DISRUPTION OF THE TEMPORAL ACTIVATION OF RAS EGFR-MAPK AXIS BY TARGETING A CRITICAL MEMBRANE PHOSPHOLIPID SECOND MESSENGER

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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The Ras-EGFR-MAPK signaling axis is chronically up-regulated in colon cancer, and pharmacological inhibition of signaling through Ras and/or EGFR has been shown to prevent colon tumor formation. Recent evidence suggests that production of the signaling lipid phosphatidic acid (PA) is essential for efficient Ras-EGFR-MAPK signaling. With respect to cancer prevention, there is a growing body of experimental, epidemiological and preclinical evidence indicating that fish oil-containing diets rich in n-3 polyunsaturated fatty acids (PUFA), e.g., DHA and its metabolic precursor EPA, are protective against colon tumorigenesis. We have demonstrated that DHA attenuates signaling of the Ras-EGFR-MAPK axis, however, the mechanism underlying this effect is not known. Therefore, we determined if the attenuation of Ras-EGFR signaling output by DHA is mediated via reduction of PA production. For this purpose, the young adult mouse colonocyte (YAMC) cell model expressing a FRET biosensor capable of monitoring cellular levels of K-Ras activation was utilized in conjunction with fluorescence microscopy techniques to monitor temporal changes of activated K-Ras levels following growth factor stimulation. Elucidating K-Ras as a molecular target of dietary bioactives is noteworthy because establishing a causal role of n-3 PUFA in colon cancer prevention would have a major translational impact due to their safety and tolerance.

CHAPTER I

INTRODUCTION

Many colorectal cancers contain KRas mutations, which confer resistance to standard therapy and have therefore been termed "undruggable"[1,2]. Since no curative treatments for KRas driven colon cancer are available, there is a critical need to develop toxicologically innocuous KRas therapeutic approaches that are free of safety problems intrinsic to drugs administered over long periods of time. One strategy for targeting oncogenic KRas driven cells, which has gained momentum recently, is to attenuate KRas activation[3,4].

There is a growing body of experimental, epidemiological and preclinical evidence indicating that fish oil-containing diets rich in n-3 PUFA, e.g., docosahexaenoic acid (DHA) and its metabolic precursor eicosapentaenoic acid (EPA), are protective against colon tumorigenesis[5,6]. Establishing a causal role of n-3 PUFA in colon cancer prevention would have a major translational impact because these dietary nutrients are safe and well tolerated[7]. For example, human doses as high as 17.6 gram per day are free of toxicity[8].

Research in the Chapkin lab has determined that DHA reduces epidermal growth factor (EGF) stimulated Ras activation *in vitro* and *in vivo*[9,10]. However, the mechanism underlying this attenuation has not been fully resolved. The epidermal growth factor receptor (EGFR) is primarily localized to the plasma membrane[11]. Upon EGF stimulation, this receptor induces a signaling pathway that recruits certain proteins to the plasma membrane including the enzymes

Phospholipase D (PLD2) and Diacylglycerol Kinase (DGK). These two enzymes generate phosphatidic acid (PA)[12].

Following EGFR activation, the guanine exchange factor, SOS1, is subsequently recruited to the plasma membrane via the production of phosphatidic acid (PA), where it activates Ras[13]. This triggers a chain reaction of signaling events that ultimately lead to cell survival[14]. Interestingly, EGFR signaling is up-regulated in many cancer cells, and the PA-generating enzymes, PLD and DGK are considered critical for proliferation, migration, and survival of cancer cells[12].

DHA is known to reduce the association of Ras with its guanine exchange factor, SOS1[15]. DHA has also been shown to reduce levels of the signaling lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2)[16]. Alterations to the spatiotemporal production of the signaling lipid PA is known to affect Ras signaling[12]. Therefore, we proposed that DHA attenuates Ras activation by disrupting the spatiotemporal production of the critical signaling lipid, PA.

CHAPTER II

METHODS

Cell culture

Young Adult Mouse Colonocytes (YAMC cells)

Young adult mouse colonocytes (YAMC) cells, conditionally immortalized colonocytes, were originally obtained from R.H. Whitehead, Ludwig Cancer Institute (Melbourne, Australia). YAMC cells (passages 12-17) were cultured under permissive conditions, 33° C and 5% CO_2 in RPMI 1640 media (Mediatech, Manassas, VA) supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM GlutaMax (Gibco, Grand Island, NY), 5 μg/mL insulin, 5 μg/ml transferrin, 5 ng/ml selenious acid (Collaborative Bomedical Products, Bedford, MA), and 5 IU/mL of murine interferon-γ (Roche, Mannheim, Germany). In select cultures, for the final 16-18 h, complete media was replaced with low-serum (0.5% FBS) media. Cells were then stimulated with 0-25 ng/mL recombinant mouse epidermal growth factor EGF (Sigma, St. Louis, MO).

Sw48 K-Ras G12D Cells (human cancer cells)

The Sw-48 isogenic cell lines (human cancer cells) are the recently developed cell models to investigate endogenous isoform-specific Ras signaling in an identical genetic background. The model has been generated using adeno-associated virus (AAV) somatic homologous recombination-based technology to directly modify endogenous Ras genes via introduction of activating mutations [17]. A panel of isogenic Sw48 cell lines expressing oncogenically mutated K-Ras variants (G12V, G12D, G13D) from the endogenous K-Ras locus has been developed and

used extensively. These K-Ras variants are chosen because one third of colorectal tumors harbor K-Ras mutations [18]. Sw48 K-Ras G12D cells (passages 12-17) were cultured under permissive conditions, 37° C and 5% CO_2 in RPMI 1640 media (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM GlutaMax (Gibco, Grand Island, NY), 5 μg/mL insulin, 5 μg/ml transferrin, and 5 ng/ml selenious acid (Collaborative Bomedical Products, Bedford, MA). In select cultures, for the final 16-18 h, complete media was replaced with low-serum (0.5% FBS) media. Cells were then stimulated with 0-25 ng/mL epidermal growth factor EGF (Sigma, St. Louis, MO).

