhibitors, since inhibited cell growth in soft agar is one of the phenotypes for blocking K-Ras signaling in K-Ras-driven cancer cells. To eliminate any non-specific effects chemical inhibitors, we will also knock down *PI4KB* in these human pancreatic cancer cells by shRNA and perform proliferation and soft-agar assays. Our ultimate goal for the first aim of project is to perform *in vivo* study, where we will inject these *PI4KB* knockdown human pancreatic cancer cells to immunosuppressed mice and harvest the tumors after 6-8 weeks to measure their size. If PI4KB is indeed a target for blocking K-Ras activity, we anticipate to see slower tumor growth in *PI4KB* knockdown tumors in comparison to control tumors where the *PI4KB* is not knockdown.

As well as possible future therapeutic outcomes described above, our study unrevealed some aspects of complicated PM anchoring of small GTPases containing PBD. Extensive studies have reported that K-Ras binds to the PM via electrostatic interactions between K-Ras PBD and PS in the inner PM leaflet, and the PM PS reduction dissociates K-Ras from the PM. We showed that PI4KB inhibition translocates PS and K-Ras to mitochondria. Thus, we hypothesized that upon PI4KB inhibition, K-Ras translocation to mitochondria is due to PS redistribution to mitochondria, and K-Ras binds mitochondrial membranes via electrostatic interactions between K-Ras PBD and mitochondrial PS. To test this hypothesis, we tested four other small GTPases, which contain a PBD for their PM localization, and we observed an enhanced mitochondrial localization only for RalA after PI4KB inhibition. Our data suggest a more complicated role of PBD, beyond simple electrostatic interactions in the PM anchoring. For this purpose, we aim to identify whether this PBD-mediated interaction is based on the positively charged amino acids within the PBD for electrostatic interactions, or the cryptic primary sequence of the PBD matters. We will address this question by introducing mutations in hexa-Lys PBD of K-Ras and study the PM mislocalization of K-Ras in PI4KB-inhibited cell. We will replace the Lys in K-Ras PBD with either Arg (the other positively charged amino acid) or Ala (uncharged amino acid), and study cellular localization of K-Ras by confocal and electron microscopy.

Moreover, aside from lipid species, we cannot rule out the possibility of contributions of proteins to the small GTPases localization to membranes, where proteins can recruit, directly bind or affect the ability or affinity of small GTPases interactions with membrane components. Furthermore, seeking for any protein-mediating interactions perhaps may provide rationales for the mitochondrial translocation preferences by K-Ras and RalA observed in PI4KB-inhibited cells, while PS was redistributed to other endomembranes in addition to mitochondria. In this regard, to

identify protein or lipid targets interacting with small GTPases at the PM and/or mitochondrial membrane, we can benefit from mass spectrometry techniques, after purification of membrane components isolated by subcellular fractionation from PI4KB inhibited and control cells.

Based on our observation for K-Ras and RalA mitochondrial translocation, another future research area is to consider mitochondrial dynamic changes in PI4KB inhibited cells. Our previous study indicted a higher glucose consumption and inhibited glycolysis while ATP synthesis remained unchanged (Miller et al., 2019). Therefore, it would be beneficial to investigate other parameters reflecting dysfunctionality of mitochondria such as production levels of reactive oxygen species (ROS), or mitochondrial fusion and fission.

V. References

Abankwa, D., & Gorfe, A. A. (2020). Mechanisms of Ras Membrane Organization and Signaling: Ras Rocks Again. *Biomolecules, 10*(11). doi:10.3390/biom10111522

Abankwa, D., Gorfe, A. A., & Hancock, J. F. (2008). Mechanisms of Ras membrane organization and signalling: Ras on a rocker. *Cell Cycle, 7*(17), 2667-2673. doi:10.4161/cc.7.17.6596

Abdelkarim, H., Banerjee, A., Grudzien, P., Leschinsky, N., Abushaer, M., & Gaponenko, V. (2019). The Hypervariable Region of K-Ras4B Governs Molecular Recognition and Function. *Int J Mol Sci, 20*(22). doi:10.3390/ijms20225718

