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INHIBITION OF RAS AS A NOVEL THERAPEUTIC APPROACH IN  
LUMINAL B BREAST CANCER

By

Raphael Ngozichi Suarez Jigo

BHS Medical Laboratory Science, University of Kentucky, 2017

A Thesis

Submitted to the Faculty of the

School of Medicine of the University of Louisville

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Master of Science in Pharmacology and Toxicology

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A Thesis Approved on

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## DEDICATION

This thesis is dedicated to my parents, Oti and Bregidita Jigo, my family, and all my friends back home in Papua New Guinea who have provided me with encouragement and support so far on this journey.

## ACKNOWLEDGMENTS

I would like to acknowledge Dr. Clark for believing in me and giving me this opportunity despite having no prior research experience. Thank you for your continued support both with my research efforts and in my personal life. To Dr. Howard Donninger, Rachel Ferrill, and Becca Von Baby – thank you for making me feel welcome in the lab and teaching me all the technical skills I know to date. To Dr. Beverly, Dr. Ceresa, and Dr. Mitchell – thank you for your assistance in the completion and defense of this thesis.

## ABSTRACT

### INHIBITION OF RAS IN LUMINAL B BREAST CANCER AS A NOVEL THERAPEUTIC APPROACH

Raphael Ngozichi Suarez Jigo

November 29<sup>th</sup>, 2022

Historically, the RAS oncoprotein has not been implicated in breast cancer due to less than 1% of breast cancer cases bearing oncogenic RAS mutations. Recently, however, it has been reported that greater than 60% of Luminal B breast cancer cases have a decreased expression of negative RAS regulators RASAL2 and DAB2IP. Thus, in many Luminal B breast cancers, RAS remains in a constitutively active state without mutation, in turn driving oncogenesis. To date, there are no FDA approved direct inhibitors of wild type RAS. We have developed a direct RAS inhibitor that acts on wild type RAS using in silico drug library screening. Using in vitro and in vivo model systems as proof-of-principle experiments, we have demonstrated our compound to inhibit anchorage-independent growth and RAS-mediated signaling in vitro, as well as decrease Luminal B cell tumor growth rate in vivo.

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## CHAPTER 1: BACKGROUND AND INTRODUCTION - BREAST CANCER

### 1.1 Breast Cancer Overview

Cancer is a leading contributor to global morbidity and mortality, with the annual rates of new cancer diagnoses and deaths continuing an upward trend. In 2020 alone, 19.3 million new cancer diagnoses were reported worldwide in addition to 10 million cancer-related deaths [1]: a significant increase from the 18 million diagnoses and 8.9 million deaths reported in 2018 [2]. In the US, cancer is the second leading cause of death after cardiovascular diseases, with the number of mortalities expected to follow worldwide trends and increase in 2022 [3, 4]. Thus, the development of new and more effective cancer treatments is an urgent unmet clinical need. Discovery of novel targeted therapeutics, however, is complicated by the heterogenous nature both within and between tumor types, presenting challenges in combatting reduced treatment sensitivity, treatment resistance and cancer recurrence [5].

As of 2020, female breast cancer has become the most diagnosed cancer type worldwide. [6]. Heterogenous in nature, these tumors are characterized by the abnormal growth of malignant cells in the ducts or glands of the breast. The heterogeneity of breast tumors means that each tumor is biologically different. At least 21 distinct histological classifications exist based on the tumor's point of origin within the organ, and the advent of gene array analysis has allowed for the classification of 4 main molecular subtypes: Luminal A, Luminal B, Triple Negative, and HER2-overexpressed breast cancers [8]. These unique biological fingerprints within and between each tumor further complicate

patient care and clinical outcome, as each subtype has a different presentation, response to therapeutic intervention, and prognosis [8].

Most breast tumors ultimately become invasive in nature, proliferating and eventually penetrating surrounding breast tissue with the possibility of metastasis to surrounding lymph nodes and distant organs [8]. Despite the percentage of breast cancer mortalities declining over the years in part due to increased early detection and treatment [8], the problem with tumor aggression and recurrence, whether local or distant, presents clinicians with problems in long-term patient care. Studies have demonstrated that despite being disease-free after initial diagnosis and treatment, about 30% of invasive breast carcinoma patients present with tumor recurrence [7]. The probability of recurrence significantly increases 5 years post-treatment and is especially true in estrogen-receptor positive breast tumors [7, 9]. Although this has spurred interest into studying the molecular mechanisms behind tumor aggression and recurrence, to date no good treatment options exist for aggressive tumors with a higher probability of relapse. Treatment resistance in certain molecular subtypes (such as Luminal B and Triple Negative Breast Cancer) is a continued problem for clinicians and patients alike. For this reason, novel targeted therapies apart from currently available treatment methods are required to alleviate this problem and improve patient care.

## 1.2 Risk Factors

### *Inherited Genetic Predisposition*

As is the case with other cancer types, disease-causing genetic variations in certain cancer susceptibility genes have been shown to increase the risk of developing breast malignancies. First discovered in the 1990s, one of the most widely studied and

tested is mutation in the Breast Cancer 1 and 2 Protein genes (BRCA1/2), which increases the risk of both breast and ovarian cancer development [8, 10, 11].

BRCA1 or BRCA2 mutations account for 5 – 10% of female breast cancers, and 15 – 20% of inherited breast cancers [8]. These mutations greatly increase the risk for breast cancer development especially in females at 80 years of age or over, where the risk increases from 10% to 70% with a BRCA1/2 mutation. BRCA1, located on chromosome 17q2, and BRCA2, located on chromosome 13q12, genes normally code for tumor suppressive proteins involved in DNA Damage Repair (DDR) and the perseveration of genetic material [10]. BRCA1 is a mediator in the repair of DNA double strand breaks through Homologous Recombination repair (HR) and cell cycle checkpoint regulation [10, 11]. The development of pathogenic BRCA1 variants due to insertion mutations, or the decrease in BRCA1 expression through deleterious mutations, increases the risk of carcinogenesis due to a decrease in the DNA-damage response, dysregulated cell cycle control, and increased propensity for cellular DNA mutations [10, 11]. This, in turn, may lead to uncontrolled cellular division, proliferation and growth: all hallmarks of carcinogenesis. Like BRCA1, BRCA2 is also involved in HR repair of DNA double strand breaks, and deleterious or pathogenic mutations have been shown to cause development of higher-grade invasive breast carcinomas when compared to BRCA1 [10].

Other cancer-susceptibility genes have been also shown to increase risk of invasive ductal carcinoma development. Mutations in ATM, CHEK2, TP53 and PTEN (genes involved in the repair of DNA double strand breaks, regulation of cellular proliferation, survival, and apoptosis) have been shown to increase breast cancer risk [12]. Functional loss of PALB2, an interacting protein of BRCA1/2, has been shown to

increase risk of breast cancer development from 10% to 35% in women aged 70 and older [8, 12]. Together with BRCA1/2, mutations in these genes are routinely included in hereditary breast cancer gene testing panels to determine lifetime breast cancer development risk.

### *Sex*

Although men can develop breast cancer, women are 100 times more likely to develop some sort of breast malignancy [8, 10, 13]. This is due to factors such as higher breast tissue density and the presence of hormones such as estrogen, which drives estrogen-receptor positive tumors and is also an approved therapeutic target. The chances of men developing breast cancer, as is the case with women, is increased with mutations in the BRCA1/2 genes.

### *Age*

Apart from sex and genetic predisposition, age drastically increases the risk of breast cancer development. Of all invasive breast cancer diagnoses made in 2019, about 71% were diagnosed in women between 50 and 80 years of age, and 90% of breast cancer mortalities were reported in women 50 years of age or older [8]. At age 70, this risk increases to 70% in females with a BRCA1/2 mutation, and 35% in females with a PALB2 mutation. For this reason, mammography screenings are recommended for women beginning at 40 years of age.

## 1.3 Breast Cancer Classification

### Clinical Breast Cancer Staging

Clinical staging of breast tumors allows for the determination of tumor burden extent and allows clinicians to determine treatability of the cancer, appropriate treatment options, and prognoses. In the US, breast cancer is staged according to the American Joint Committee on Cancer (AJCC) system, which considers three main anatomical factors: tumor size, lymph node status, and metastasis to distant organs.

#### *Tumor Size (T)*

Determined after primary diagnosis, this describes the size and extent of the tumor. Tumor size is measured in centimeters and is graded from ranges *Tis* (ductal carcinoma in situ) to *T4* [14]. Ductal carcinoma in situ (DCIS) is a pre-invasive phase tumor with the potential to penetrate and proliferate into surrounding breast tissue. *T2* to *T4* is measured from 2cm to 5cm in tumor size, with the latter being further divided to establish whether malignant cell growth has spread to either the chest wall or skin [14]. Higher grade correlates with a later stage of tumor growth and is often accompanied with increased treatment resistance.

#### *Lymph Node Status (N)*

Lymph node staging describes whether malignant cells have spread to lymph nodes near the breast and collar bones. Metastasis is assessed on a scale of *N0* to *N3*: *N0* correlating to no metastasis detected in nearby lymph nodes, and *N3* correlating to spread of cancer cells to 10 or more axillary lymph nodes, with metastasized cells detected in lymph nodes above the collar bone [14]. As with tumor size, higher staged lymph node status is indicative of a later, more advanced stage of tumor growth and spread

### *Metastasis (M)*

Breast cancer most commonly metastasizes to the brain, lung, liver, and bone [15]. Metastasis staging, therefore, is determined by imaging tests and assesses whether malignant cells have spread from their point of origin in the breast to distant organs. Tumors are graded from a scale of *M0* to *M1*: *M0* corresponding to no detectable malignant cells, and *M1* indicating detectable spread to a distant organ with a tumor at least 0.2mm in size [14]. Metastasis to distant organs is indicative of more aggressive, late-stage cancer and increased likelihood of treatment resistance and mortality.

### Prognostic Breast Cancer Staging

Apart from determination of stage through anatomical staging, clinicians also consider the presence of molecular markers to determine prognoses and appropriate care and treatment. In addition to the hereditary gene panel testing discussed earlier, the presence of hormone receptors for estrogen and/or progesterone, as well as the status of HER2, aids in clinical decision making [14]. The absence or presence of these markers forms the basis for the 4 molecular subclassifications of breast cancer.