Transfection

Spo-20 GFP PA Probe

YAMC cells and Sw48 K-Ras G12D cells were seeded and transfected with the a plasmid encoding for the Spo-20 GFP PA binding probe (Zhang, 2014). Transfection conditions were optimized to minimize the amount of DNA and lipofection reagent used to avoid nonspecific cytotoxicity. Prior to transfection, cells were seeded at a density of 25,000 cells/well into a Lab-Tek (# 154461) two chamber slide of 4.0 cm^2 /well. YAMC cells and Sw48 K-Ras G12D were transfected using 4 μL Lipofectamine 3000, 5μL P3000 reagent, 250 μL Opti-MEM Medium, and 2 μg DNA/well (Lipofectamin 3000 Reagent Protocol, Invitrogen, Life Technologies). Approximately 32 h after transfection, cells were incubated in low serum media (0.5% FBS) and then imaged 48 h after transfection.

Use of the Spo GFP PA probe

The design of our study was based on previous working documenting the properties of the Spo GFP PA probe in EGF-stimulated HCC1806 breast cancer cells[12]. HCC1806 breast cancer cell imaging results were taken into consideration and compared with YAMC and Sw48 GFP cells transfected with the Spo20 GFP PA probe and stimulated with EGF.

K-Ras Raichu FRET Biosensor

The plasmid encoding for the K-Ras domain targeted KRas-Raichu (Raichu-124x) biosensor (Mochizuki, 2001) was utilized. Transfection conditions were optimized to minimize the amount of DNA and lipofection reagent used to avoid nonspecific cytotoxicity. Prior to transfection, cells were seeded at a density of 25,000 cells/well into MatTek (Ashland, MA) glass bottom 35mm dishes. Cells were transfected using 4 μL Lipofectamine 3000, 5μL P3000 reagent, 250 μL Opti-MEM Medium, and 2 μg DNA (Lipofectamin 3000 Reagent Protocol, Invitrogen, Life Technologies). Approximately 32 h after transfection, cells were incubated in low serum media (0.5% FBS). Cells were imaged 48 h after transfection.

PLD2 Inhibitor (FIPI) and Diacylglycerol Kinase Inhibitor R59949 (DGKi)

The phosphpolipase D inhibitor 5-fluoro-2-indolyl-deschlorohalopemide (FIPI), and Diacylglycerol Kinase inhibitor R59949 (DGKi) have been described in previous studies[12]. YAMC cells were treated with PLD2 inhibitor (FIPI, 1uM) and/or the diacylglycerol kinase inhibitor (DGKi, 20uM). Cells were pretreated with these inhibitors 1 h before imaging.

Imaging and Quantification

YAMC Cells – Spo-20 GFP PA Probe

Young Adult Mouse Colonocytes were transfected at a density of 25,000 cells/well with plasmid encoding for Spo-20 GFP PA probe (Zhang, 2014). Cells were then serum starved in Phenol free-RPMI (0.5%, FBS), 1% Glutamax, 1% Pen/Strep, with IFN-γ, no ITS, for 24 h before stimulation with EGF (25 ng/ml). Upon EGF stimulation, cells were fixed at 4% PFA for 10 min and then washed with PBS. Following this, YAMC cells were incubated with glycine (100 mM) for another 10 min and then washed again with PBS. Last, cells were mount on Prolong Gold mounting medium. The positive control cells were transfected and fixed YAMC cells with no EGF stimulation. Microscope images were taken at 40X magnification in select fixed cells.

Sw48 K-Ras G12D Cells – Spo-20 GFP PA Probe

Sw48 K-Ras G12D cells were transfected at a density of 25,000 cells/well with plasmid encoding for Spo-20 GFP PA probe (Zhang, 2014). Cells were then serum starved in Phenol free-RPMI (0.5%, FBS), 1% Glutamax, 1% Pen/Strep, with IFN-γ, no ITS, for 24 h before stimulation with EGF (25 ng/ml). Microscope images were taken at 40X magnification every 1 min over a 30 min time frame. EGF was directly added to the dish 10 min after the first image was acquired. Unlike YAMC cells, this human cancer cell line was *in vitro* and *in vivo.* Translocation of the Spo-20 GPF PA probe to the plasma membrane was analyzed according to the protocol of Feng, 2014 et al[12].

YAMC Cells – Ras-Raichu FRET Biosensor

YAMC cells were seeded at a density of 25,000 cells into MatTek (Ashland, MA) glass bottom 35mm dishes and transfected with plasmid encoding for the K-Ras domain targeted KRas-Raichu (Raichu-124x) biosensor (Mochizuki, 2001). Cells were then serum starved in Phenol free-RPMI (0.5%, FBS), 1% Glutamax, 1% Pen/Strep, with IFN-γ, no ITS, for 24 h before stimulation with EGF (25 ng/ml), and images were taken at 40X magnification every 2 min over a 26 min time frame. EGF was directly added to the dish 10 min after the first image was acquired. Images were processed with NIS Elements software and FRET ratios between 3 and 4.5 were determined as described previously (Aoki, 2009). Briefly, cells were imaged every two minutes, with a Nikon TE 300 Nikon Eclipse fluorescent microscope equipped with a programmable XY stage. The FRET image was acquired by detecting YFP signal during CFP excitation. The FRET image was then divided by the corresponding CFP image to generate FRET/CFP images used for visualization purposes. Quantification of Ras activation was determined by dividing the average FRET signal by the average CFP signal on a per cell basis.