Adhikari, H., Kattan, W. E., Kumar, S., Zhou, P., Hancock, J. F., & Counter, C. M. (2021). Oncogenic KRAS is dependent upon an EFR3A-PI4KA signaling axis for potent tumorigenic activity. *Nat Commun, 12*(1), 5248. doi:10.1038/s41467-021-25523-5

Agliarulo, I., & Parashuraman, S. (2022). Golgi Apparatus Regulates Plasma Membrane Composition and Function. *Cells, 11*(3). doi:10.3390/cells11030368

Apolloni, A., Prior, I. A., Lindsay, M., Parton, R. G., & Hancock, J. F. (2000). H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. *Mol Cell Biol, 20*(7), 2475-2487. doi:10.1128/MCB.20.7.2475-2487.2000

Arrazola Sastre, A., Luque Montoro, M., Galvez-Martin, P., Lacerda, H. M., Lucia, A. M., Llavero, F., & Zugaza, J. L. (2020). Small GTPases of the Ras and Rho Families Switch on/off Signaling Pathways in Neurodegenerative Diseases. *Int J Mol Sci, 21*(17). doi:10.3390/ijms21176312

Balla, T. (2020). Rushing to maintain plasma membrane phosphoinositide levels. *J Gen Physiol, 152*(12). doi:10.1085/jgp.202012793

Barcelo, C., Paco, N., Beckett, A. J., Alvarez-Moya, B., Garrido, E., Gelabert, M., . . . Agell, N. (2013). Oncogenic K-ras segregates at spatially distinct plasma membrane signaling platforms according to its phosphorylation status. *J Cell Sci, 126*(Pt 20), 4553-4559. doi:10.1242/jcs.123737

Bhattacharjee, A., & Gurung, A. B. (2015). Significance of Ras Signaling in Cancer and Strategies for its Control. *Oncology & Hematology Review (US), 11*(02). doi:10.17925/ohr.2015.11.02.147

Boura, E., & Nencka, R. (2015). Phosphatidylinositol 4-kinases: Function, structure, and inhibition. *Exp Cell Res, 337*(2), 136-145. doi:10.1016/j.yexcr.2015.03.028

Canon, J., Rex, K., Saiki, A. Y., Mohr, C., Cooke, K., Bagal, D., . . . Lipford, J. R. (2019). The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity. *Nature, 575*(7781), 217-223. doi:10.1038/s41586-019-1694-1

Casalou, C., Faustino, A., & Barral, D. C. (2016). Arf proteins in cancer cell migration. *Small GTPases, 7*(4), 270-282. doi:10.1080/21541248.2016.1228792

Castellano, E., & Downward, J. (2011). RAS Interaction with PI3K: More Than Just Another Effector Pathway. *Genes Cancer, 2*(3), 261-274. doi:10.1177/1947601911408079

Chardin, P., & Tavitian, A. (1986). The ral gene: a new ras related gene isolated by the use of a synthetic probe. *EMBO J, 5*(9), 2203-2208. doi:10.1002/j.1460-2075.1986.tb04485.x

Chavan, T. S., Muratcioglu, S., Marszalek, R., Jang, H., Keskin, O., Gursoy, A., . . . Gaponenko, V. (2015). Plasma membrane regulates Ras signaling networks. *Cell Logist, 5*(4), e1136374. doi:10.1080/21592799.2015.1136374

Chen, K., Zhang, Y., Qian, L., & Wang, P. (2021). Emerging strategies to target RAS signaling in human cancer therapy. *J Hematol Oncol, 14*(1), 116. doi:10.1186/s13045-021-01127-w

Chen, K. J., Chiang, T. C., Yu, C. J., & Lee, F. S. (2020). Cooperative recruitment of Arl4A and Pak1 to the plasma membrane contributes to sustained Pak1 activation for cell migration. *J Cell Sci, 133*(3). doi:10.1242/jcs.233361

Cherfils, J., & Zeghouf, M. (2013). Regulation of small GTPases by GEFs, GAPs, and GDIs. *Physiol Rev, 93*(1), 269-309. doi:10.1152/physrev.00003.2012

Cho, K. J., van der Hoeven, D., Zhou, Y., Maekawa, M., Ma, X., Chen, W., . . . Hancock, J. F. (2016). Inhibition of Acid Sphingomyelinase Depletes Cellular Phosphatidylserine and Mislocalizes K-Ras from the Plasma Membrane. *Mol Cell Biol, 36*(2), 363-374. doi:10.1128/MCB.00719-15