### Molecular Subclassifications

The advent of microarray gene expression analysis in 1995 allowed for the intrinsic subtyping of breast tumors, further increasing understanding of the heterogeneity that exists between each subtype. Originally based strictly on the presence or absence of estrogen receptor (ER), progesterone receptor (PR), or HER2 by immunohistochemistry, gene array analysis has allowed for detection of gene expression levels of these historic



markers in addition to known genetic alterations that drive breast cancer progression [16, 17]. Understanding the underlying genetic fingerprint of these tumors has drastically improved patient care and treatment. The discovery of more molecular drivers of breast carcinogenesis is required to combat the inequalities in treatment resistance that exists between the different molecular subtypes through the development of novel targeted therapies.

### *Triple Negative Breast Cancer (TNBC)*

TNBC is characterized by the lack of expression of both hormone receptors and HER2. Representative of about 10 - 15% of breast cancer cases, these tumors are further subclassified into basal-like (about 70% of TNBC) and non-basal (about 30% of TNBC), each with different presentation and prognoses [17]. Unlike non-basal TNBC, basal-like tumors express cytokeratins CK5/6 and CK14 normally expressed in the basal layer of the mammary gland, in addition to EGFR overexpression and a high TP53 mutation rate (about 80% of basal-like tumors) [16]. Breast cancer patients who present with a BRCA1 mutation have a higher likelihood TNBC development [16, 17].

Their aggressive nature, together with the absence of currently accepted molecular markers, makes these tumors difficult to treat. TNBCs have the worst prognoses of all 4 subtypes, and this is in part due to the high rates of proliferation, metastasis, and relapse [17]. Research into therapeutic vulnerabilities has lagged significantly behind other subtypes and, to date, no targeted therapies exist. Standard of care relies solely on lumpectomy and radiation, rendering research into targeted therapies important for the improvement of long-term care.

### *HER2 overexpression*

The HER2-overexpression subtype makes up about 4% of breast cancer cases. They frequently fail to express hormone receptors [16] but have a high mutation rate of TP53 and PIK3CA. These tumors tend to be aggressive in nature, show a high proliferation rate, and exhibit poor prognosis in comparison to most luminal tumors. The advent of monoclonal antibodies directed against HER2 (Trastuzumab), however, has significantly improved prognosis of HER2-overexpressed tumors, and combination therapies with tyrosine kinase inhibitors has demonstrated improved tumor sensitivity [17]. Considerable overlap exists in the classification of HER2-overexpressed and Luminal B tumors. Although over half of HER2-enriched tumors are negative for hormone receptors, the remaining can be positive for ER expression and therefore classified as Luminal B tumors. Gene array analysis allows for the differentiation of the two, with ER-negative tumors having a higher mutation rate of TP53 and expression of tyrosine kinase receptors [16].

### *Luminal tumors*

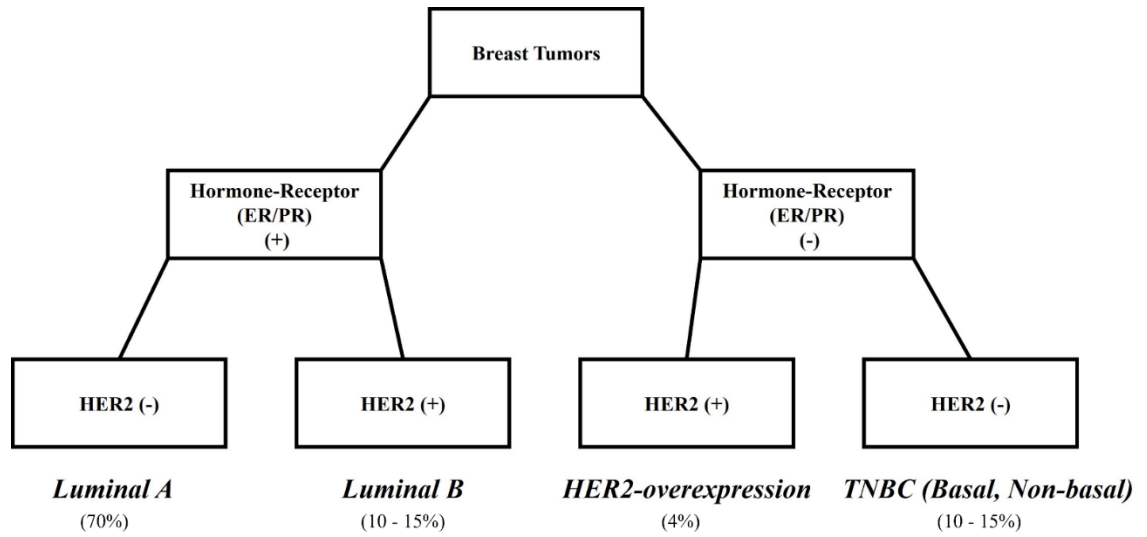
Luminal breast tumors account for most breast cancers. They arise from the lumen and share expression profiles reminiscent of the luminal epithelial cells that line the mammary breast duct, which includes the expression of ER and luminal cytokeratins 8/18 [7]. Luminal cancers are further subtyped into Luminal A and Luminal B tumors, the former accounting for about 70% of breast cancers and the latter about 10 - 15% of cases. Luminal A tumors are positive for ER and/or PR, negative for HER2 expression, and have the best prognosis and rates of survival of all 4 subtypes. These tumors have a relatively slow rate of growth, respond well to hormone-targeted therapies, and have low

incidence of tumor recurrence and cancer-related mortality [16, 17]. About 45% of Luminal A cancers have an activating mutation in PIK3CA but exhibit less genomic instability when compared to Luminal B tumors.

On the other hand, Luminal B cancers have survival rates and prognoses comparable to TNBCs, in part due to their aggressive nature, high resistance to current standards of treatment, and lack of targeted therapies. These cancers are positive for ER and vary as to whether they express or lack PR and HER2 [17]. When compared to their Luminal A counterpart, Luminal B tumors exhibit elevated genomic instability, possessing a high frequency of activating mutations in PIK3CA, as well as mutations in TP53 and genes involved in p53 activation (for example, loss of ATM expression and overexpression of MDM2) [16, 18]. A key discriminator between Luminal A and Luminal B tumors is the expression levels of Ki-67. Localized in the nucleus, Ki-67 has long been used as a marker for proliferation in cancer cells and shares a positive correlation with rates of metastasis, relapse after treatment, and overall survival [16]. Luminal B tumors have an elevated expression for Ki-67 (more than 20%) when compared to Luminal A cancers [16, 17]. High Ki-67 expression, together with an elevated expression of proliferation-related genes, contributes to the high rates of proliferation and growth, as well as their aggressive nature. This in part may explain the decreased treatment sensitivity to currently available hormone and HER2-targeted therapies seen in this subtype.

## 1.4 Current Standards of Treatment

Surgical resection followed by radiation is currently the standard for breast cancer treatment [8]. Patients can elect to have the affected breast completely removed (mastectomy) or malignant tumors removed by lumpectomy, with the majority electing for the former to avoid post-surgery radiation [8]. The efficacy of currently available chemotherapies and targeted therapies is dependent on tumor stage, as well as expression of hormone receptors and HER2. ER-positive luminal tumors may benefit from hormone-targeted therapies (such as Tamoxifen) and CDK4/6 inhibitors, although response to treatment is decreased in the Luminal B subtype. In tumors expressing HER2 (HER2-overexpression, Luminal B tumors), patients may benefit from treatment with monoclonal antibodies targeted against HER2 (Trastuzumab) together with tyrosine kinase inhibitors (such as Lapatinib). Subtypes lacking expression of markers (such as TNBC) may benefit from immunotherapies (PD-L1 inhibitors) and chemotherapies (such as Taxol), however, surgical resection remains the gold standard due to the lack of effective treatment methods. The rates of recurrence 5 years post-treatment, coupled with the aggressive nature and treatment resistance seen in certain subtypes such as Luminal B cancers, increases the need for more targeted therapies to combat tumor growth and progression.



**Figure 1. Molecular Classification of Breast Tumors.**

## CHAPTER 2: RAS ONCOPROTEIN AND ITS ROLE IN CANCER

### 2.1 RAS Overview

The discovery of the Rat Sarcoma virus (RAS) gene in 1964 gave rise to a field of oncology that, to date, remains under much investigation. RAS is an oncogene that drives the progression of about 30% of human cancers including colon, pancreatic, and lung. Each year, 3.4 million global and 19% of US cancer diagnoses are driven in part by a RAS gene mutation [19]. Yet, to date, only one FDA-approved drug exists that targets a specific mutant RAS isoform, KRASG12C, present in 13% of non-small cell lung adenocarcinoma (NSCLC) patients [20]. Although this discovery took over 30 years to come to fruition, it demonstrated that targeting the correct RAS mutation or isoform harbored in a tumor has therapeutic potential. It also brought to light the need for more RAS-targeted therapies despite decades of being deemed “undruggable”, as multiple oncogenic mutations, as well as wild type RAS, have been reported to drive tumor progression [21, 22].

There are four main RAS proteins: KRAS4A, KRAS4B, HRAS, and NRAS. Although unique gene products, all share 85% sequence homology, each containing a G-domain involved in GDP/GTP cycling through guanine nucleotide binding and GTP hydrolysis, as well as a hypervariable region at the C-terminus that contains sites for posttranslational modifications (PTMs) important for membrane association [23]. The Switch I and Switch II regions are conserved among all 3 isoforms and regulate RAS-protein interactions by undergoing changes in structural conformation during the

GTP/GDP cycle [23]. KRAS is far more frequently mutated in human cancer than the other 2 isoforms [21]. Different mutant RAS isoforms each have differing clinical presentation and prognoses, further complicating therapeutic targeting.

### *Mutant RAS in Cancer*

RAS gene mutations drive about 1/3 of human cancers, including NSCLC, colorectal and pancreatic. These mutations occur at a high frequency at codons 12, 13, and 61 across the 3 isoforms [24] and render RAS in a constitutively active state due to the loss of negative regulation, driving uncontrolled cellular proliferation and growth. The frequency at which these mutations occur varies by tissue and cancer type [24]. Mutations in codon 12 of KRAS occur at a high frequency and drive pancreatic (86%), lung (32%), and colorectal cancers (41%) [24]. Mutations in HRAS are more common in bladder (6%) and head and neck cancers (5%) at a lesser rate at codons 12 and 61, while NRAS mutations at codon 61 have been indicated in driving 29% of melanomas [24].

