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Determining the mechanism of C-Raf driven metastasis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular and Medical Pharmacology

by

Lisa Hieu Ta

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ABSTRACT OF THE DISSERTATION

Determining the mechanism of C-Raf driven metastasis

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Doctor of Philosophy in Molecular and Medical Pharmacology

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Professor Owen N. Witte, Chair

Mutated Ras and Raf kinases are well-known to promote cancer metastasis via flux through the Ras/Raf/MEK/ERK (MAPK) pathway. A role for non-mutated Raf in metastasis is also emerging, but the driving mechanisms remain unclear. Elevated expression of any of the three wildtype Raf family members (C, A or B) can drive metastasis. Here, we utilized an *in vivo* model to show that wildtype C-Raf overexpression can promote metastasis of immortalized prostate cells in a gene dosage dependent manner. Analysis of the transcriptomic and phospho-proteomic landscape indicated that C-Raf driven metastasis is accompanied by upregulated MAPK signaling. Use of C-Raf mutants demonstrated that the dimerization domain, but not its kinase activity is essential for metastasis. Endogenous Raf monomer knockouts revealed C-Raf's ability to form heterodimers with A-Raf and B-Raf are important for promoting metastasis. Taken together, these data identify wildtype C-Raf heterodimer signaling as a potential target for treating metastatic disease.

The dissertation of Lisa Hieu Ta is approved.

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DEDICATION

This dissertation is dedicated to my mom, Lieu Ta and my sisters, Christina and Linda Ta who have been and continue to be my pillars in life. They have taken turns taking me to school for 27 years – that's a really long time. Finally, no more school drop offs. Their constant support, warmth, laughter, and love have made this work possible.

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ACKNOWLEDGEMENTS

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Lastly and most importantly I want to thank my family. I hold immeasurable gratitude for my mom and my dad for always supporting me even though we may not walk the same path anymore. When I was younger, my mom always tried to sneak reading in her busy schedule to learn new things. She was a student of life and taught me the beauty of pursuing the unknown. My mom's fight with cancer was the reason why I got into cancer research. Despite the tragedy of her death, my drive to learn and understand what took her from me transformed to a fascination for science. I hope that she will be proud of her youngest daughter. I am grateful for my dad who encouraged me consistently to be curious, remain curious and most importantly, to stay humble. His lessons and our shared experience have shaped a large part of who I am today. Finally, I want to thank my sisters, Christina, and Linda. They have been with me through the moments in life where no light seemed to penetrate the suffocating blackness. They have held my hand through the whirlwind that is our lives and have accepted me wholeheartedly. Their encouragement and love have bolstered me throughout my Ph.D studies and life. We had no choice to be family, but they are the ones I choose to be my close friends. I think our mom would be very happy that her three little bears support and love each other.

Without these people, I would not be the person I am today. To paraphrase Carl Sagan, in the finite time that we have on this earth, I am so immensely thankful to have passed time, even if briefly, with all of you.

Lisa H. Ta

Education

UCLA Ph.D Molecular and Medical Pharmacology — Expected June 2023
UCLA B.S. Molecular, Cell and Developmental Biology — 2013
(Minor: Biomedical Research)

Research Experience

Graduate Researcher | Pharmacology — 2016 – present
Dr. Owen Witte Lab, UCLA

- Discovered mechanisms of Raf driven metastasis leading to first author manuscript by leading study design, execution, and analysis of experiments
- Communicated science to diverse audience by presenting findings at 3 national and international conferences and co-authoring 10 publications, with 2 first author publications in peer-reviewed journals
- Spearheaded 2 institutional collaborations, including cross-disciplinary fields resulting in key scientific findings (Stanford and UCLA)
- Successfully wrote grant proposal for $500,000 to study small cell ovarian cancer
- Awarded $70,000 in competitive fellowships & scholarships
- Initiated in vivo TCR therapy arm by functionally testing TCR and CAR-T candidates in various mouse models