Chung, J., Torta, F., Masai, K., Lucast, L., Czapla, H., Tanner, L. B., . . . De Camilli, P. (2015). INTRACELLULAR TRANSPORT. PI4P/phosphatidylserine countertransport at ORP5- and ORP8-mediated ER-plasma membrane contacts. *Science, 349*(6246), 428-432. doi:10.1126/science.aab1370

Cox, A. D., & Der, C. J. (2010). Ras history: The saga continues. *Small GTPases, 1*(1), 2-27. doi:10.4161/sgtp.1.1.12178

Cox, A. D., Der, C. J., & Philips, M. R. (2015). Targeting RAS Membrane Association: Back to the Future for Anti-RAS Drug Discovery? *Clin Cancer Res, 21*(8), 1819-1827. doi:10.1158/1078-0432.CCR-14-3214

D'Angelo, G., Vicinanza, M., Di Campli, A., & De Matteis, M. A. (2008). The multiple roles of PtdIns(4)P -- not just the precursor of PtdIns(4,5)P2. *J Cell Sci, 121*(Pt 12), 1955-1963. doi:10.1242/jcs.023630

Das, S., Yin, T., Yang, Q., Zhang, J., Wu, Y. I., & Yu, J. (2015). Single-molecule tracking of small GTPase Rac1 uncovers spatial regulation of membrane translocation and mechanism for polarized signaling. *Proc Natl Acad Sci U S A, 112*(3), E267-276. doi:10.1073/pnas.1409667112

David, Y., Castro, I. G., & Schuldiner, M. (2021). The Fast and the Furious: Golgi Contact Sites. *Contact (Thousand Oaks), 4*, 1-15. doi:10.1177/25152564211034424

De Matteis, M. A., Di Campli, A., & Godi, A. (2005). The role of the phosphoinositides at the Golgi complex. *Biochim Biophys Acta, 1744*(3), 396-405. doi:10.1016/j.bbamcr.2005.04.013

De, P., Rozeboom, B. J., Aske, J. C., & Dey, N. (2020). Active RAC1 Promotes Tumorigenic Phenotypes and Therapy Resistance in Solid Tumors. *Cancers (Basel), 12*(6). doi:10.3390/cancers12061541

De Tito, S., Hervas, J. H., van Vliet, A. R., & Tooze, S. A. (2020). The Golgi as an Assembly Line to the Autophagosome. *Trends Biochem Sci, 45*(6), 484-496. doi:10.1016/j.tibs.2020.03.010

Dickson, E. J., Jensen, J. B., & Hille, B. (2014). Golgi and plasma membrane pools of PI(4)P contribute to plasma membrane PI(4,5)P2 and maintenance of KCNQ2/3 ion channel current. *Proc Natl Acad Sci U S A, 111*(22), E2281-2290. doi:10.1073/pnas.1407133111

Donaldson, J. G., & Jackson, C. L. (2011). ARF family G proteins and their regulators: roles in membrane transport, development and disease. *Nat Rev Mol Cell Biol, 12*(6), 362-375. doi:10.1038/nrm3117

Dunn, K. W., Kamocka, M. M., & McDonald, J. H. (2011). A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol, 300*(4), C723-742. doi:10.1152/ajpcell.00462.2010

Eser, S., Schnieke, A., Schneider, G., & Saur, D. (2014). Oncogenic KRAS signalling in pancreatic cancer. *Br J Cancer, 111*(5), 817-822. doi:10.1038/bjc.2014.215

Galmes, R., Houcine, A., van Vliet, A. R., Agostinis, P., Jackson, C. L., & Giordano, F. (2016). ORP5/ORP8 localize to endoplasmic reticulum-mitochondria contacts and are involved in mitochondrial function. *EMBO Rep, 17*(6), 800-810. doi:10.15252/embr.201541108

Gentry, L. R., Nishimura, A., Cox, A. D., Martin, T. D., Tsygankov, D., Nishida, M., . . . Der, C. J. (2015). Divergent roles of CAAX motif-signaled posttranslational modifications in the regulation and subcellular localization of Ral GTPases. *J Biol Chem, 290*(37), 22851-22861. doi:10.1074/jbc.M115.656710