Despite the large number of oncogenic RAS mutants, only one RAS-targeted therapy exists to date. Sotorasib, a potent allele-specific inhibitor of KRASG12C (a transversion mutation in KRAS from a glycine to cysteine), was approved in 2020 for use in NSCLC patients, 13% of which harbor this specific mutation [20, 24]. It takes advantage of the highly reactive nature of mutant cysteine at codon 12, irreversibly binding it in an allosteric binding pocket behind the Switch II region [20]. This binding blocks RAS function by locking the molecule in its inactive, GDP-bound state, disallowing the cycling to its active GTP-state which is normally mediated by guanine nucleotide exchange factors (GEFs). Although the majority of KRASG12C tumors are GTP-bound at steady state, cancers with this mutation exhibit intrinsically high GTPase

activity, which normally aids in the cycling of RAS to its inactive state together with GTPase Activating Proteins (GAPs). This intrinsic nature together with the reactive nature of cysteine proved to have therapeutic utility for Sotorasib in lung adenocarcinomas.

## 2.2 RAS Model of Action

RAS is a small GTPase involved in cellular signaling through the transduction of extracellular signals from cellular membrane receptors to intracellular signaling pathways. Inactive in the cytosol, RAS first undergoes prenylation to enable anchoring to the cell membrane. This is mediated by farnesyl transferases which attach a farnesyl group to the cysteine residue of the CAAX motif located in the carboxy terminal conserved across all 3 RAS isoforms [23]. Farnesyl attachment allows RAS to be trafficked to the endoplasmic reticulum (ER), where the AAX motif is cleaved by RAS Converting Enzyme 1 (RCE1), and subsequent methylation of the farnesylated cysteine residue by isoprenylcysteine carboxymethyltransferase (ICMT) occurs [23, 25]. Trafficking to the plasma membrane is achieved through unique mechanisms dependent on the RAS isoform. HRAS and NRAS are first transported to the Golgi apparatus and then shuttled to the plasma membrane for anchoring via vesicular transport, while KRAS membrane anchoring is mediated by transport direct to the plasma membrane in a microtubule-dependent manner [23-25].

Once at the membrane, RAS activation occurs downstream of an activated receptor tyrosine kinase (RTK) such as HER2. RTKs are activated through receptor-specific ligand binding, leading to the autophosphorylation of tyrosine residues located



within the cytoplasmic domain [23, 26]. Phosphorylated tyrosine residues subsequently recruit the binding of proteins containing Src homology domain (SH) signaling motifs. The adaptor protein GRB2 (containing a SH2 domain and two SH3 domains), binds the receptor phospho-tyrosine residues via its SH2 domain, leading to the recruitment and binding of the guanine nucleotide exchange factor (GEF) Son-of-Sevenless (SOS) to its SH3 domain [23]. The GRB2-SOS complex brings SOS in a position to activate RAS by stimulating the replacement of GDP for GTP. Once bound by GTP, RAS is in its active state and able to bind downstream effector proteins to promote signaling.

### 2.3 Regulation of RAS Activity and Signaling

#### *RAS Guanine Nucleotide Exchange Factors (RASGEFs)*

RAS is in its active form when bound by GTP. Although cytosolic concentrations of GTP are about 10 times higher than GDP, and RAS has a higher affinity for GTP than GDP binding, the intrinsic disassociation of GDP from RAS is a rather slow process [27]. Additionally, nucleotide binding to RAS is very strong in nature and exhibits picomolar affinity [27-29]. For this reason, RASGEFs are essential for RAS activation by stimulating the cycling of GDP for GTP binding. They achieve this by weakening nucleotide binding to RAS, rendering the molecule in a nucleotide-free state [27-29]. RAS is then turned “on” through preferential association and loading with GTP. GTP-bound RAS can associate with downstream effector proteins through subsequent Switch I and Switch II region binding and conformational changes [27]. Although at least six RASGEFs have been identified that can mediate RAS activation, perhaps the most studied are members of the SOS family, SOS1 and SOS2 [30]. The involvement of

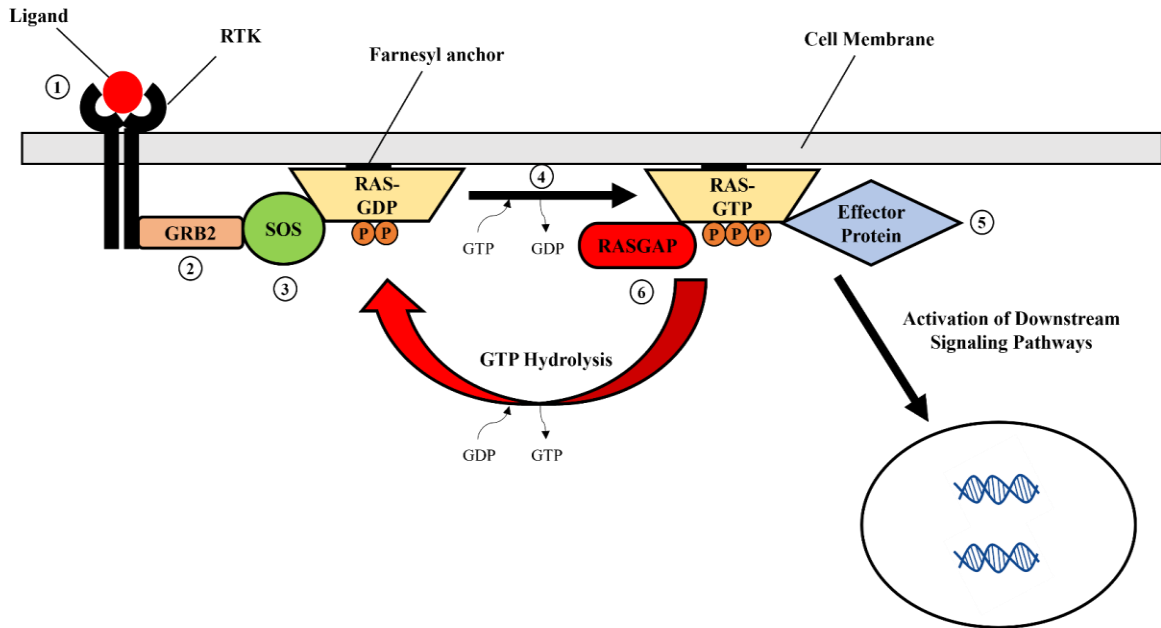
RASGEFs in cancer primarily works downstream of oncogenic RTK activation, as oncogenic mutations/amplifications of SOS1 as well as other RASGEF family members is rare in human cancer [30]. Upstream RTK hyperactivation due to oncogenic mutation is a driver of multiple cancer types and leads to elevated downstream RAS signaling.

#### *RAS GTPase Activating Proteins (RASGAPs)*

RASGAPs mediate the transition of RAS from its active, GTP-bound state to its inactive GDP-bound state. Although RAS itself has intrinsic GTPase activity, it hydrolyzes GTP at a very slow rate [31, 32]. An increase in the rate of GTP hydrolysis is mediated by the interaction with RASGAPs, leading to almost a 100-fold increase in GTPase activity and RAS effectively being cycled to its “off” state [23]. Mechanistically, a conserved arginine finger in a RASGAP is bound by RAS-GDP in its Switch regions [33]. The arginine residues of the RASGAP interact with phosphate groups on RAS involved in phosphoryl transfer, causing its stabilization in a transition state conformation that promotes the hydrolysis of GTP [31, 33]. Mutant RAS isoforms (such as those with missense mutations in codons 12, 13, and 61) exhibit GAP-independence due to their inability to form a stable transition state complex with a RASGAP [31]. This causes a decrease in the efficiency of GTP hydrolysis and leads to RAS being in a constitutively active state.

Due to their function in RAS inactivation, RASGAPs exhibit characteristics of tumor suppressors [34]. Loss of function mutations or decreased RASGAP expression, therefore, could promote RAS-driven tumorigenesis due to the lack of negative regulation. Indeed, this has been documented in several cancer types. For example, loss

of RASA2 is implicated in melanoma progression [35], while loss of function mutations in the NF1 gene occurs in about 23% of glioblastomas [21, 30]. In breast cancer, loss of expression of RASGAPs DAB2IP and RASAL2 has been shown to drive Luminal B breast cancers and increase the risk of metastasis [34, 36]. Indeed, although not directly involved in tumorigenesis, loss of RASGAP function or expression can indirectly drive cancer progression.



**Figure 2. RAS Model of Action.** (1) Ligand-mediated activation of a RTK such as HER2 leads to the autophosphorylation of tyrosine residues in its intracellular domain. (2) Bridging protein GRB2 binds phospho-tyrosine residues of the activated receptor via its SH2 domain, leading to the recruitment and binding of RASGEF SOS to its SH3 domain (3). The RTK-GRB2-SOS complex allows SOS to stimulate the cycling of GDP for GTP on RAS (4), leading to its activation. (5) GTP-bound RAS can then bind downstream effector proteins such as RAF of the MAPK pathway to cause transcription of genes involved in cell cycle progression, PI3K to activate downstream proteins involved in cell survival and growth, and RALGDS to activate proteins involved in endocytosis, exocytosis, cell proliferation and survival. Active RAS undergoes GTP hydrolysis mediated by RASGAP binding (7), causing its inactivation.

## 2.4 RAS Signaling Pathways

RAS can bind at least seven distinct downstream effector proteins of unique cellular signaling cascades to promote its biologic effects. Perhaps the most studied signaling pathways are the RAS/RAF/MAPK and RAS/PI3K pathways, and to a lesser extent the RAS/RALGDS pathway. Despite over three decades of research, RAS itself has been difficult to directly target and inhibit. This is evidenced by only the recent approval of the first RAS-targeted drug for use in NSCLC. Studies into signaling pathways downstream of RAS has led to the development of targeted inhibitors of both the MAPK and PI3K pathways. Although significant milestones, they have shown to be of little benefit to long term patient care [37]. This may suggest a larger involvement than previously thought of the other RAS signaling cascades in cancer progression.