CHAPTER III

RESULTS

Validation of the Spo-20 GFP PA binging probe

YAMC cells – Spo-20 GFP PA binging probe

Young adult mouse colonocytes transfected with the Spo-20 GFP PA probe and stimulated with EGF did not express significant changes compared to the non-EGF-stimulated cells (control). There was no clear translocation of the PA-binding probe to the plasma membrane, as shown in **Figure 2**. Prior scientific studies have described a rapid translocation of the PA-binding probe to the plasma membrane within 1 min of EGF stimulation, reaching maximal levels at 3 min (**Figure 2)**. However, in this study, this process was not observed over time.

Validation of the probe Spo-20 GFP PA was not successful in YAMC cells, and hence, measurement of PA levels in EGF-stimulated cells were not going to be feasible for this study. Some possible identified problems include the flattened shape of YAMC cells, a morphological factor that affects the ability to differentiate the cytosol from the plasma membrane; the microscope resolution might not have been high enough to distinguish cellular cytosol from the plasma membrane; the affinity of the Spo-20 GFP may be lacking; and human errors. An alternative approach will be to utilize a FRET based biosensor capable of determining PA levels (Nishioka, 2010).

Sw-48 K-Ras G12D cells - Spo-20 GFP PA binging probe

Sw-48 K-Ras G12D human colon cancer cells were selected as an alternative approach to validate the Spo-20 GFP probe primarily for their rounded shape (**Figure 1**), and malignant transformation phenotype. Similar to the YAMC cells results (described above), transfected human cancer cells did not exhibit PA-binding probe translocation to the plasma membrane after stimulation with EGF compared to non-EGF- stimulated cells (control), as shown in **Figure 2**. The expected immediate plasma membrane localization of the Spo-20 GFP PA probe within 1 min of EGF stimulation with maximal levels at 3 min was not observed (**Figure 2**). These findings lead us to conclude that validation of the probe Spo-20 GFP PA was not successful in Sw-48 K-Ras G12D. Similar to YAMC cells, there was no translocation of the PA-binding probe to the plasma membrane in the Sw48 K-Ras G12D cell model. Plausible problems related to the lack of translocation of the PA-binding probe include lack of research evidence of K-Ras activation reduction on Sw48 K-Ras G12D cells pre-treated with DHA and EGFR stimulated; mutation on the K-Ras domain of Sw48 K-Ras G12D cells, which suggests that the phospholipid second messenger, PA is not involved in the activation of K-Ras; low efficiency of the selected PA-binding probe, Spo-20 GFP PA; and human errors.

PLD2 and DGK Inhibitor Effects on Transfected YAMC Cells

For analysis purposes, all of the data obtained in the experiments were compared to the positive control, i.e., YAMC cells stimulated with EGF and no inhibitors. According to previous studies in the Chapkin Lab, YAMC cells stimulated with EGF generate a rapid peak FRET ratio after 10 min of imaging (experiment 2). Mr. Robert Fuentes, a graduate student working in the Chapkin Lab, observed a peak FRET ratio of over 1.4 increase (**Figure 3**). However, during this

experiment, YAMC cells with EGF and no inhibitors expressed a FRET ratio increase delay of 5 min (experiment 1). Errors must have occurred when adding EGF to non-treated YAMC cells under the microscope. Therefore, these data were excluded from the analysis. The high FRET ratio of the positive control seen in cells transfected with the K-Ras Raichu probe is represented by red fluorescence as shown in **Figure 4**.

As mentioned above, the Chapkin Lab has demonstrated that DHA attenuates signaling of the Ras-EGFR-MAPK axis in YAMC cells[9]. **Figure 5** indicates that DHA reduced the activation of K-Ras represented as demonstrated by a reduction in the FRET ratio levels in EGF stimulated cells. With regard to EGF stimulated cells (positive control), DHA suppression of K-Ras was assessed relative to YAMC cells treated with inhibitors (**Figure 5).** In comparison, YAMC cells pre-treated with FIPI (PLD inhibitor) followed by stimulation with EGF exhibited a significant ($P<0.005$) decrease in K-Ras activation as represented by the low FRET ratio expression after 10 min of imaging. From a quantitative perspective, the K-Ras Raichu probe fluorescence levels were reduced by approximately 40 % as shown in **Figure 6**.

YAMC cells pre-treated with DGKi and stimulated with EGF exhibited a blunted increase in the FRET ratio compared to EGF alone (positive control) treated cells. (**Figure 6**). These findings suggest that both PLD2 and DGK play a pivotal role in activating K-Ras in the presence of EGF. Even though both enzymes are independently regulated, their inhibition generated similarly reduced FRET ratios over time.

With regard to co-treatment with DGKi and FIPI inhibitors, YAMC cells responded similarly to previous treatments, yet after 6 min of EGF simulation, cells expressed a lower FRET ratio value that was maintained over time (**Figure 6)**. Examination of the FRET slope for cells treated with both inhibitors revealed a noticeable decrease of K-Ras activation in comparison with the addition of independent inhibitors. In addition, 10 min following YAMC stimulation with EGF, there was a modest and steady drop of the FRET ratio similar to the DHA K-Ras suppression in YAMC cells. These findings suggest that inhibition of both PLD2 and DGK generated a greater dampening effect on K-Ras (**Figure 6**). It is noteworthy that some cells pre-incubated with inhibitors died upon EGF stimulation (data not shown). These cells were excluded from the analysis.