Gerwert, K., Mann, D., & Kotting, C. (2017). Common mechanisms of catalysis in small and heterotrimeric GTPases and their respective GAPs. *Biol Chem, 398*(5-6), 523-533. doi:10.1515/hsz-2016-0314

Goswami, D., Chen, Yang, Y., Gudla, P. R., Columbus, J., Worthy, K., . . . Turbyville, T. (2020). Membrane interactions of the globular domain and the hypervariable region of KRAS4b define its unique diffusion behavior. *Elife, 9*. doi:10.7554/eLife.47654

Graham, T. R., & Burd, C. G. (2011). Coordination of Golgi functions by phosphatidylinositol 4-kinases. *Trends Cell Biol, 21*(2), 113-121. doi:10.1016/j.tcb.2010.10.002

Gray, J. L., von Delft, F., & Brennan, P. E. (2020). Targeting the Small GTPase Superfamily through Their Regulatory Proteins. *Angew Chem Int Ed Engl, 59*(16), 6342-6366. doi:10.1002/anie.201900585

Guin, S., & Theodorescu, D. (2015). The RAS-RAL axis in cancer: evidence for mutation-specific selectivity in non-small cell lung cancer. *Acta Pharmacol Sin, 36*(3), 291-297. doi:10.1038/aps.2014.129

Hancock, J. F. (2003). Ras proteins: different signals from different locations. *Nat Rev Mol Cell Biol, 4*(5), 373-384. doi:10.1038/nrm1105

Harada, A., Matsumoto, S., Yasumizu, Y., Shojima, K., Akama, T., Eguchi, H., & Kikuchi, A. (2021). Localization of KRAS downstream target ARL4C to invasive pseudopods accelerates pancreatic cancer cell invasion. *Elife, 10*. doi:10.7554/eLife.66721

Hayes, T. K., Neel, N. F., Hu, C., Gautam, P., Chenard, M., Long, B., . . . Der, C. J. (2016). Long-Term ERK Inhibition in KRAS-Mutant Pancreatic Cancer Is Associated with MYC Degradation and Senescence-like Growth Suppression. *Cancer Cell, 29*(1), 75-89. doi:10.1016/j.ccell.2015.11.011

Henkels, K. M., Rehl, K. M., & Cho, K. J. (2021). Blocking K-Ras Interaction With the Plasma Membrane Is a Tractable Therapeutic Approach to Inhibit Oncogenic K-Ras Activity. *Front Mol Biosci, 8*, 673096. doi:10.3389/fmolb.2021.673096

Heo, W. D., Inoue, T., Park, W. S., Kim, M. L., Park, B. O., Wandless, T. J., & Meyer, T. (2006). PI(3,4,5)P3 and PI(4,5)P2 lipids target proteins with polybasic clusters to the plasma membrane. *Science, 314*(5804), 1458-1461. doi:10.1126/science.1134389

Hobbs, G. A., Der, C. J., & Rossman, K. L. (2016). RAS isoforms and mutations in cancer at a glance. *J Cell Sci, 129*(7), 1287-1292. doi:10.1242/jcs.182873

Hofmann, I., Thompson, A., Sanderson, C. M., & Munro, S. (2007). The Arl4 family of small G proteins can recruit the cytohesin Arf6 exchange factors to the plasma membrane. *Curr Biol, 17*(8), 711-716. doi:10.1016/j.cub.2007.03.007

Jacobs, S., Schilf, C., Fliegert, F., Koling, S., Weber, Y., Schurmann, A., & Joost, H. G. (1999). ADP-ribosylation factor (ARF)-like 4, 6, and 7 represent a subgroup of the ARF family characterization by rapid nucleotide exchange and a nuclear localization signal. *FEBS Lett, 456*(3), 384-388. doi:10.1016/s0014-5793(99)00759-0

Jou, T. S., & Nelson, W. J. (1998). Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. *J Cell Biol, 142*(1), 85-100. doi:10.1083/jcb.142.1.85