### *RAS/RAF/MAPK Pathway*

The RAS/RAF/MAPK pathway is perhaps the most widely studied signaling cascade due to its hyperactivation in a multitude of human cancers. RAS activation of this pathway is mediated by RAS-GTP binding directly to the RAF kinase, leading to its localization to the cell membrane [23, 38]. This binding promotes a conformation of RAF which exposes Serine/Threonine residues to be phosphorylated, leading to RAF activation [23]. Subsequent downstream phosphorylation events cause the activation of downstream signaling proteins, with RAF phosphorylating and activating the kinases MEK1/2, and MEK1/2 activating ERK1/2. The activation of ERK leads to the transcription of genes within the nucleus that are involved in cellular proliferation and survival [23, 39]. Hyperactivation of this pathway can occur due to oncogenic mutations in RAS, leading to uncontrolled cellular growth and cancer progression. To date,

inhibitors for BRAF and MEK have been approved for use in cancers such as melanoma and neurofibromatosis type 1 (NF1). Crosstalk between this pathway and signaling proteins of different cascades occurs frequently, further complicating its therapeutic targeting.

#### *RAS/PI3KCA Pathway*

RAS activation of PI3KCA is mediated by its binding to the PI3KCA RBD of its p110 catalytic region, leading to its recruitment to the plasma membrane. Once PI3KCA is activated, PDK1 and AKT are localized to the membrane by PIP3, where AKT is phosphorylated and activated by the dual activity of PDK1 and mTORC2 on unique Serine/Threonine residues [23]. Activated AKT promotes cellular survival and proliferation through unique mechanisms, including the inhibition of proteins involved in apoptosis (Bcl-2 proteins) and activation of negative regulators of p53 such as MDM2 [23]. Currently, FDA-approved inhibitors of this pathway target the p110 subunit of PI3KCA and mTORC and have been used for applications in chronic lymphocytic leukemia (CLL) and lymphomas. However, the clinical utility of these drugs has proven to be limited [23, 37]. Treatment resistance may in part be since PI3K can be activated independently of RAS by RTKs through its p85 regulatory domain [23]. Additionally, although PI3KCA is the most mutated family member, two additional PI3K families exist, further complicating therapeutic targeting in addition to potential crosstalk with proteins of other pathways

### *RAS/RALGEF Signaling Pathway: Implications in Tumor Progression*

The RAS/RALGEF signaling pathway is a significantly lesser studied arm of RAS-effector signaling. However, growing evidence continues to support its importance in RAS-driven tumorigenesis. RAS is connected to this pathway through its binding to the RAS Association (RA) domain of RALGEFs (RAS-like Guanine Nucleotide Exchange Factors) such as RALGDS, leading to its recruitment to the cell membrane [23]. Here it activates downstream RAL proteins (RALA and RALB) through the cycling GDP for GTP. Activated RAL can then activate downstream proteins (e.g. RALBP1) to influence biologic processes essential for tumorigenesis such as actin organization, gene transcription, and cellular proliferation and survival [23, 40].

The importance of the RALGEF pathway in cancer progression has and continues to be well documented. In pancreatic cancer, when compared to the MAPK and PI3K pathways, hyperactivation of the RAL pathway is more frequently observed in patient tumors [41]. Further, suppression of RALA in pancreatic models of cancer demonstrated a decrease in anchorage-dependent growth [40]. High RALA expression in prostate cancer has been shown to correlate with tumor aggression in prostate cancer patients, and studies have shown its expression to correlate with bone metastasis [40, 42]. In models of skin cancer, RALGDS-knockout mice demonstrated delayed tumor development by up to 4 weeks after being induced for squamous cell carcinoma, while knockout of RALGDS in vitro resulted in no cellular transformation without having an effect on cellular proliferation [43]. In breast cancer, in vivo knockout of RALA in a patient-derived xenograft (PDX) model of TNBC inhibited tumor growth and metastasis [44]. Indeed, the involvement of the RAL pathway in the progression of multiple cancer types makes it a

promising therapeutic target. Unfortunately, to date no approved inhibitors exist for this pathway.

## 2.5 Role of wild type RAS in Luminal B Breast Cancer

Since the discovery of RAS and its involvement in cancer, the majority of RAS-targeted therapies have been focused on targeting its oncogenic mutant forms and downstream signaling pathways. The push for mutant-specific therapies was in part due to toxicity concerns when targeting the wild type isoform, as knockout of KRAS in mice has demonstrated to cause lethality during embryogenesis [45, 46]. However, increased findings of wild type RAS driven cancers makes the development of pan-RAS inhibitors an attractive therapeutic option.

RAS has historically been neglected as a potential driver of cancers that lack RAS mutations. However, studies have demonstrated that despite the lack of RAS mutations, loss of function of its negative regulators (i.e. RASGAPs) results in a tumor promoting role for wild type RAS. This has been shown to be true in cancers such as glioblastomas, melanomas, and some cases of NSCLC, where loss of function mutations in the RASGAP NF1 causes a hyperactivation of wild type RAS signaling without RAS mutation [47-50]. In cancers such as breast and medulloblastomas that lack RAS mutations, epigenetic inactivation of the RASGAP DAB2IP occurs at a high rate [22]. Increased upstream signaling from RAS can also result in hyperactivation of RAS signaling. In cancers such as lung adenocarcinomas [22] where RTKs (such as HER2) are overexpressed or suffer oncogenic mutations, signaling through RAS is subsequently elevated due to decreased upstream negative regulation.



Luminal B cancer is an aggressive breast cancer subtype that accounts for about 10-15% of breast cancer cases. Historically, RAS has not been implicated in breast cancer progression due to the low rates of oncogenic mutations, accounting for only 1% in breast tumors [52, 53]. Despite this, RAS signaling has been reported to be amplified in luminal breast cancer patients and correlate with increased rates of metastasis [54]. Although the availability of HER2 and hormone-targeted therapies has improved the treatment of luminal tumors, this subtype shares rates of relapse like the more aggressive TNBC subtype in part due to low sensitivity to treatment [51]. This suggests the involvement of other molecular drivers in the progression of Luminal B tumors. Studies by Olsen et. al. demonstrated that there is decreased mRNA and protein expression of either DAB2IP or RASAL2 in up to 62% of Luminal B cancers [36]. Mechanistically, this is due to epigenetic inactivation caused by promoter hypermethylation, and loss of both RASGAPs was found to correlate with elevated RAS activation and signaling, as well as increased metastasis and invasion [36]. Hyperactivated RAS signaling, therefore, is indirectly caused by decreased RASGAP-mediated negative regulation. This makes RAS a promising therapeutic target in this subtype.

We hypothesized, therefore, that Luminal B breast cancers are sensitive to the inhibition of wild type RAS. We sought to test this hypothesis by using a novel direct RAS inhibitor developed in-lab in collaboration with the Molecular Modeling Facility and Medicinal Chemistry Core at the University of Louisville. Our lead compound, coined F3860, was tested against model systems of Luminal B cancer to determine its efficacy against this subtype.

## MATERIALS AND METHODS

### *Tissue Culture, Cell Lines, and Plasmids*

BT474, MDA-MB-361, S462.TY, and HEK-293T cells were purchased from ATCC (Manassas, VA) and maintained in culture with DMEM (Dulbecco's Modified Eagle Medium - Corning) supplemented with 1% penicillin/streptomycin and 10% or 20% (MDA-MB-361) fetal bovine serum (FBS - Sigma). MCF-10A cells obtained from ATCC were maintained in DMEM/F12 medium (Corning) supplemented with 1% penicillin/streptomycin, 10 $\mu$ g/mL insulin, 0.5mg/mL hydrocortisone, 20ng/mL EGF, and 5% horse serum. All cell lines were maintained at 37 degrees Celsius and 5% CO<sub>2</sub>. GFP-tagged RALGDS construct was purchased from Addgene. pCGN-HA-HRAS12V construct was made as previously described [55]. Full length HRAS12V cDNA was cloned into a pCGN vector under control of a CMV promoter.

### *Western Blot Analysis*

Cell lysates were quantified using BioRad protein quantification assay. Equal amounts of cell lysates (20 $\mu$ g) and immunoprecipitates were combined with LDS buffer, vortexed, and boiled at 100 degrees Celsius for 5 minutes to enable protein denaturation. Samples were then subjected to SDS-PAGE electrophoresis through loading on a NuPAGE 4-12% polyacrylamide gel. Electrophoresis was performed at 120V for 90 minutes in NuPAGE MOPS SDS Running Buffer, after which proteins were transferred to a 0.2 $\mu$ M nitrocellulose membrane using wet transfer technique at 10V for 3 hours in 1X transfer buffer mixed with methanol and MilliQ water. Membrane with transferred proteins was then blocked for 1 hour in 5% nonfat dry milk dissolved in 1X

TBST, after which overnight incubation took place with respective antibodies.

Membranes were washed 3 times with 1X TBST then incubated at room temperature with mouse or rabbit secondary antibody. After a second wash protocol, blots were subjected to West Pico PLUS solution for 5 minutes and bands detected using autoradiography and chemiluminescence film.

#### *Soft Agar Colony Formation Assays*

1.8g of powdered agar was dissolved in 100mL 1X PBS, autoclaved and allowed to equilibrate to 42 degrees Celsius to create a 1.8% agar solution. To determine the effect of drug treatment on anchorage-independent growth, a 1mL bottom agar layer consisting of a 0.6% agar mix (1.6mL FBS, 32mL DMEM or DMEM/F12, and 16mL 1.8% agar solution) was first added to a 12-well plate. Cells were then seeded at appropriate seeding densities and resuspended in 3mL of agar/medium mix (1mL DMEM or DMEM/F12, 2mL 0.6% agar mix) in the absence or presence of inhibitor. Effect on anchorage-independent growth was determined after 2 – 6 weeks of incubation by manual counting of colony formation. Assays are performed in duplicate for each concentration of drug tested.

#### *2D Growth Curves*

2D standard growth curves were performed by plating cells at the appropriate seeding density for each cell line tested in a 12-well plate. 24 hours after plating, cells were trypsinized and counted, corresponding to “Day 0”. Media was replaced with or without the presence of 1 $\mu$ M F3860, and cells were trypsinized and manually counted at the same time each subsequent day for a total of 7 days. Cell counts each day were taken as an

average of two duplicate counts.