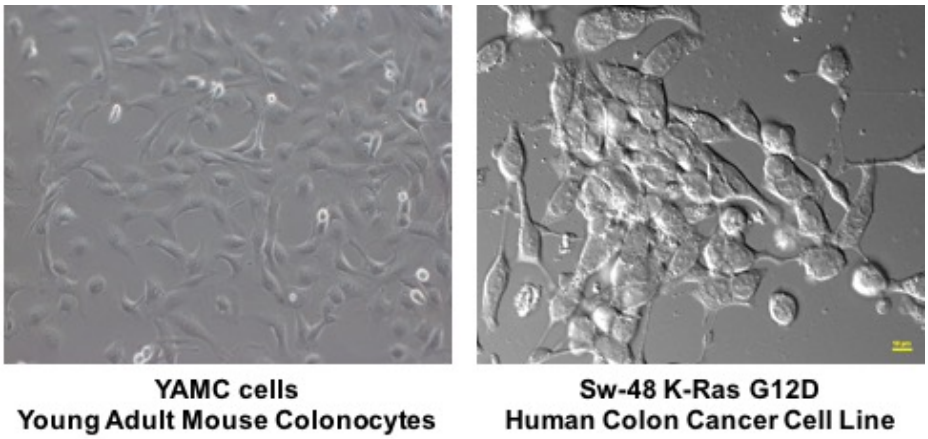

Figure 1. Morphology of YAMC cells and Sw-48 K-Ras G12D cells. YAMC cells have a flattened oval shape. Human colon cancer cells exhibit a more rounded shape.

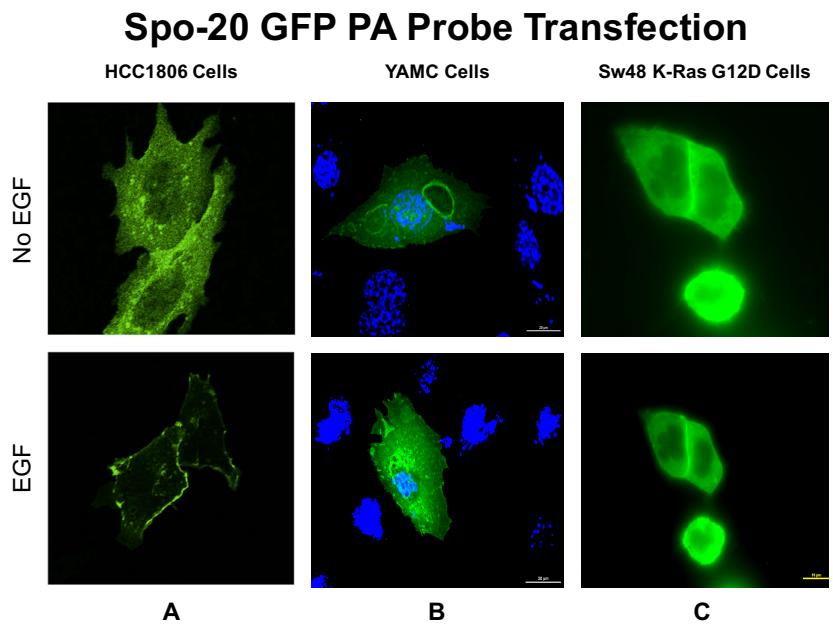

Figure 2. Validation of the Spo-20 GFP PA Probe. A) HCC1806 breast cancer cell images demonstrate a noticible plasma membrane localization of the Spo-20 GFP Pa probe[12]. B) YAMC cells expressed no clear membrane localization by the PA-binding probe. These two images represent the control and EGF-stimuted cells. C) Sw-48 K-Ras G12D human cancer cells showed no translocation of the Spo-20 GFP PA probe to the plasma membrane. These two cell images were taken from cultures stimulated with EGF after 10 min of imaging.

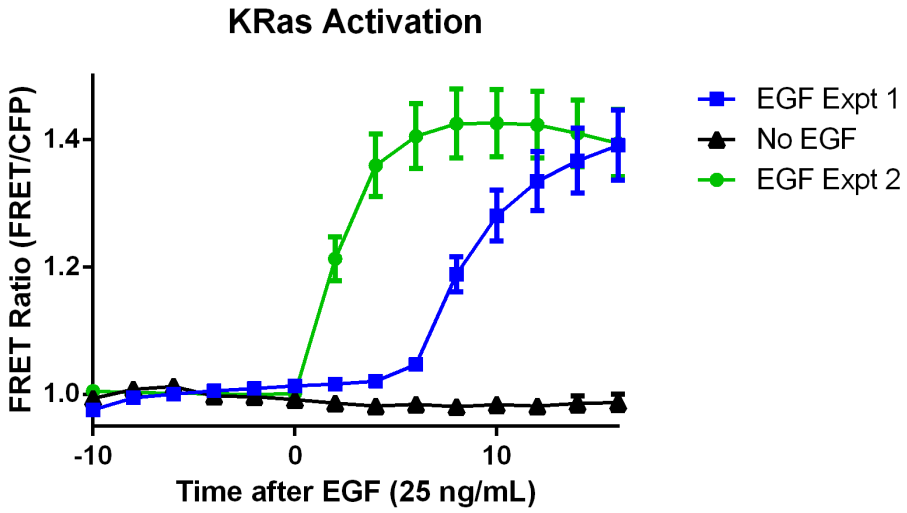

Figure 3. EGF activates K-Ras. YAMC cells stimulated with EGF exhibited a delayed K-Ras activation response (experiment 1). Research studies indicate that K-Ras activation should be instantaneous, within 1 min following the addition of EGF (experiment 2).