Lab Manager/Staff Research Associate II | Pharmacology, UCLA — 2013 – 2016
Dr. David Nathanson Lab, UCLA

- Addressed the lack of glioblastoma patient derived models by creating a *in vivo* representative patient derived orthotopic mouse model for glioblastoma and streamlining clinic-to-mouse project and workflow
- Established a new laboratory compliant with UCLA health and safety standards
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Undergraduate Independent Researcher | Pharmacology, UCLA — 2010-2013
Dr. Caius Radu Lab, UCLA

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- Effectively mentored and empowered undergraduate students from disadvantaged backgrounds to pursue and excel in STEM careers.
- Lead 5-10 workshops per quarter to teach scientific communication skills for undergraduates (e.g. presentations, abstracts and posters)
- Spearheaded and collaborated with a graduate student team to devise and coordinate presentation assembly for undergraduate workshops, resulting in a 50% increase in workshop attendance and participant engagement.

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- Supervised students in final projects, graded exams, and weekly assignments

Publications

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Chapter 1: Introduction

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1.1 Introduction to metastasis

Metastasis is the development of secondary malignant growths at sites distant from the primary cancer and is responsible for >90% of cancer-related deaths [1]. Tumor cells that spread to form metastases must undergo a complex multi-step process, including the degradation of the basement membrane, escaping from the primary organ of diagnosis, intravasation into the bloodstream and/or lymph, extravasation into the secondary organ, survival, adaptation in the secondary organ, and, finally, the formation of micro and macro metastases. Although many cancer cells are shed from primary tumors continuously, metastasis is still a highly inefficient process [2,3]. To understand how this inefficient process still claims thousands of lives each year, we can take a reductionist approach and categorize metastasis as dependent on two main categories that mirror the classic nature vs. nurture argument: 1) environmental factors that affect and influence the cancer cell, and 2) intrinsic alterations that favor their survival. Likely, both points contribute to the success of a disseminated cancer cell. Over the years, concerted efforts have extensively explored the effect of the tumor microenvironment (TME) on metastasis. For this thesis, we will focus on the intrinsic alterations in the cancer cell that favor its survival.

1.2. Alteration in the Ras/Raf/MEK/ERK pathway is a major driver of cancer metastasis.

Cancer is primarily a genomic disease driven by alterations in tumor suppressor and oncogenes [4]. Over the decades, DNA and RNA sequencing studies with human samples have been able to pinpoint frequent alterations that confer malignant cancer phenotypes. Many of these alterations are in the Ras/Raf/MEK/ERK pathway, also known as the MAPK cascade.

The primary role of the MAPK pathway is to convey mitogenic signals from the cell surface via the signaling cascade to the nucleus to produce widescale transcriptomic changes [5]. This occurs through a relay of signals from ligand interaction to tyrosine kinase receptors, such

as the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), resulting in the recruitment of Ras and the activation of the MAPK cascade.

Dysregulation of this pathway has been heavily implicated in cancer progression. Genetic amplifications in upstream controls of the pathway, such as receptor tyrosine kinases (altered in approximately 10% of cancers [6]), and the Ras family (altered in 19% of cancers, [7]) have been reported in various cancers including colorectal cancer, melanoma, and breast invasive ductal carcinoma. The frequency of mutations progressively decreases among the pathway's downstream components. [8,9]. Several prevalent mutations result in the constitutive activation of the pathway, which subjects the cells to continual growth promoting signals that facilitate unregulated proliferation. Prevalent mutations such as KRASG12C or BRAFV600E have been prime target of for small molecule development [10,11]. Other genomic rearrangement events, such as activated fusion products in B-Raf and C-Raf, have also been demonstrated [12]. Cancer dependency on the MAPK signaling is apparent through the acquired resistance mechanisms. Melanomas with activated B-Raf can genetically amplify MEK or ERK in response to B-Raf inhibitors as a compensatory mechanism to maintain flux through the pathway [13,14]. Other ways cancers maintain flux through the MAPK pathway include dysregulation of negative feedback mechanisms, such as the activation of phosphatases [15,16]. These biological findings highlight the MAPK cascade as a prominent signal transduction cascade driving various stages of cancer progression. These examples also demonstrate cancer's ability to hijack a highly conserved signaling cascade for survival and growth and explain the normal function of this pathway in biology. The Ras/Raf/MEK/ERK cascade is crucial for regulating complex processes in normal cell such as cell fate, proliferation, and differentiation [17].