### *Co-Immunoprecipitation Experiments*

HEK-293T cells were grown to 70% confluence in 60mm dishes then transiently transfected using jetPRIME® transfection reagent. Briefly, 250ng and 500ng of pCGN-HA-HRAS12V and GFP-RALGDS, respectively, were diluted in 200µL transfection buffer, then added dropwise together with jetPRIME® transfection reagent to the cells. Media was refreshed after 8 hours, and transfection allowed to continue for 24 hours. To determine the effect of RAS inhibition, cells were treated with 10µM F3860 for 1 hour and lysed in a modified NP40 lysis buffer (10mM Tris-Hcl pH 7.5, 150mM NaCl, 0.5mM EDTA, 0.5% NP40, mixed together in MilliQ water) together with a protease and phosphatase inhibitor cocktail. Lysates were passed through a 21-gauge needle 3 times then cleared by centrifugation for 3 minutes at 13,000rpm at 4 degrees Celsius. Cleared lysates were quantified using BioRad protein quantification. 1mg of protein was then immunoprecipitated for GFP using GFP-Trap® agarose for 30 minutes at 4 degrees Celsius, washed 3 times using a modified wash buffer (10mM Tris-Hcl pH 7.5, 150mM NaCl, 0.5mM EDTA in MilliQ water), then subjected to SDS-PAGE electrophoresis for Western Analysis. For endogenous co-immunoprecipitation, BT474 cells were treated with F3860 and similar lysis methods followed. Cells were immunoprecipitated for 30 minutes using rat monoclonal pan-RAS conjugated beads. Protein association was determined through blotting with HA or c-RAF antibodies in overexpression or endogenous experiments, respectively.

### *RAL Activation Assays*

Determination of active RALA levels was performed according to protocols set by the RAL Activation Assay Kit (MilliporeSigma). Briefly, cells were grown to 80% confluence in 60mm plates and treated for 1 hour with F3860. Treated cells were then lysed with RAL Activation Buffer (RAB) together with protease and phosphatase inhibitors, cleared with glutathione agarose, then quantified using BioRad protein detection assay. 1mg of protein was then immunoprecipitated with 15 $\mu$ L of RALBP1 glutathione agarose conjugated beads for 30 minutes. After collecting beads through pulsing for 5 seconds at 14,000rpm, immunoprecipitates were washed three times with ice cold RAB. Lysates and immunoprecipitates were subjected to SDS-PAGE and subsequently probed with the provided RALA antibody.

### *Xenograft Experiment*

2x10<sup>6</sup> BT474 cells were grafted through IP injection into the mammary fat pad of NOD scid gamma (NSG) immunodeficient mice in a 50:50 Matrigel mix. Once tumors reached an appropriate volume of 50mm<sup>3</sup>, mice were treated with carrier or 1mg/kg F3860 by IP administration every other day for 12 days. Mice were euthanized after 2 weeks after which fold increase in tumor volume was determined in carrier vs. treatment group.

### *Statistical Analysis*

All statistical analyses were performed using GraphPad Prism. One-way ANOVA was used for soft agar colony formation and xenograft experiments. Student t-test was used to determine significance in western blot analyses. p-values < 0.05 were considered significant.

## RESULTS

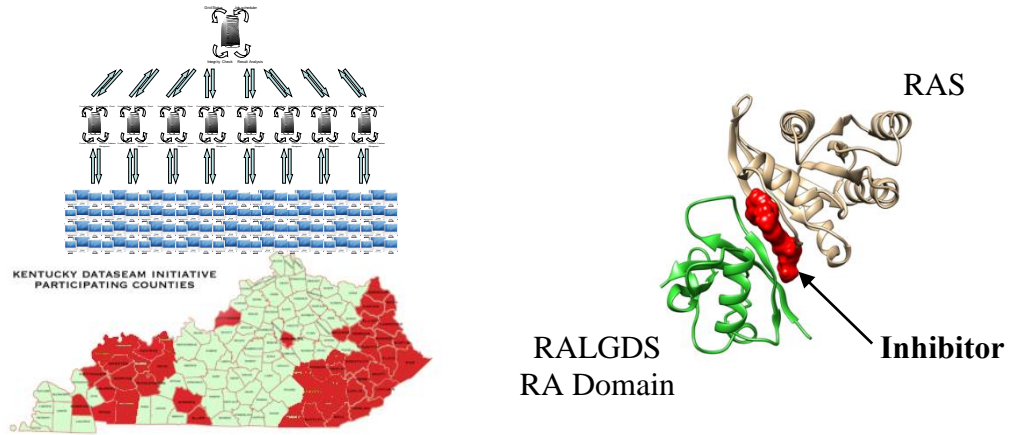
### *Identification of F3860: A Novel RAS Inhibitor*

In collaboration with the Kentucky DataSeam Initiative and Dr. John O. Trent of the University of Louisville Molecular Modeling Facility, 24,000 computers were placed in 52 K-12 Kentucky school districts to facilitate learning in underprivileged communities. This allowed for a continuous *in silico* screening of over 1 million compounds contained within SurflexDock and Molplex screening libraries (Figure 3A). Screening was modeled for compounds to target and inhibit binding of RALGDS to RAS-GTP in a binding groove near the effector binding region of RAS (Figure 3A). Top candidates were then screened *in vitro* by Dr. Geoffrey J. Clark using 3D soft agar assays against RAS-driven cancer cell lines to determine inhibitory effects on anchorage-independent growth: a hallmark of malignant transformation. Cancer cells can avoid anoikis (a form of apoptotic cell death induced in 3D suspension upon removal from extracellular matrix), achieving this in part due to oncogenic RAS signaling [56]. Hence, compounds that caused inhibition in 3D but not in 2D cell culture systems at the same concentration were selected for further characterization to avoid drugs that exhibited non-specific toxicity. This led to the identification of our parent compound F3. Collaboration with the University of Louisville Medicinal Chemistry Core and several rounds of optimization led to the development of our lead compound F3860: an increased activity variant of F3. Binding of F3860 to wild type KRAS was confirmed by analytical ultracentrifugation (AUC) and microscale thermophoresis (MST) assays (figures not shown). AUC confirmed the preferential co-sedimentation of F3860 with GTP-loaded KRAS, while MST assays revealed a  $K_d$  of around 2 - 15 $\mu$ M for KRAS-GTP. Nuclear

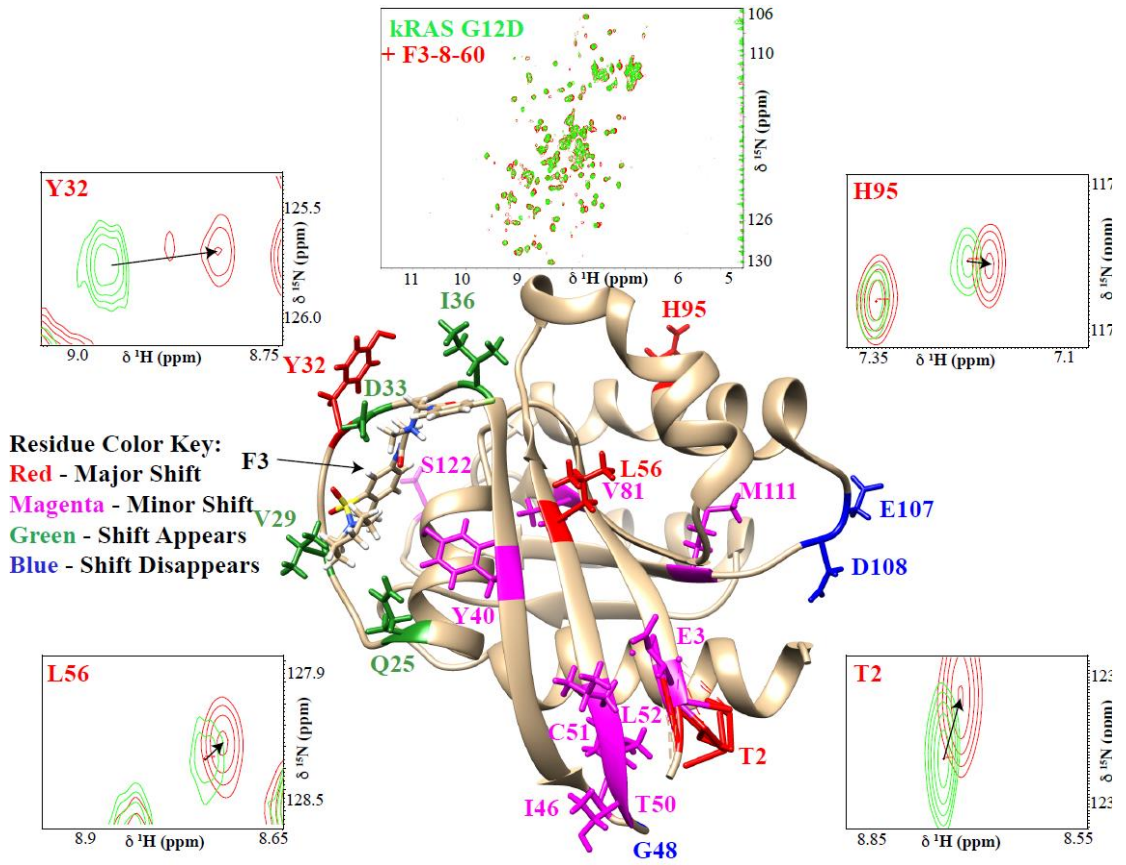
Magnetic Resonance (NMR) studies performed by Dr. T. Michael Sabo demonstrated F3860 disruption of RAS mutant effector binding as evidenced by emission spectra shift in residues of the RAS effector binding domain necessary for RAS effector interaction (Figure 3B). Endogenous co-immunoprecipitation of RAS and c-RAF in wild type RAS-driven BT474 human Luminal B cells further proved the binding of F3860 to RAS, as evidenced by a loss in RAS/c-RAF association with F3860 treatment (Figure 3C).

**Virtual Screening**  
**Kentucky Dataseam University of Louisville**  
**Public-Private Partnership**

3A.