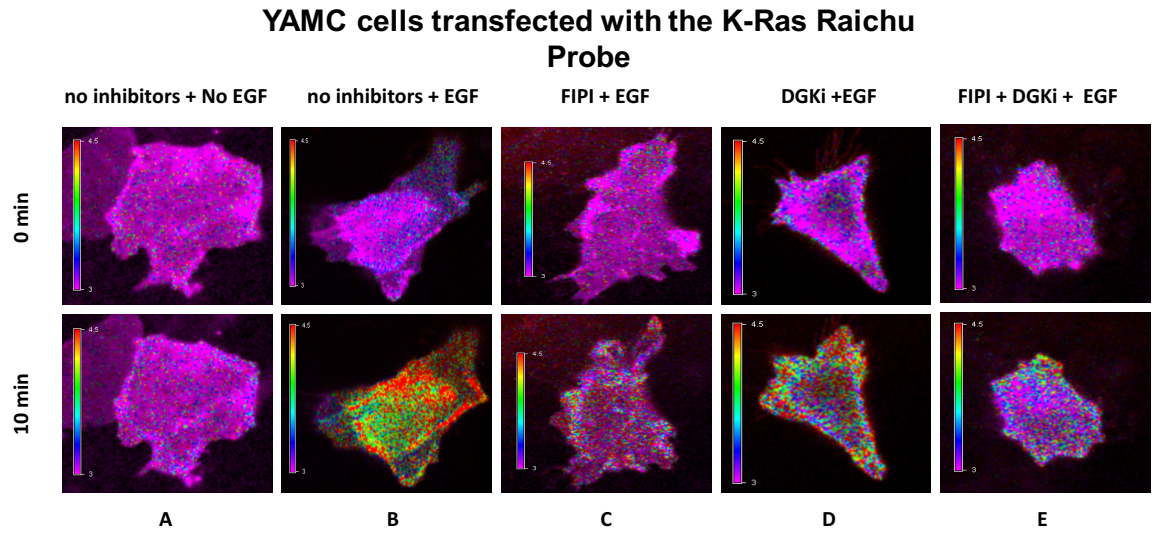

Figure 4. YAMC cells transfected with a K-Ras Raichu probe. A) Non-EGF-stimulated cells with no inhibitors did not express FRET fluorescence (pseudo colored purple). B) Cells with no inhibitors and stimulated with EGF (positive control) expressed high red fluorescence expression in the FRET ratio scale. This indicates "high" K-Ras activation. C) YAMC cells pre-treated with FIPI (PLD2 inhibitor) and stimulated with EGF expressed little fluorescence. D) The FRET fluorescence signature of YMAC cells pre-treated with DGKi (DGK inhibitor) was similar to FIPI treated cells. E) Pre-treated cells with combined FIPI & DGKi and EGF generated the lowest fluorescence FRET signature compared to the individual inhibitor treatments.

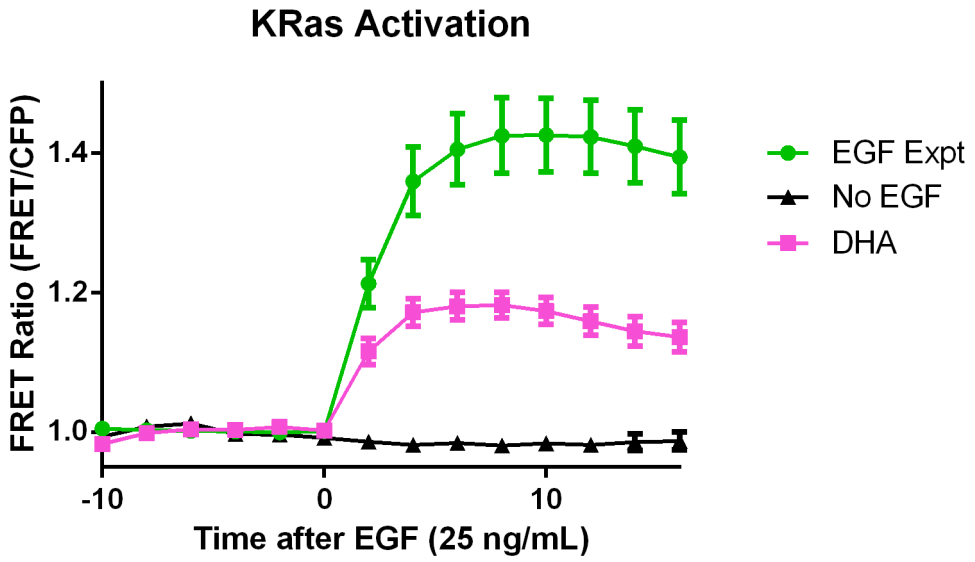

Figure 5. DHA suppresses K-Ras activation. Prior research on YAMC cells has shown that DHA attenuates K-Ras activation following stimulation with EGF for 10 min[9]. YAMC cells with no EGF, serves as a negative control. DHA reduced K-Ras activation by approximately 50% after 4 min of EGF stimulation ($P<0.005$).