1.3. The underappreciated role of wildtype (WT) proteins in cancer

While mutations in genes are often associated with the development and progression of cancer, it is also possible for wild-type, non-mutated genes to play a role. Firstly, many non-mutated

genes regulate critical cellular processes that are involved in tumor development, such as cell cycle regulation, DNA repair, and apoptosis. When these genes are altered or deregulated, cells can grow and divide uncontrollably, leading to the development and progression of cancer. For instance, the HER2 gene, which encodes a growth factor receptor, is overexpressed in about 25% of breast cancers, leading to increased signaling through the HER2 pathway and promoting tumor growth [18]. Alterations can occur in the regulation of gene expression, post translational regulation and epigenetic modifications (such as changes in DNA methylation patterns or alterations in histone acetylation). Some mutated genes are dependent on wild-type genes to contribute to cancer progression. Multiple examples have been described of critically retained signal transduction pathways that can be targeted by affecting non-mutated genes such as wild-type Ras in oncogene Ras-driven cancers, PDGFRa in non-small cell lung cancer, and BTK chronic myelogenous leukemia [19-23]. Therefore, alterations in the expression or regulation of normal genes can contribute to the development and progression of cancer. Understanding the role of wild-type genes in cancer progression is crucial for developing effective treatments and prevention strategies.

1.4. Kinases as successful drug targets in cancer and metastasis

Here, we focus our attention on protein kinases, which are enzymes that transfer a γ-phosphate group from ATP to serine, threonine, or tyrosine residues. Protein kinases play a vital role in regulating numerous cellular processes, and mutations in their genes or changes in their expression can lead to cancer and other diseases. The human genome encodes 538 protein kinases, many of which are linked to cancer initiation and progression. Consequently, kinase-inhibitors have proven successful in clinical therapy. Protein kinases are the second most targeted group of proteins, following G-protein-coupled receptors. Since the approval of imatinib in 2001, over 70 kinase inhibitors have been approved by the FDA. Given their success, dysregulated kinases are promising targets for cancer treatment. Therefore, it is crucial to

understand and investigate their mechanistic contribution to cancer, which could facilitate downstream therapeutic development.

1.5. WT Raf family kinases drive metastasis in an *in vivo* screen.

Though comprehensively studied, many of Raf's contributions to malignant phenotypes have only been explored in the context of mutated Raf in *in vitro* systems and have yet to be evaluated in *in vivo* metastasis models. In fact, non-mutated Raf can also drive metastasis. A previous study from our group screened 125 non-mutated kinases and identified all three Raf kinases can independently drive metastasis in a mouse prostate cancer *in vivo* model [24]. Interrogation of human tissue microarray samples demonstrated that there was increased Raf family accumulation in metastatic samples compared to localized disease and normal prostate tissue. C-Raf drove the most pronounced and malignant metastatic disease followed by B-Raf and A-Raf when overexpressed. Interestingly, other kinases part of the Ras/Raf/MEK/ERK pathway or kinases that feed into this pathway were also tested but did not drive metastasis when overexpressed. These data point to the uniqueness of Raf's contribution to cancer progression in the context of metastasis and suggests its MAPK independent roles may be a contributor to Raf driven metastasis. Although much focus has been on Ras, this chapter aims to take a closer look at Raf signaling and its contribution to metastasis. Fully understanding the functions of Raf signaling in metastasis requires a thorough appreciation of its role and contribution to normal biology and its function in cancer development.