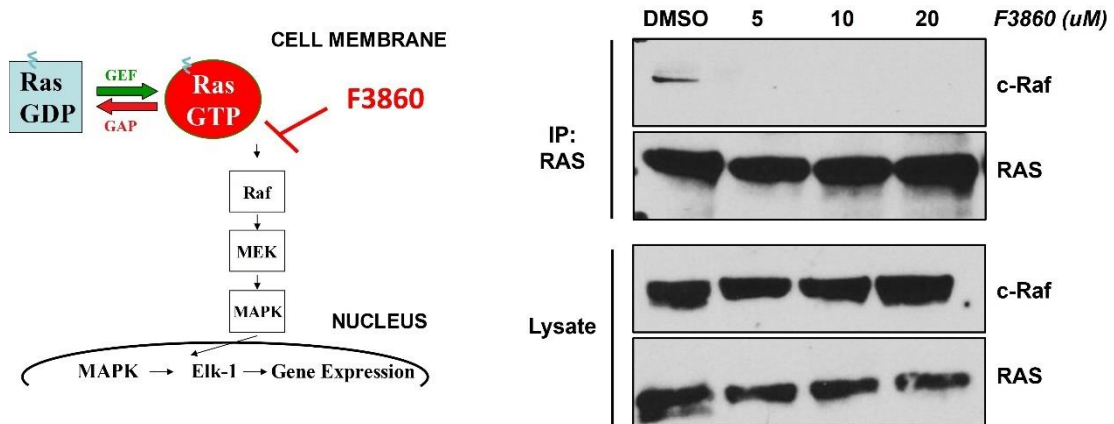


3B.





3C.

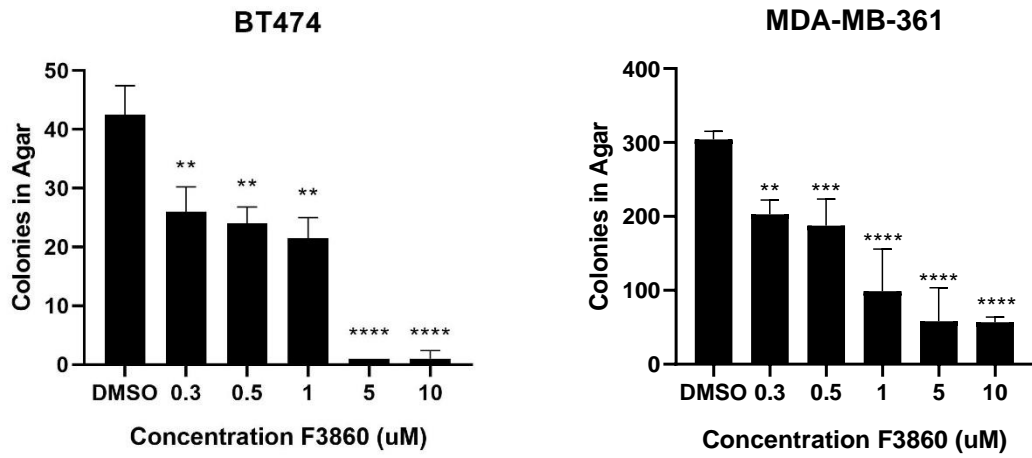


**Figure 3. Identification of novel RAS inhibitor F3860.** A) Schematic representation of in silico screening. The inhibitor was modeled to bind RAS and disrupt its association with the RAS Association (RA) domain of RALGDS. B) NMR of F3860 binding of mutant KRASG12D. F3860 was found to bind and inhibit RAS-effector interaction as evidenced by emission spectra shift in residues required for downstream effector binding. C) F3860 binds and inhibits RAS/RAF association. BT474 cells were treated with 5, 10, or 20 $\mu$ M of F3860 and immunoprecipitated using pan-RAS conjugated beads to determine effect on RAS/RAF association. F3860 effectively blocked this interaction.

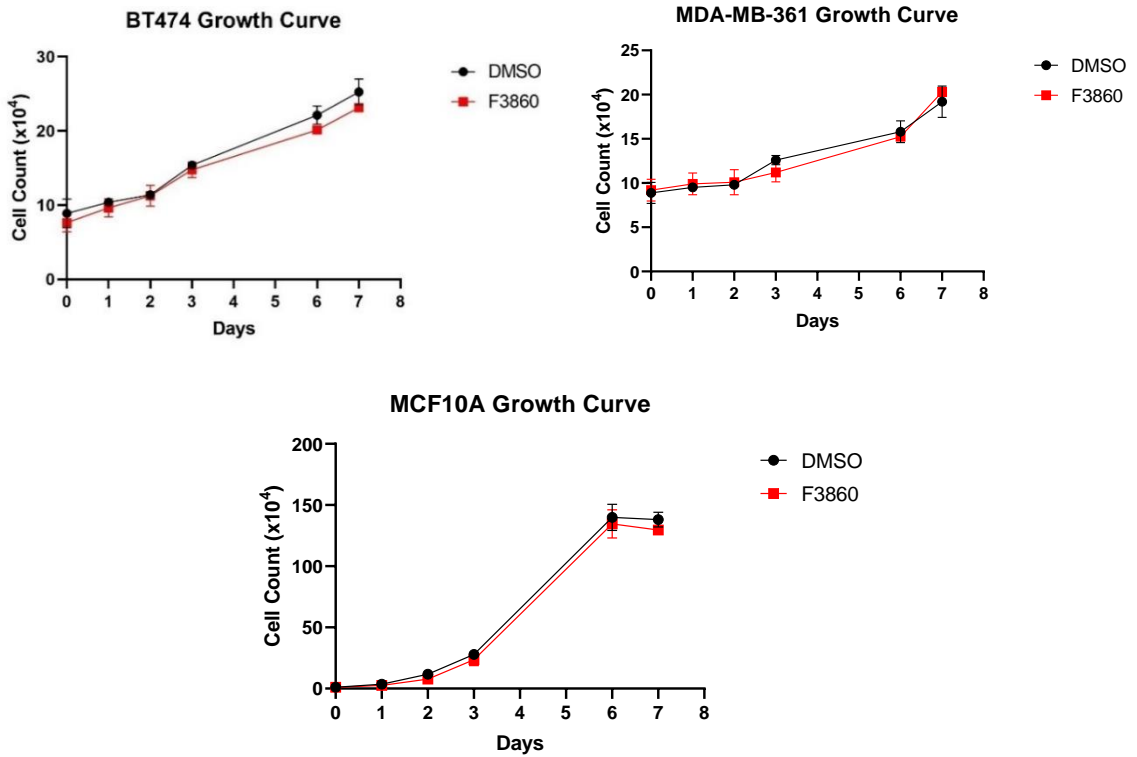
*F3860 inhibits 3D Tumor Colony Growth without exhibiting 2D Growth Toxicity in Luminal B Breast Cancer Cells*

To investigate the effects of F3860 treatment on anchorage-independent growth, human BT474 and MDA-MB-361 Luminal B cells were treated at concentrations ranging from 0.3 to 10  $\mu$ M of F3860 in 3D soft agar colony formation assays. These cell lines have been characterized previously, with MDA-MB-361 exhibiting joint loss of expression of both RASGAPs DAB2IP and RASAL2, while BT474 cells exhibit RASAL2 loss but maintain DAB2IP levels [36]. We found that at all concentrations tested, F3860 caused significant inhibition of 3D colony formation when compared to control, with an IC<sub>50</sub> of around 1  $\mu$ M (Figure 4A). In 2D growth curves for each cell line, treatment with 1  $\mu$ M F3860 demonstrated no significant effect on cell proliferation when compared to DMSO control (Figure 4B). To determine the effect of F3860 treatment on normal breast epithelial cells, MCF10A cells were treated at 1  $\mu$ M F3860 in a similar 2D growth assay. Consistent with the Luminal B cell lines, no significant effect on cell proliferation was observed (Figure 4B).

4A.



4B.

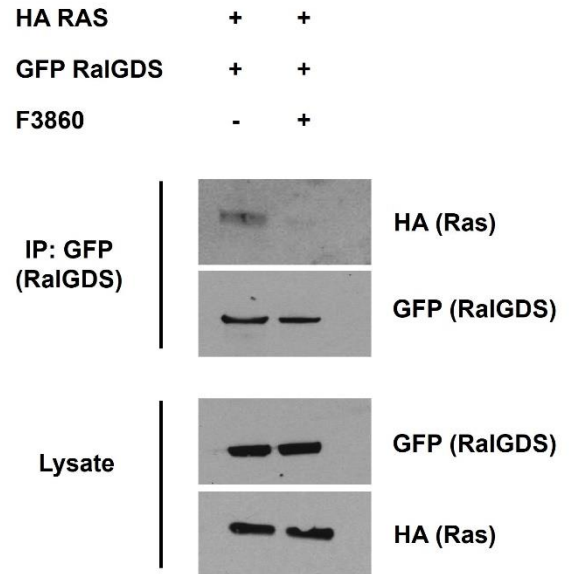
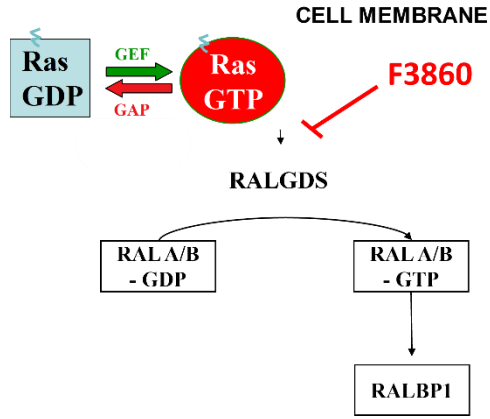


**Figure 4. F3860 inhibits 3D anchorage-independent growth in Luminal B cell lines without affecting cell proliferation in 2D.** **A)** BT474 and MDA-MB-361 Luminal B cells were treated with F3860 at concentrations of 0.3, 0.5, 1, 5 and 10 $\mu$ M in soft agar colony formation assays. The number of colonies formed were scored after 4 weeks for BT474 cells and 6 weeks for MDA-MB-361. Significant inhibition as compared to DMSO control was seen at all concentrations, with an IC<sub>50</sub> of around 1 $\mu$ M. **B)** 2D growth curves in the presence or absence of 1 $\mu$ M F3860 for BT474, MDA-MB-361, and MCF10A cells. Treatment with F3860 saw no significant difference between control and treated cells.