Kattan, W. E., Chen, W., Ma, X., Lan, T. H., van der Hoeven, D., van der Hoeven, R., & Hancock, J. F. (2019). Targeting plasma membrane phosphatidylserine content to inhibit oncogenic KRAS function. *Life Sci Alliance, 2*(5). doi:10.26508/lsa.201900431

Kay, J. G., & Fairn, G. D. (2019). Distribution, dynamics and functional roles of phosphatidylserine within the cell. *Cell Commun Signal, 17*(1), 126. doi:10.1186/s12964-019-0438-z

Lenoir, G., D'Ambrosio, J. M., Dieudonne, T., & Copic, A. (2021). Transport Pathways That Contribute to the Cellular Distribution of Phosphatidylserine. *Front Cell Dev Biol, 9*, 737907. doi:10.3389/fcell.2021.737907

Leventis, P. A., & Grinstein, S. (2010). The distribution and function of phosphatidylserine in cellular membranes. *Annu Rev Biophys, 39*, 407-427. doi:10.1146/annurev.biophys.093008.131234

Lim, J. K. M., & Leprivier, G. (2019). The impact of oncogenic RAS on redox balance and implications for cancer development. *Cell Death Dis, 10*(12), 955. doi:10.1038/s41419-019-2192-y

Lim, K. H., Baines, A. T., Fiordalisi, J. J., Shipitsin, M., Feig, L. A., Cox, A. D., . . . Counter, C. M. (2005). Activation of RalA is critical for Ras-induced tumorigenesis of human cells. *Cancer Cell, 7*(6), 533-545. doi:10.1016/j.ccr.2005.04.030

Lin, Y. C., Chiang, T. C., Liu, Y. T., Tsai, Y. T., Jang, L. T., & Lee, F. J. (2011). ARL4A acts with GCC185 to modulate Golgi complex organization. *J Cell Sci, 124*(Pt 23), 4014-4026. doi:10.1242/jcs.086892

Magee, T., & Marshall, C. (1999). New Insights into the Interaction of Ras with the Plasma Membrane. *Cell, 98*(1), 9-12. doi:10.1016/s0092-8674(00)80601-7

MANDERS, E. M. M., VERBEEK, F. J., & ATEN, J. A. (1993). Measurement of co-localization of objects in dual-colour confocal images. *169*(3), 375-382. doi:https://doi.org/10.1111/j.1365-2818.1993.tb03313.x

Marei, H., & Malliri, A. (2017). Rac1 in human diseases: The therapeutic potential of targeting Rac1 signaling regulatory mechanisms. *Small GTPases, 8*(3), 139-163. doi:10.1080/21541248.2016.1211398

Martin, T. D., & Der, C. J. (2012). Differential involvement of RalA and RalB in colorectal cancer. *Small GTPases, 3*(2), 126-130. doi:10.4161/sgtp.19571

Matos, P. (2021). Small GTPases in Cancer: Still Signaling the Way. *Cancers (Basel), 13*(7). doi:10.3390/cancers13071500

Matsubara, K., Kishida, S., Matsuura, Y., Kitayama, H., Noda, M., & Kikuchi, A. (1999). Plasma membrane recruitment of RalGDS is critical for Ras-dependent Ral activation. *Oncogene, 18*(6), 1303-1312. doi:10.1038/sj.onc.1202425

Maxwell, K. N., Zhou, Y., & Hancock, J. F. (2018). Clustering of Rac1: Selective Lipid Sorting Drives Signaling. *Trends Biochem Sci, 43*(2), 75-77. doi:10.1016/j.tibs.2017.11.007

Michaelson, D., Abidi, W., Guardavaccaro, D., Zhou, M., Ahearn, I., Pagano, M., & Philips, M. R. (2008). Rac1 accumulates in the nucleus during the G2 phase of the cell cycle and promotes cell division. *J Cell Biol, 181*(3), 485-496. doi:10.1083/jcb.200801047

Miller, T. E., Henkels, K. M., Huddleston, M., Salisbury, R., Hussain, S. M., Sasaki, A. T., & Cho, K. J. (2019). Depletion of phosphatidylinositol 4-phosphate at the Golgi translocates K-Ras to mitochondria. *J Cell Sci, 132*(16). doi:10.1242/jcs.231886