1.6. Raf history, biology, regulation, and role in cancer

1.6.1 The discovery of Raf

Raf kinases are key gatekeepers of the MAPK cascade. The summers of the 1980s were historical times as the first Raf kinase, C-Raf or Raf1, was discovered in murine and avian viral variants by two different groups as retroviral oncogenes.

In 1983, Ulf Rapp and colleagues found that the murine retroviral variant 3611 was able to transform rodent fibroblasts and consequently named it Virus-induced Rapidly Accelerated Fibrosarcoma (v-raf) [25]. The summer following the discovery of v-raf, the avian homolog, v-mil, was also discovered via the help of viruses [26]. Rapp and Bister further report the cellular homologs are the same for v-mil and v-raf [27]. Raf1 or C-Raf was soon discovered to be a serine/threonine kinase cellular homolog in which may be the reason it adopted the name C-Raf [28,29]. Shortly after, A-Raf and B-Raf were identified by Rapp and Kosei Toyoshima respectively [30,31]. Following these discoveries, cloning of the cellular homolog was performed to elucidate C-Raf function. C-Raf was shown to be important in mediating growth factor induced effects [32].

1.6.2. Establishing the framework of the MAPK pathway

Early studies of invertebrate systems including c. elegans, drosophila and mammalian cell lines helped elucidate Raf's role in the Ras/Raf/MEK/ERK pathway. In C. elegans and Drosophila, only one homolog of Raf was found. Work pioneered by Norbert Perrimon's group found two Raf homologs in Drosophila via homology of the kinase domain sequence by *in situ* hybridization of v-raf to Drosophila DNA [33]. Perrimon's group was also the first to identify Raf to be downstream of Ras signaling in Drosophila [34]. This was later confirmed in C. Elegans, where genetic knock out studies of Lin-45, the C. Elegan homolog for Raf, was shown to be downstream of let60 (the c. Elegan ortholog to Ras) [35]. Following this discovery, Ernst Hafen and colleagues spearheaded many elegant molecular studies that uncovered the intricate Raf signaling network [36-38]. Hafen and co-authors demonstrated that D-Raf was part of a pathway in Drosophila, downstream of Sevenless and Torso (drosophila tyrosine kinase receptor homologs) [38]. Genetic knock out of D-Raf showed that it played a crucial role in embryogenesis [34] and this function was later shown in mouse embryogenesis[39].

The basic structure of the MAPK cascade was delineated with Raf being oriented in the EGF receptor-Grb2-SOS-Ras-Raf pathway mainly through work in Drosophila. Hafen also

uncovered some of Raf's mode of negative regulation via binding to 14-3-3 protein [40]. Surprisingly, there are no Raf orthologs in yeast. Later, MEK1/2 were confirmed by multiple groups to be Raf's substrates and the Ras/Raf/MEK/ERK cascade was established in the mammalian systems [41-45].

1.6.3. Raf family kinases

Raf isoforms play a crucial role in early biological processes. Knockouts of any of the three paralogs can lead to embryonic and post-natal lethality in mice [39,46,47]. In the late 1990s, Ulf Rapp, Andreas Zimmer, and Manuela Bacarrini independently investigated the role of all three Raf paralogs in mouse embryogenesis. C-Raf was the first to be heavily studied of the three kinases. C-Raf knockout mice showed embryonic lethality and poor development of the placenta, liver, and hematopoietic organs [39,48]. This suggested that the presence of C-Raf counteracted apoptosis. B-Raf homozygous knockout mice demonstrated significant defects in neuronal, vascular, and endothelial cell development as well as irregular apoptosis compared to wildtype mice in the developing embryo [49]. When knocked out, A-Raf, often deemed the "black sheep" of the Raf family, did not result in embryonic lethality when knocked out [46]. However, A-Raf knock out mice exhibited shortened post-natal survival, dying usually within a month after birth. Additionally, A-Raf-/- exhibited severe gastrointestinal defects and neurological abnormalities. Though these studies suggest that Raf family paralogs have distinct roles in development, combination knockouts highlight the compensatory roles that the paralogs possess. Double knock out studies of A-Raf and C-Raf exhibited the most severe phenotype [50]. These compensatory roles among the Raf isoforms are observed beyond the scope of development. For example, regulatory mechanisms that are activated via dimerization will be addressed in section 1.3.6. In early 2000, Zimmer's group demonstrated that all three paralogs are expressed ubiquitously throughout the developing embryo [47]. Additionally, individual Raf

isoform knockouts did not alter the expression or activation of downstream MAPK, further supporting redundancy within the Raf family.