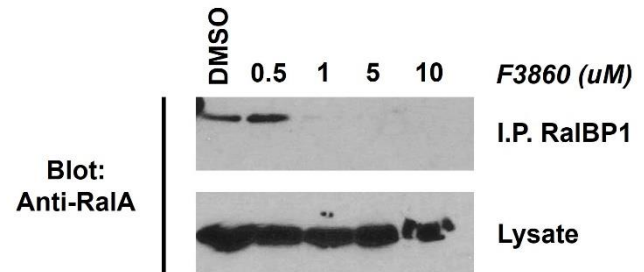
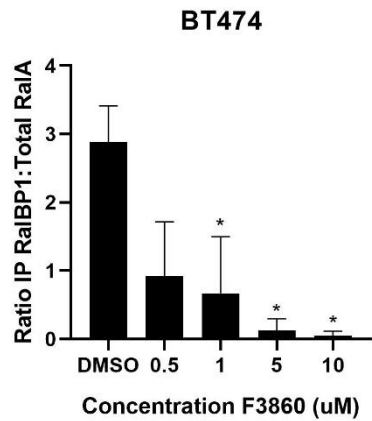
*F3860 blocks the interaction of RAS with RALGDS and inhibits downstream RAL Signaling*

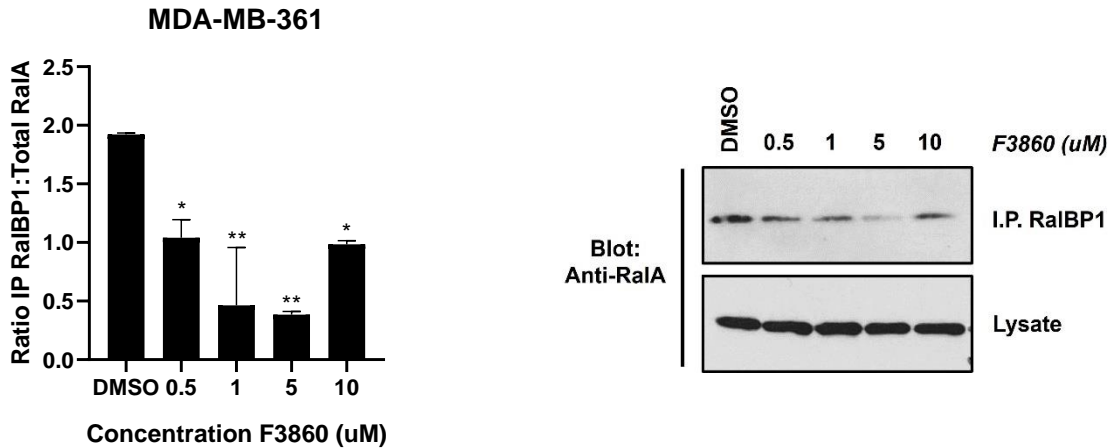
Our novel RAS inhibitor F3860 was modeled to inhibit the binding of active RAS to its downstream effector RALGDS of the RAL signaling pathway. To confirm this, an in vitro co-immunoprecipitation experiment using tagged HRAS12V (a mutant HRAS construct) and RALGDS constructs was performed. Constructs were overexpressed in HEK-293T cells, subjected to 1 hour of 10 $\mu$ M F3860 treatment, and immunoprecipitated to determine the presence or absence of protein association. We found that, when compared to control, F3860 inhibited the association of RAS with RALGDS (Figure 5A). Since RAS/RALGDS association was inhibited, we next wanted to determine the effect of F3860 on downstream RAL signaling. BT474 and MDA-MB-361 cells were treated with concentrations of 0.5 to 10 $\mu$ M F3860 for 1 hour, then immunoprecipitated for RALBP1 (a downstream effector of RALA) to determine levels of active RAL when compared to lysate control. We found that in both cell lines, significant inhibition of RAL activation was seen at concentrations as low as 500nM (Figure 5B).

5A.



5B.

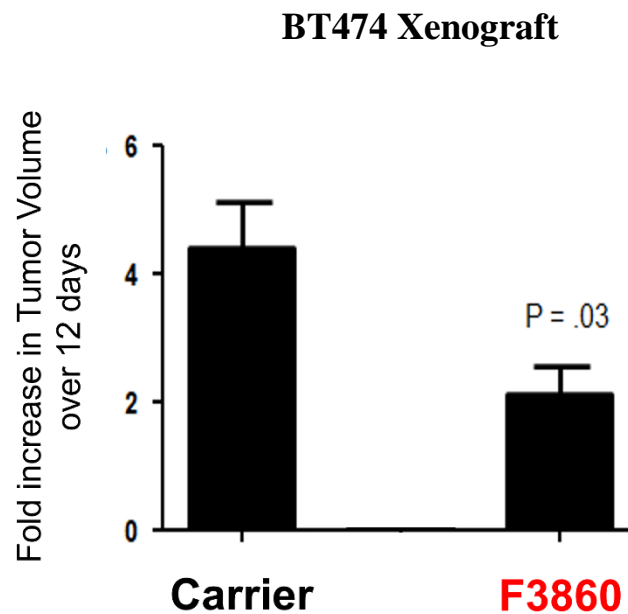




**Figure 5. F3860 blocks RAS/RALGDS association and RALA activation in Luminal B cells.** **A)** HEK293-T cells were co-transfected overnight with pCGN-HA-HRAS12V and GFP-RALGDS DNA constructs. After 24 hours, cells were treated with DMSO control or 10μM F3860, immunoprecipitated for RALGDS using GFP-Trap agarose beads, then blotted for RAS with HA antibody to determine protein association. RAS/RALGDS association was blocked with F3860 treatment. **B)** BT474 and MDA-MB-361 cells were treated with F3860 at concentrations of 0.5, 1, 5, and 10μM F3860. Cells were then immunoprecipitated for RALBP1 as an indicator of active RAL, then blotted for RALA to determine effect on its activation status. F3860 caused significant inhibition of activation at all concentrations tested across both cell lines as evidenced by densitometric analysis. Blots are representative of 2 independent experiments. Student t-test was performed to determine statistical significance ( $p < 0.05$ ).

*F3860 inhibits Luminal B tumor growth rate in vivo*

To determine whether F3860 exhibited activity in vivo, we tested it in an orthotopic xenograft assay. NSG mice were grafted with  $2 \times 10^6$  BT474 Luminal B cells and treated every other day with IP injection of 1mg/kg F3860 over a 12-day period. We found that, when compared to control, F3860 caused a significant reduction in the rate of tumor growth with no apparent toxicities (Figure 6).



**Figure 6. F3860 decreases the rate of tumor growth in an in vivo model of Luminal B breast cancer.** Statistical significance determined by one-way ANOVA followed by post-hoc Tukey test. Data is representative of mean +/- standard error of the mean



## DISCUSSION

Historically, RAS has not been considered in the context of breast cancer due to its low rate of oncogenic mutations. Despite this, hyperactivation of RAS signaling is observed in greater than 50% of breast tumors [34]. In Luminal B breast cancers, loss of expression of either DAB2IP or RASAL2, which normally function as RASGAPs to negatively regulate RAS function, is seen in 62% of tumors [34]. This may confer a tumor promoting role for wild type RAS as loss of negative regulation causes RAS to be in an unregulated activated state. To date, only one approved RAS-targeted therapy exists for the RAS mutant KRASG12C in NSCLC. Therapeutic targeting of wild type RAS in Luminal B tumors has not been explored.

In this study, we have identified a novel RAS inhibitor coined F3860, modeled to inhibit RAS/RALGDS effector association. NMR studies confirmed binding of F3860 to mutant KRASG12D in the groove in which it binds its downstream effectors. This was evidenced by emission spectra shifts in conserved residues of the RAS effector binding domain. Tyrosine residue 32 of RAS is conserved across all isoforms and has been shown to be essential for RAS-effector interactions. In one study, phosphorylation of Tyr32 by Src inhibited RAS/RAF binding [57]. The tyrosine phosphatase SHP2 dephosphorylates RAS at Tyr32, and pharmacological inhibition of SHP2 suppresses tumor growth [58]. A major emission spectra shift in this residue by F3860 demonstrates a disruption of RAS-effector binding through conformational change in structure. Likewise, the appearance of a shift in residues such as Asp33 further confirms drug binding. This residue is part of the flexible effector binding loop of RAS, so the appearance of a shift suggests F3860 binding and stabilization of the groove to interrupt effector interaction. The binding of

F3860 to wild type RAS has also been proven through MST and AUC studies (data not shown), as well as in an endogenous co-immunoprecipitation experiment of RAS/c-RAF in BT474 Luminal B cells (Figure 3C).

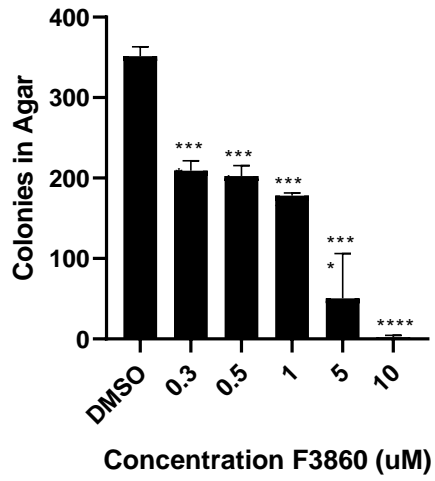
The use of soft agar colony formation assays allows for the determination of malignant cell transformation in vitro as determined by anchorage-independent growth. Cancer cells develop resistance to anoikis, a form of apoptosis induced upon detachment from basement membrane, to promote tumor survival and metastasis. This assay therefore allows for the screening of potential drugs in a system that more closely recapitulates the 3D environment seen in vivo. In BT474 and MDA-MB-361 cells, F3860 caused significant inhibition of tumor colony formation at concentrations as low as 0.5 $\mu$ M (Figure 4A), with 50% inhibition seen at around 1 $\mu$ M. 2D growth curves tested at the same concentration had no significant effect on cellular proliferation both in Luminal B and normal breast epithelial cells, boding well for toxicity concerns (Figure 4B). Treatment group mice from our in vivo experiment also showed no apparent toxicity while still demonstrating a decrease in tumor growth rate (Figure 6).