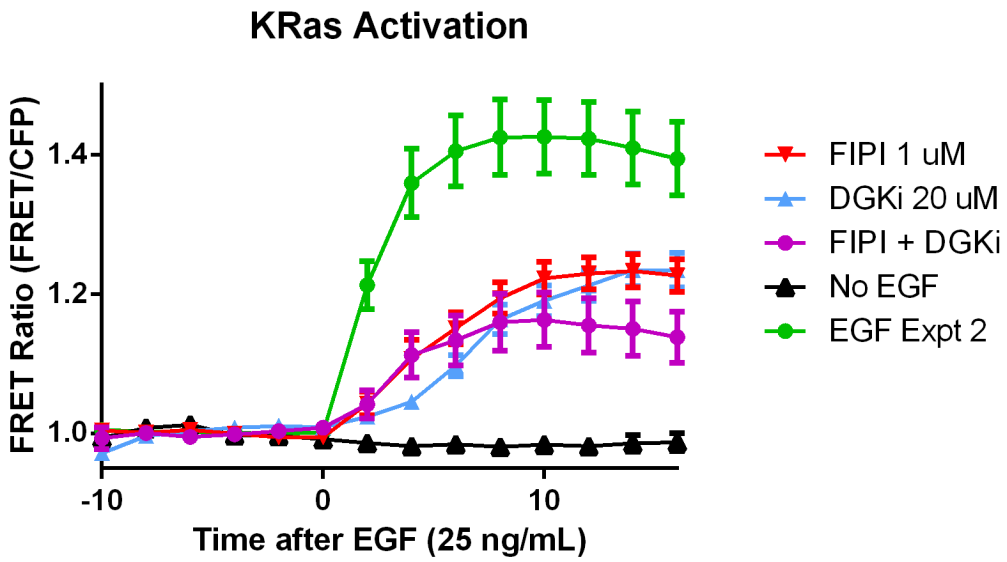

Figure 6. FIPI and DGKi suppress K-Ras activation. FIPI inhibitor disrupted the EGFR-MAPK signaling pathway and reduced K-Ras activation. The inhibition was slow and constant over time. The DGK inhibitor also suppressed K-Ras activation. Although the observed reduction was slightly greater than FIPI, the DGK FRET ratio slope eventually reached the same slope as FIPI. This finding suggests equivalent reduction in K-Ras. FIPI and DGKi combination exhibited a greater K-Ras compared to the independent respective treatment ($P<0.05$).

CHAPTER IV

CONCLUSION

Validation of the Spo-20 GFP PA probe

The validation of the Spo-20 GFP PA probe in both YAMC and Sw48 K-Ras G12D cells was not successful due to multiple possible factors, including cell morphological shape, microscope resolution, Spo-20 GFP PA suitability, lack of relevant evidence on human cancer cells for this study, and human errors. Nonetheless, based on the multiple experimental trials to validate the Spo-20 GFP PA probe[12], we believe that the efficacy and efficiency of this PA-binding probe needs to be re-evaluated. The Spo-20 GFP PA might not be effective, so selecting an alternative PA-binding probe is recommended for future DHA-PA related studies. One suggestion is to utilize the FRET biosensor for PA named Pii. This probe contains a lipid-binding domain sandwiched with the cyan fluorescent protein and yellow fluorescent protein and is tagged with the plasma membrane-targeting sequence of K-Ras[19]. The use of this FRET-based PA probe might afford greater affinity for PA and better results since this PA biosensor binds to cells' plasma membrane. For this study, the K-Ras Raichu probe and the inhibitors FIPI and DGKi were adopted as an alternative approach to address the initial hypothesis.

PLD2 and DGK Inhibitor Effects on Transfected YAMC Cells

Signaling by EGFR is up-regulated in many colon cancer cells[12]. This process involves two critical PA-generating enzymes (PLD2 and DGK) for the activation of K-Ras, a G protein that promotes cell growth and proliferation. Data from the present study demonstrated the effects of PLD2 and DGKi on K-Ras inhibition. In general, YAMC cells pre-treated with FIPI (PLD2

inhibitor) and/or DGKi exhibited a similar effect on K-Ras activation. Overall, the co-treatment (FIPI & DGKi) resulted in the greatest K-Ras reduction as represented by the lowest FRET ratio. Interestingly, the FRET ratio data analysis showed that DHA exhibited a similar suppressive effect on K-Ras activation.

PLD2 and DGK are enzymes that produce phosphatidic acid in response to an actived EGFR-MAPK signaling pathway. This signaling pathway then recruits SOS, a guanine nucleotide exchange factor that activates Ras[9]. Since PA is the product of PLD2 and DGK, our findings suggest that PA is pivotal for SOS recruitment following activation of K-Ras. Further studies are needed in order to determine precisely how PA levels affect Ras activation in YAMC cells. If PA is correlated with K-Ras inhibition, this experiment will open a new window into DHA-PA related studies for cancer prevention.

The purpose of this study was to demonstrate the relationship between PA-producing enzymes and K-Ras activation. However, the initial hypothesis which involved DHA as the extrinsic factor responsible for PA attenuation and subsequent K-Ras reduction still needs to be studied. Cell viability experiments using YAMC cells treated with DHA, FIPI, DGKi and FIPI & DGKi are ongoing. In addition, it is critical that intracellular PA levels be measured using the PA-binding probe Spo20-GFP-PA (or probes with a greater binding affinity for PA) in combination with total internal reflection fluorescence (TIRF) microscopy.

REFERENCES

1. Cox AD, Fesik SW, Kimmelman AC, Luo J, Der CJ. Drugging the undruggable RAS: Mission Possible? *Nat Rev Drug Discov*. 2014;13(11):828-851. doi:10.1038/nrd4389.

2. Stephen AG, Esposito D, Bagni RG, McCormick F. Dragging ras back in the ring. *Cancer Cell*. 2014;25(3):272-281. doi:10.1016/j.ccr.2014.02.017.

3. Spiegel J, Cromm PM, Zimmermann G, Grossmann TN, Waldmann H. Small-molecule modulation of Ras signaling. *Nat Chem Biol*. 2014;10(8):613-622. doi:10.1038/nchembio.1560.

4. Winter JJG, Anderson M, Blades K, et al. Small molecule binding sites on the Ras:SOS complex can be exploited for inhibition of Ras activation. *J Med Chem*. 2015;58(5):2265-2274. doi:10.1021/jm501660t.

5. Hall MN, Chavarro JE, Lee I-M, Willett WC, Ma J. A 22-year Prospective Study of Fish, n-3 Fatty Acid Intake, and Colorectal Cancer Risk in Men. *CANCER Epidemiol BIOMARKERS Prev*. 2008;17(5):1136-1143. http://lib-ezproxy.tamu.edu:2048/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=edsbl&AN=RN230773693&site=eds-live.