Mechanistic studies determining the role of Raf isoforms conducted in eukaryotic cell culture models have mainly interrogated the effects of manipulating B-Raf and C-Raf. These studies have found that Raf regulates various cellular functions, including cell cycle progression, cell proliferation, and regulation of apoptosis [51,52]. Initially, Raf's function was thought to be confined to the MAPK linear cascade. However, subsequent work from multiple groups demonstrated that intricate post-translational modifications, protein-protein interactions, and feedback mechanisms render this pathway much more complex. This thesis will also focus on the uniqueness of C-Raf function and role in disease.

1.6.4. C-Raf structure

The activation mechanism of Raf has been the subject of a decade-long hunt. Many of the initial studies focused on C-Raf regulation. The three mammalian Raf members have three main conserved regions in their protein structure:

1. CR1 - Contains the Ras binding domain, also annotated as RBD. It also contains a cysteine-rich domain (CRD) that doubles as a secondary Ras binding site and interacts with the kinase domain to enforce autoinhibition (part of the N-Terminal regulatory region) [53,54].
2. CR2 – Contains inhibitory phosphorylation sites for regulating activation (part of the N-terminal regulatory region), specifically phosphorylation of S259 (on C-Raf) facilitates binding with 14-3-3, an inhibitory scaffolding protein.
3. CR3 – Contains the kinase domain, dimerization domain and the second phosphorylation site for 14-3-3 binding (S621 on C-Raf).

1.6.5 Ras interaction

First observed in v-Raf, deletion of CR1 and CR2 (N-terminal regulatory domains) renders the protein constitutively active [55,56]. Data from subsequent studies demonstrated that C-Raf's N-terminus interacts with the kinase domain in an autoinhibitory loop [57,58]. This inactivated state was hypothesized to be reinforced and stabilized through inhibitory binding of 14-3-3 proteins on the S259 and S621 phosphorylation sites [59,60]. S259 is phosphorylated by Protein Kinase A (PKA) and Akt [61-64]. The Raf proteins mainly resides in the cytoplasmic space. Upon GTP loading of Ras, Raf gets recruited to plasma membrane microdomains where MAPK signaling components are present at higher concentrations [65]. Ras can interact with Raf via two regions: the RBD and the cysteine rich domain (CRD). The CRD can interact with farnesyl groups attached to Ras' C-terminus [66,67]. Additionally, Raf's CRD interacts directly with phosphatidyl-serine, stabilizing it at the plasma membrane [68,69]. Ras activation promotes dephosphorylation of S259 and S621 by protein phosphatase 2A (PP2A) and PP1, thereby releasing 14-3-3 from the N-terminus [70,71]. Ras has also been shown to promote dimerization [72]. These interactions secure Raf, poising it for activation.

1.6.6. Raf Dimerization

The next crucial step in Raf's activation sequence is dimerization with other Raf monomers. The importance of Raf dimerization became apparent when forced oligomerization of Raf via fusion with FKBP12 domains was found to promote Raf activity [73]. The role of dimerization in Raf activation was further supported by the discovery that catalytically inactive B-Raf mutants in melanoma were able to promote downstream MAPK signaling via association with endogenous kinase competent C-Raf [74-76]. These findings showed that dimerization was important for downstream activation and that B-Raf was able to induce C-Raf catalytic function independent of B-Raf's intrinsic kinase activity. Dimerization can be accomplished via the association of homodimers or heterodimers. Through intricate molecular studies, Walter Kolch and colleagues demonstrated that heterodimers and homodimers can spontaneously form

under physiological conditions; however, B-Raf:C-Raf heterodimers had higher activity [77]. The work was further supported by Deborah Morrison and colleagues in the context of cancer and normal Raf signaling [78].