Mechanistically, F3860 was found to inhibit the interaction of RAS with RALGDS in overexpression experiments (Figure 5A), although future biologic experiments with RALGDS shRNA need to be performed as confirmation of F3860 working through this pathway. This inhibition, however, coincided with a decrease in downstream active RALA levels in BT474 and MDA-MB-361 cells (Figure 5B). To date, no inhibitors of the RAS/RAL interaction exist despite evidence suggesting its importance in RAS-driven tumorigenesis and malignant cell transformation [40-44]. Previous studies have demonstrated that breast tumors have an elevated expression of

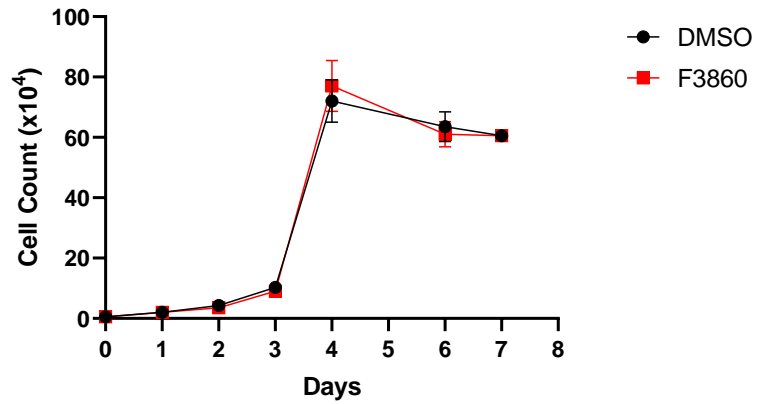
HRAS when compared to other RAS isoforms [59-61]. HRAS expression has also been shown to be present in malignant breast tumors and absent in benign and normal breast tissue, suggesting a role for HRAS in breast cancer progression [59]. For this reason, a HRAS12V DNA construct was used for overexpression studies, representative of constitutively active HRAS. The presence of RAS in malignant lesions, and its absence in normal breast tissue, coincides with results seen in 2D growth assays performed in MCF10A cells and bodes well for toxicity concerns.

Apart from breast cancer, wild type RAS signaling can drive the progression of other tumors with loss of RASGAP activity. Malignant Peripheral Nerve Sheath Tumors (MPNST) are lethal sarcomas lacking effective treatment options. About half of MPNSTs arise from cases of Neurofibromatosis Type 1 – a genetic condition caused by mutation in the GAP NF1 [63]. These tumors lack RAS mutations and may therefore be driven in part by wild type RAS. Using the MPNST cell line S462.TY, we found F3860 to inhibit 3D soft agar colony formation and RAL signaling without affecting cell proliferation in 2D (Figure 7). This demonstrates a potential therapeutic option for MPNSTs, and strengthens the case for wild type RAS as a target in cancers with loss of RASGAP activity.

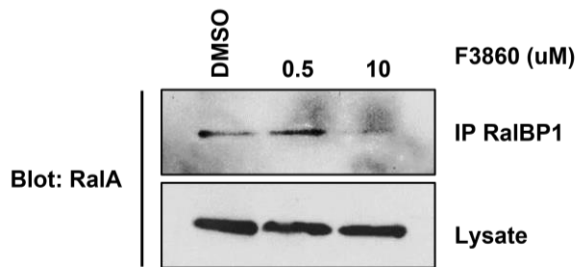
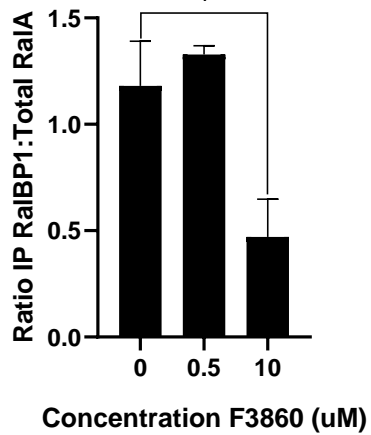
### S462.TY



### S462.TY Growth Curve



### S462.TY



**Figure 7. F3860 demonstrates efficacy against MPNST cells.** (*Top*) S462.TY cells were grown in 3D soft agar assays in the absence or presence of F3860 at concentrations of 0.3, 0.5, 1, 5, and 10 $\mu$ M. After 2 weeks of incubation, plates were manually scored to determine effect on colony formation. F3860 caused significant inhibition at all concentrations tested without an effect on proliferation in 2D at 1 $\mu$ M F3860 treatment (*Middle*). (*Bottom*) Cells were treated for 1 hour with F3860, immunoprecipitated for RALBP1, then blotted for RALA to determine effect on RALA activation levels. Densitometric analysis revealed significant inhibition at 10 $\mu$ M.

## SUMMARY AND CONCLUSIONS

Numerous cancer types can be driven by constitutively active wild type RAS due to loss of its negative regulators. This is true in Luminal B breast cancers which exhibit a joint loss of RASGAPs DAB2IP and RASAL2 in 62% of cases. Loss of RASGAP expression or function is also commonly seen in melanomas, MPNSTs, and NSCLC, where NF1 is selectively mutated, and in medulloblastomas where DAB2IP inactivation occurs at a high rate. Indeed, although the focus has historically been on targeting and inhibiting mutant RAS, hyperactivation of wild type RAS has also demonstrated to play an important role in tumor progression.

In this study, we have identified a novel RAS inhibitor, F3860, that binds and inhibits the interaction of RAS with its downstream effectors. Proof-of-principle experiments demonstrated its efficacy in Luminal B and MPNST model systems where RAS lacks oncogenic mutations and has not been considered as a therapeutic target. These findings provide rationale for considering wild type RAS as a novel therapeutic option in non-mutant RAS cancers

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## LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
AKT	Protein Kinase B
ATM	Ataxia Telangiectasia Mutated
AUC	Analytical Ultracentrifugation
BCL-2	B-Cell Lymphoma 2
BRAF	v-Raf Murine Sarcoma Viral Oncogene Homolog B1
BRCA1/2	Breast Cancer Gene 1/2
CAAX	Cysteine-Aliphatic-Aliphatic-any amino acid
CDK4/6	Cyclin-dependent Kinases 4 and 6
CHEK2	Checkpoint Kinase 2
CK14	Cytokeratin 14
CK5/6	Cytokeratin 5 and 6
CMV	Cytomegalovirus
DAB2IP	Disabled Homolog 2-Interacting Protein
DCIS	Ductal Carcinoma In Situ
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
ER	Endoplasmic Reticulum
ERK1/2	Extracellular Signal-Regulated Kinase 1 and 2
GDP	Guanosine Diphosphate

GFP	Green Fluorescent Protein
GTP	Guanosine-5'-Triphosphate
HA	Human Influenza Hemagglutinin
HER2	Human Epidermal Growth Factor Receptor 2
HR	Homologous Recombination
HRAS	Harvey Rat Sarcoma Virus
IC50	Half-maximal Inhibitory Concentration
ICMT	Isoprenylcysteine Carboxymethyltransferase
IP	Intraperitoneal
Ki-67	Kiel-67
KRAS	Kirsten Rat Sarcoma Virus
LDS	Lithium Dodecyl Sulfate
MAPK	Mitogen-Activated Protein Kinase
MDM2	Mouse Double Minute 2 Homolog
MEK1/2	Mitogen-Activated Protein Kinase Kinase 1 and 2
MPNST	Malignant Peripheral Nerve Sheath Tumor
MST	Microscale Thermophoresis
mTOR	Mammalian Target of Rapamycin
NF1	Neurofibromatosis Type 1
NMR	Nuclear Magnetic Resonance
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
NSCLC	Non-Small Cell Lung Cancer
NSG	NOD Scid Gamma

PALB2	Partner and Localizer of BRCA2
PBS	Phosphate Buffered Saline
PDK1	3'-Phosphoinositide-Dependent Kinase 1
PD-L1	Programmed Death-Ligand 1
PDX	Patient Derived Xenograft
PI3K	Phosphoinositide 3-Kinase
PI3KCA	Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha
PIP3	Phosphatidylinositol (3,4,5)-Triphosphate
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homolog
PTMs	Post Translational Modifications
RAF	Rapidly Accelerated Fibrosarcoma
RALA	RAS-Like Proto-Oncogene A
RALB	RAS-Like Proto-Oncogene B
RALBP1	RALA Binding Protein 1
RALGDS	RAL Guanine Nucleotide Dissociation Stimulator
RALGEF	RAL Guanine Nucleotide Exchange Factor
RAS	Rat Sarcoma Virus
RASA2	RAS p21 Protein Activator 2
RASAL2	RAS Protein Activator Like 2
RASGAP	RAS GTPase-Activating Protein
RASGEF	RAS Guanine Nucleotide Exchange Factor

RBD	RAS Binding Domain
RCE1	RAS Converting Enzyme 1
RTK	Receptor Tyrosine Kinase
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SH2	Src Homology Domain 2
SH3	Src Homology Domain 3
SOS	Son of Sevenless
TBST	Tris-Buffered Saline with Tween
TNBC	Triple Negative Breast Cancer
TP53	Tumor Protein p53



## CURRICULUM VITA

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(May 22 – 25, 2022)  
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Awarded: October 26, 2021

### PRESENTATIONS:

September 20, 2022:

Jigo, Raphael N, Donninger, Howard, Ferrill, Rachel, Von Baby, Becca, Burlison, Joe, Trent, John, Sabo, Michael, Clark, Geoffrey J. *A Novel RAS Inhibitor for Luminal B and Triple Negative Breast Cancers*. Poster presented at Research!Louisville Conference; Louisville, KY.

June 30, 2022:

Jigo, Raphael N., Burlison, Joe, Trent, John, Clark, Geoffrey J. *RAS Inhibition of Luminal B Breast Cancer as a Novel Therapeutic Approach*. Seminar presented at Pharmacology and Toxicology Departmental Seminar, University of Louisville; Louisville, KY.

May 22, 2022:

Jigo, Raphael N, Donninger, Howard, Ferrill, Rachel, Von Baby, Becca, Burlison, Joe, Trent, John, Sabo, Michael, Clark, Geoffrey J. *A Novel RAS Inhibitor for Luminal B and Triple Negative Breast Cancers*. Poster presented at Keystone Symposia Meeting: Modern Phenotypic Drug Discovery: From Chemical Biology to Therapeutics; Denver, CO.

October 26, 2021:

Jigo, Raphael N., Burlison, Joe, Trent, John, Clark, Geoffrey J. *RAS Inhibition of Luminal B Breast Cancer as a Novel Therapeutic Approach*. Poster presented at Research!Louisville Conference; Louisville, KY.

June 25, 2021:

Jigo, Raphael N., Burlison, Joe, Trent, John, Clark, Geoffrey J. *RAS Inhibition of Luminal B Breast Cancer as a Novel Therapeutic Approach*. Poster presented at Twisted Pink Metastatic Breast Cancer Symposium; Louisville, KY.

March 18, 2021:

Jigo, Raphael N., Burlison, Joe, Trent, John, Clark, Geoffrey J. *RAS Inhibition of Luminal B Breast Cancer as a Novel Therapeutic Approach*. Seminar presented at Pharmacology and Toxicology Departmental Seminar, University of Louisville; Louisville, KY.