6. Cockbain AJ, Toogood GJ, Hull MA. Omega-3 polyunsaturated fatty acids for the treatment and prevention of colorectal cancer. *Gut*. 2012;61(1):135-149. doi:10.1136/gut.2010.233718.

7. Lien EL. Toxicology and safety of DHA. *Prostaglandins, Leukot Essent Fat Acids*. 2009;81(Workshop on DHA as a Required Nutrient):125-132. 10.1016/j.plefa.2009.05.004.

8. Skarke C, Alamuddin N, Lawson JA, et al. Bioactive products formed in humans from fish oils. *J Lipid Res*. 2015;56(9):1808-1820. doi:10.1194/jlr.M060392.

9. Turk HF, Barhoumi R, Chapkin RS. Alteration of EGFR spatiotemporal dynamics suppresses signal transduction. *PLoS One*. 2012;7(6):e39682. doi:10.1371/journal.pone.0039682.

10. Ma DWL, Seo JM, Davidson LA, et al. n-3 PUFA Alter caveolae lipid composition and resident protein localization in mouse colon. *FASEB J*. 2004;18(6):1040 - +. http://lib-ezproxy.tamu.edu:2048/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=edswsc&AN=000221108800014&site=eds-live.

11. Lajoie P, Partridge EA, Guay G, et al. Plasma Membrane Domain Organization Regulates EGFR Signaling in Tumor Cells. *J Cell Biol VO - 179*. 2007;(2):341. http://lib-ezproxy.tamu.edu:2048/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=edsjsr&AN=edsjsr.30050474&site=eds-live.

12. Zhang F, Wang Z, Lu M, et al. Temporal production of the signaling lipid phosphatidic acid by phospholipase D2 determines the output of extracellular signal-regulated kinase signaling in cancer cells. *Mol Cell Biol*. 2014;34(1):84-95. doi:10.1128/MCB.00987-13.

13. Zhao C, Du G, Skowronek K, Frohman MA, Bar-Sagi D. Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. *Nat Cell Biol*. 2007;9(6):706-712. http://lib-ezproxy.tamu.edu:2048/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=mdc&AN=17486115&site=eds-live.

14. Walker F, Kato A, Gonez LJ, et al. Activation of the Ras/mitogen-activated protein kinase pathway by kinase-defective epidermal growth factor receptors results in cell survival but not proliferation. *Mol Cell Biol*. 1998;18(12):7192-7204. http://lib-ezproxy.tamu.edu:2048/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=mdc&AN=9819406&site=eds-live.

15. Rogers KR, Kikawa KD, Mouradian M, et al. Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association. *Carcinogenesis*. 2010;31(9):1523-1530. 10.1093/carcin/bgq111.

16. Hou TY, Monk JM, Fan Y-Y, et al. n-3 polyunsaturated fatty acids suppress phosphatidylinositol 4,5-bisphosphate-dependent actin remodelling during CD4+ T-cell activation. *Biochem J*. 2012;443(1):27-37. doi:10.1042/BJ20111589.

17. Inoue N. Introduction of Single Base Substitutions at Homologous Chromosomal Sequences by Adeno-associated Virus Vectors. *Mol Ther*. 2001;3(4):526-530. doi:10.1006/mthe.2001.0283.

18. Prior I a, Lewis PD, Mattos C. UKPMC Funders Group UKPMC Funders Group Author Manuscript A comprehensive survey of Ras mutations in cancer. *Cancer Res*. 2012;72(10):2457-2467. doi:10.1158/0008-5472.CAN-11-2612.A.

19. Nishioka T, Frohman MA, Matsuda M, Kiyokawa E. Heterogeneity of phosphatidic acid levels and distribution at the plasma membrane in living cells as visualized by a F??rster Resonance Energy Transfer (FRET) biosensor. *J Biol Chem*. 2010;285(46):35979-35987. doi:10.1074/jbc.M110.153007.

APPENDIX A

CULTURING PROTOCOLS

YAMC Cell Culture: Seeding and Passaging

Purpose: To pass and seed YAMC cell culture.

Preparation:

Use sterile hood conditions for the procedure.
Warm 50 ml of complete RPMI 1640 and 5 ml of trypsin-EDTA (Gibco #25300-054) to room temperature.

Procedure:

For any 70% confluent cell culture in a T-75 flask:

1. Add 50 ml of complete media plus 5 μl γ-IFN to a 50 ml conical tube (to grow cells).
2. Add 5 ml of Trypsin-EDTA to a 15 ml conical tube.
3. Warm both media and trypsin in water bath at 37°C.
4. Aspire old media from flask with YAMC cells.
5. Rinse monolayer of cells with ~10 ml HBSS (Sigma, H-6648) by adding and aspiring gently without disturbing the cell monolayer.
6. Add 5 ml Trypsin-EDTA to the flask and incubate cells at 37°C for 5 minutes. Gently tap the bottom of the flask to assist in cell lifting.
7. Add 10 ml complete media (step 1) from the conical tube to the flask of trypsinized cells to neutralize trypsin (add by rapidly dispensing the media and moving the pipette in a back and forth motion across the flask to dislodge any stuck cells).
8. Transfer trypsinized cells into a 15 ml conical tube to spin down cells.
9. Centrifuge the cells in the tube at 200 x g in the tabletop centrifuge for 5 minutes.
10. Use a pipette to transfer 10 μl of the cell suspension onto each end of the hemacytometer. Count the number of cells.
11. Add 5 ml of complete RPMI 1640 (with γ-IFN) and resuspend the pellet by gently pipetting up and down to make a homogenous suspension. It is recommended to gently tap the "almost dry" pellet before resuspending with medium to help resuspension.
12. Seeds cell according to desired density in complete RPMI 1604 with γ-IFN. Remember to open all flasks and dishes under the sterile hood.