Moghadam, A. R., Patrad, E., Tafsiri, E., Peng, W., Fangman, B., Pluard, T. J., . . . Farassati, F. (2017). Ral signaling pathway in health and cancer. *Cancer Med, 6*(12), 2998-3013. doi:10.1002/cam4.1105

Moser von Filseck, J., Vanni, S., Mesmin, B., Antonny, B., & Drin, G. (2015). A phosphatidylinositol-4-phosphate powered exchange mechanism to create a lipid gradient between membranes. *Nat Commun, 6*, 6671. doi:10.1038/ncomms7671

Munoz-Maldonado, C., Zimmer, Y., & Medova, M. (2019). A Comparative Analysis of Individual RAS Mutations in Cancer Biology. *Front Oncol, 9*, 1088. doi:10.3389/fonc.2019.01088

Navarro-Lerida, I., Pellinen, T., Sanchez, S. A., Guadamillas, M. C., Wang, Y., Mirtti, T., . . . Del Pozo, M. A. (2015). Rac1 nucleocytoplasmic shuttling drives nuclear shape changes and tumor invasion. *Dev Cell, 32*(3), 318-334. doi:10.1016/j.devcel.2014.12.019

Navarro-Lerida, I., Sanchez-Perales, S., Calvo, M., Rentero, C., Zheng, Y., Enrich, C., & Del Pozo, M. A. (2012). A palmitoylation switch mechanism regulates Rac1 function and membrane organization. *EMBO J, 31*(3), 534-551. doi:10.1038/emboj.2011.446

Neel, N. F., Martin, T. D., Stratford, J. K., Zand, T. P., Reiner, D. J., & Der, C. J. (2011). The RalGEF-Ral Effector Signaling Network: The Road Less Traveled for Anti-Ras Drug Discovery. *Genes Cancer, 2*(3), 275-287. doi:10.1177/1947601911407329

Neely, A., & Hidalgo, P. (2014). Structure-function of proteins interacting with the alpha1 pore-forming subunit of high-voltage-activated calcium channels. *Front Physiol, 5*, 209. doi:10.3389/fphys.2014.00209

Ostrem, J. M., Peters, U., Sos, M. L., Wells, J. A., & Shokat, K. M. (2013). K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature, 503*(7477), 548-551. doi:10.1038/nature12796

Parker, J. A., & Mattos, C. (2015). The Ras-Membrane Interface: Isoform-specific Differences in The Catalytic Domain. *Mol Cancer Res, 13*(4), 595-603. doi:10.1158/1541-7786.MCR-14-0535

Pasqualato, S., Renault, L., & Cherfils, J. (2002). Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for 'front-back' communication. *EMBO Rep, 3*(11), 1035-1041. doi:10.1093/embo-reports/kvf221

Phuyal, S., & Farhan, H. (2019). Multifaceted Rho GTPase Signaling at the Endomembranes. *Front Cell Dev Biol, 7*, 127. doi:10.3389/fcell.2019.00127

Pollock, S. R., Schinlever, A. R., Rohani, A., Kashatus, J. A., & Kashatus, D. F. (2019). RalA and RalB relocalization to depolarized mitochondria depends on clathrin-mediated endocytosis and facilitates TBK1 activation. *PLoS One, 14*(4), e0214764. doi:10.1371/journal.pone.0214764

Prior, I. A., & Hancock, J. F. (2001). Compartmentalization of Ras proteins. *J Cell Sci, 114*(Pt 9), 1603-1608. doi:10.1242/jcs.114.9.1603

Prior, I. A., & Hancock, J. F. (2012). Ras trafficking, localization and compartmentalized signalling. *Semin Cell Dev Biol, 23*(2), 145-153. doi:10.1016/j.semcdb.2011.09.002

Prior, I. A., Hood, F. E., & Hartley, J. L. (2020). The Frequency of Ras Mutations in Cancer. *Cancer Res, 80*(14), 2969-2974. doi:10.1158/0008-5472.CAN-19-3682

Qu, L., Pan, C., He, S. M., Lang, B., Gao, G. D., Wang, X. L., & Wang, Y. (2019). The Ras Superfamily of Small GTPases in Non-neoplastic Cerebral Diseases. *Front Mol Neurosci, 12*, 121. doi:10.3389/fnmol.2019.00121