Sw48 K-Ras G12D Cell Culture: Seeding and Passaging

Purpose: To pass and seed Sw48 K-Ras G12D cell culture.

Preparation:

Use sterile hood conditions for the procedure.
Warm 50 ml of complete RPMI 1640 and 5 ml of trypsin-EDTA (Gibco #25300-054) to room temperature.

Procedure:

For any 70% confluent cell culture in a T-75 flask or T-175 flask:

1. Add 50 ml of complete media to a 50 ml conical tube (to grow cells).
2. Add 5 ml of Trypsin-EDTA to a 15 ml conical tube.
3. Warm both media and trypsin in water bath at 37°C.
4. Aspire old media from flask with YAMC cells.
5. Rinse monolayer of cells with ~10 ml HBSS (Sigma, H-6648) by adding and aspiring gently without disturbing the cell monolayer.
6. Add 5 ml Trypsin-EDTA to the flask and incubate cells at 37°C for 5 minutes. Gently tap the bottom of the flask to assist in cell lifting.
7. Add 10 ml complete media (step 1) from the conical tube to the flask of trypsinized cells to neutralize trypsin (add by rapidly dispensing the media and moving the pipette in a back and forth motion across the flask to dislodge any stuck cells).
8. Transfer trypsinized cells into a 15 ml conical tube to spin down cells.
9. Centrifuge the cells in the tube at 200 x g in the tabletop centrifuge for 5 minutes.
10. Use a pipette to transfer 10 μl of the cell suspension onto each end of the hemacytometer. Count the number of cells.
11. Add 5 ml of complete RPMI 1640 and resuspend the pellet by gently pipetting up and down to make a homogenous suspension. It is recommended to gently tap the “almost dry” pellet before resuspending with medium to help resuspension.
12. Seed cells according to desired density in complete RPMI 1604. Remember to open all flasks and dishes under the sterile hood.

APPENDIX B

TRANSFECTION PROTOCOLS

YAMC Cell Culture: Transfection

Purpose: To transfect YAMC cells.

Preparation:

Use sterile hood conditions for the procedure.
Warm 10-30 ml of complete RPMI at room temperature depending on needed media.

DNA-lipid Complex Preparation:

1. Add 4 μl of Lipofectamine 3000 plus 250 μl of Opti-MEM Medium into a 1.5 ml conical tube. These amounts are base in a single dish or well.
2. Add 5 μl of P3000 Reagent, 250 μl of Opti-MEM Medium, and 2 μg of DNA into a 1.5 ml conical tube. These amounts are based in a single dish or well.
3. Transfer the Lipofectamin 3000 and Opti-MEM Medium solution (step1) into the P3000 reagent containing solution.

Procedure:

Fro any 70%-90% confluent cell culture:

1. Seed 25,000 to 35,000 cells in a 35 mm dish.
2. Warm needed media with γ-IFN.
3. Aspire old media from dishes or wells with YAMC cells.
4. Rinse monolayer of cells with ~2 ml HBSS (Sigma, H-6648) by adding and aspiring gently without disturbing the cell monolayer.
5. Add between 1.6 – 1.9 ml of new complete RPMI media with γ-IFN to each dish or well
6. Add the respective calculated amount of DNA complex to each dish or well.
7. Incubate cells at 37°C.

Sw48 K-Ras G12D Cell Culture: Transfection

Purpose: To transfect Sw48 K-Ras G12D cells.

Preparation:

Use sterile hood conditions for the procedure.
Warm 10-30 ml of complete RPMI at room temperature depending on needed media.

DNA-lipid Complex Preparation:

1. Add 4 μl of Lipofectamine 3000 plus 250 μl of Opti-MEM Medium into a 1.5 ml conical tube. These amounts are base in a single dish or well.
2. Add 5 μl of P3000 Reagent, 250 μl of Opti-MEM Medium, and 2 μg of DNA into a 1.5 ml conical tube. These amounts are based in a single dish or well.
3. Transfer the Lipofectamin 3000 and Opti-MEM Medium solution (step1) into the P3000 reagent containing solution.

Procedure:

For any 70%-90% confluent cell culture:

1. Seed 25,000 to 35,000 cells in a 35 mm dish.
2. Warm needed media.
3. Aspire old media from dishes or wells with YAMC cells.
4. Rinse monolayer of cells with ~2 ml HBSS (Sigma, H-6648) by adding and aspiring gently without disturbing the cell monolayer.
5. Add between 1.6 – 1.9 ml of new complete RPMI media to each dish or well.
6. Add the respective amount of DNA-lipid complex to each dish or well.
7. Incubate cells at 37°C.

APPENDIX C

CELL FIXATION PROTOCOL

YAMC Cell Culture: Transfection

Purpose: To fix YAMC cells.

Procedure:

1. Make 4% PFA at room temperature. Dilute reagents in PBS. This must be done in the chemical hood. Always make PFA fresh (i.e. the same day of the experiment). Dilute 20% PFA 1:5 and 8% GA 1:80.
2. Aspire media and rinse with PBS, then immediately add 500 μl PFA (4%) per well for 10 minutes at room temperature. This must be done in the chemical hood.
3. Rinse the samples with 2 ml of PBS 3 times each, and incubate cells with 500 μl glycine (100 mM) in PBS for 10 min at room temperature. This step is performed to quench aldehyde groups. Do first wash in chemical hood and dump into waste.
4. Wash with PBS twice to remove any residual glycine.
5. Use tool to remove plastic cover.
6. Add ~3 drop antifade reagent with DAPI. Make sure it coats the sample with the light layer.
7. Place lid on samples then cover with foil and allow to cure for 24 hours in the dark.
8. After curing, seal with clear nail polish and the samples can be stored at 4°C.