Rahajeng, J., Kuna, R. S., Makowski, S. L., Tran, T. T. T., Buschman, M. D., Li, S., . . . Field, S. J. (2019). Efficient Golgi Forward Trafficking Requires GOLPH3-Driven, PI4P-Dependent Membrane Curvature. *Dev Cell, 50*(5), 573-585 e575. doi:10.1016/j.devcel.2019.05.038

Reiner, D. J., & Lundquist, E. A. (2018). Small GTPases. *WormBook, 2018*, 1-65. doi:10.1895/wormbook.1.67.2

Remorino, A., De Beco, S., Cayrac, F., Di Federico, F., Cornilleau, G., Gautreau, A., . . . Coppey, M. (2017). Gradients of Rac1 Nanoclusters Support Spatial Patterns of Rac1 Signaling. *Cell Rep, 21*(7), 1922-1935. doi:10.1016/j.celrep.2017.10.069

Rojas, A. M., Fuentes, G., Rausell, A., & Valencia, A. (2012). The Ras protein superfamily: evolutionary tree and role of conserved amino acids. *J Cell Biol, 196*(2), 189-201. doi:10.1083/jcb.201103008

Schmick, M., Vartak, N., Papke, B., Kovacevic, M., Truxius, D. C., Rossmannek, L., & Bastiaens, P. I. H. (2014). KRas localizes to the plasma membrane by spatial cycles of solubilization, trapping and vesicular transport. *Cell, 157*(2), 459-471. doi:10.1016/j.cell.2014.02.051

Shipitsin, M., & Feig, L. A. (2004). RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells. *Mol Cell Biol, 24*(13), 5746-5756. doi:10.1128/MCB.24.13.5746-5756.2004

Spencer-Smith, R., & O'Bryan, J. P. (2019). Direct inhibition of RAS: Quest for the Holy Grail? *Semin Cancer Biol, 54*, 138-148. doi:10.1016/j.semcancer.2017.12.005

Spiegel, J., Cromm, P. M., Zimmermann, G., Grossmann, T. N., & Waldmann, H. (2014). Small-molecule modulation of Ras signaling. *Nat Chem Biol, 10*(8), 613-622. doi:10.1038/nchembio.1560

Sztul, E., Chen, P. W., Casanova, J. E., Cherfils, J., Dacks, J. B., Lambright, D. G., . . . Kahn, R. A. (2019). ARF GTPases and their GEFs and GAPs: concepts and challenges. *Mol Biol Cell, 30*(11), 1249-1271. doi:10.1091/mbc.E18-12-0820

Takai, Y., Sasaki, T., & Matozaki, T. (2001). Small GTP-binding proteins. *Physiol Rev, 81*(1), 153-208. doi:10.1152/physrev.2001.81.1.153

Tan, X., Banerjee, P., Pham, E. A., Rutaganira, F. U. N., Basu, K., Bota-Rabassedas, N., . . . Kurie, J. M. (2020). PI4KIIIbeta is a therapeutic target in chromosome 1q-amplified lung adenocarcinoma. *Sci Transl Med, 12*(527). doi:10.1126/scitranslmed.aax3772

Tokuda, E., Itoh, T., Hasegawa, J., Ijuin, T., Takeuchi, Y., Irino, Y., . . . Takenawa, T. (2014). Phosphatidylinositol 4-phosphate in the Golgi apparatus regulates cell-cell adhesion and invasive cell migration in human breast cancer. *Cancer Res, 74*(11), 3054-3066. doi:10.1158/0008-5472.CAN-13-2441

Toma-Fukai, S., & Shimizu, T. (2019). Structural Insights into the Regulation Mechanism of Small GTPases by GEFs. *Molecules, 24*(18). doi:10.3390/molecules24183308

van der Hoeven, D., Cho, K. J., Ma, X., Chigurupati, S., Parton, R. G., & Hancock, J. F. (2013). Fendiline inhibits K-Ras plasma membrane localization and blocks K-Ras signal transmission. *Mol Cell Biol, 33*(2), 237-251. doi:10.1128/MCB.00884-12

Venditti, R., Masone, M. C., Wilson, C., & De Matteis, M. A. (2016). PI(4)P homeostasis: Who controls the controllers? *Adv Biol Regul, 60*, 105-114. doi:10.1016/j.jbior.2015.09.007

Waugh, M. G. (2014). Amplification of Chromosome 1q Genes Encoding the Phosphoinositide Signalling Enzymes PI4KB, AKT3, PIP5K1A and PI3KC2B in Breast Cancer. *J Cancer, 5*(9), 790-796. doi:10.7150/jca.9794

Waugh, M. G. (2019). The Great Escape: how phosphatidylinositol 4-kinases and PI4P promote vesicle exit from the Golgi (and drive cancer). *Biochemical Journal, 476*(16), 2321-2346. doi:10.1042/BCJ20180622 %J Biochemical Journal

Yan, C., Jones, D. N., & Theodorescu, D. (2015). Drugging the Ral GTPase. *Small GTPases, 6*(3), 157-159. doi:10.1080/21541248.2015.1018403

Yan, C., & Theodorescu, D. (2018). RAL GTPases: Biology and Potential as Therapeutic Targets in Cancer. *Pharmacol Rev, 70*(1), 1-11. doi:10.1124/pr.117.014415

Yang, Y., Lee, M., & Fairn, G. D. (2018). Phospholipid subcellular localization and dynamics. *J Biol Chem, 293*(17), 6230-6240. doi:10.1074/jbc.R117.000582

Zago, G., Biondini, M., Camonis, J., & Parrini, M. C. (2019). A family affair: A Ral-exocyst-centered network links Ras, Rac, Rho signaling to control cell migration. *Small GTPases, 10*(5), 323-330. doi:10.1080/21541248.2017.1310649

Zhang, J., Zhang, Q., Sun, C., Huang, Y., Zhang, J., & Wang, Q. (2020). Clinical relevance of ARF/ARL family genes and oncogenic function of ARL4C in endometrial cancer. *Biomed Pharmacother, 125*, 110000. doi:10.1016/j.biopha.2020.110000

Zhou, Y., Gorfe, A. A., & Hancock, J. F. (2021). RAS Nanoclusters Selectively Sort Distinct Lipid Headgroups and Acyl Chains. *Front Mol Biosci, 8*, 686338. doi:10.3389/fmolb.2021.686338

Zhou, Y., & Hancock, J. F. (2015). Ras nanoclusters: Versatile lipid-based signaling platforms. *Biochim Biophys Acta, 1853*(4), 841-849. doi:10.1016/j.bbamcr.2014.09.008

Zhou, Y., & Hancock, J. F. (2018). Deciphering lipid codes: K-Ras as a paradigm. *Traffic, 19*(3), 157-165. doi:10.1111/tra.12541

Zhou, Y., & Hancock, J. F. (2020). A novel prenyl-polybasic domain code determines lipid-binding specificity of the K-Ras membrane anchor. *Small GTPases, 11*(3), 220-224. doi:10.1080/21541248.2017.1379583

Zhou, Y., Liang, H., Rodkey, T., Ariotti, N., Parton, R. G., & Hancock, J. F. (2014). Signal integration by lipid-mediated spatial cross talk between Ras nanoclusters. *Mol Cell Biol, 34*(5), 862-876. doi:10.1128/MCB.01227-13

Zhou, Y., Prakash, P., Liang, H., Cho, K. J., Gorfe, A. A., & Hancock, J. F. (2017). Lipid-Sorting Specificity Encoded in K-Ras Membrane Anchor Regulates Signal Output. *Cell, 168*(1-2), 239-251 e216. doi:10.1016/j.cell.2016.11.059

Zhou, Y., Prakash, P. S., Liang, H., Gorfe, A. A., & Hancock, J. F. (2021). The KRAS and other prenylated polybasic domain membrane anchors recognize phosphatidylserine acyl chain structure. *Proc Natl Acad Sci U S A, 118*(6). doi:10.1073/pnas.2014605118

Zhou, Y., Wong, C. O., Cho, K. J., van der Hoeven, D., Liang, H., Thakur, D. P., . . . Hancock, J. F. (2015). SIGNAL TRANSDUCTION. Membrane potential modulates plasma membrane phospholipid dynamics and K-Ras signaling. *Science, 349*(6250), 873-876. doi:10.1126/science.aaa5619