Mutation of key residues in the kinase domain elucidated the dimer interface. In Drosophila, KSR, a close relative of Raf, serves to activate D-Raf via dimerization, required the kinase domain but not kinase activity to activate Raf in Drosophila [79]. Further structural studies of human B-Raf kinase domain crystals revealed that the kinase domains formed side-to-side dimers [80]. At the center of this dimerization interface is a key arginine residue (Braf Arg509 and C-Raf Arg401) in the kinase domain that is distant from the catalytic cleft. Mutation of this residue abolished Raf activity [78].

Raf kinases are divided into the N-lobe and the C-lobe connected by a flexible hinge. The N-lobe contains a regulatory helix called alpha-C. The C-lobe contains the activation segment which presents in a key loop structure [81].

Dimerization is thought to promote allosteric activation and conformational changes that stabilize the closed conformation of the kinase structure [82]. This involves restriction of lobe movements. Generally, closed conformation for protein kinases is the active form and is more stable via alignment of two parallel columns of hydrophobic residues along the N and C-lobe, called the catalytic and regulatory spines, respectively [83]. Interrogation of inactive and active states of Raf shows that the steps to activation involve various protein interactions and phosphorylation of the activation segment, resulting in spine alignment, kinase closure, and kinase activation [83]. Upon dimerization, Raf kinases are activated upon the concerted inward movement of the alpha-C helix (containing the key Arginine residue) and the Phe residue part of the DFG motif in the N-terminal end of the activation segment [84].

1.6.7 Key phosphorylation sites for Raf activation

Raf kinases require phosphorylation for their activation, which occurs in three regions of the protein: 1) the N-terminal region of the kinase domain, 2) the activation segment in the kinase domain, and 3) the C-terminal 14-3-3 binding site.

The N-terminal region contains an SSYY motif spanning residues 338-341, and phosphorylation of Ser338 and Tyr341 is required for full activity [85,86]. This region acquires a negative charge upon phosphorylation, explaining why B-Raf has a lower threshold for activation due to its inherent negative charge from residue Asp448 [86,87]. This also explains why B-Raf has a higher mutation frequency in cancer [88]. Phosphomimic substitution in this region has shown that the negative charge can determine the direction of Raf dimer transactivation and create asymmetry [89]. Phosphorylation allows Raf to act either as an activator (transactivate via phosphorylation) or a receiver, while non-phosphorylated and therefor uncharged alternates could only behave as a recievers [89]. These residues are known substrates for SRC family kinases, PAK, and CK2 [90-94].

The activation segment requires phosphorylation at Thr491 and Ser494 for C-Raf [95].These phosphorylation sites are hard to detect due to their transient nature, low levels, and unstructured nature in crystal structures. However, mutation of these sites prevents activation, and the responsible kinases are unclear. There are some data that suggests cis-autophosphorylation may be a viable mechanism [89]

The C-terminal end is the last region to be phosphorylated for dimeric 14-3-3 binding. Phosphorylation of C-Raf at S291 promotes binding of Raf to ATP [96]. This site was previously thought to be inhibitory, but it is hypothesized that phosphorylation of this site and binding of dimeric 14-3-3 stabilizes the Raf dimer interaction [80,97].

1.6.8 C-Raf feedback inhibition

C-Raf is subjected to feedback inhibition from the MAPK pathway. As a signal transduction cascade it is tightly regulated to ensure adequate signal attenuation in normal signaling. In

diseased states, where components of MAPK are constitutively active, feedback regulation is hindered [98]. C-Raf is regulated by dephosphorylation of specific activation residues. All the phosphatases responsible for this process is still unclear, however there is some evidence that identifies protein phosphatase 5 (PP5) to be responsible for dephosphorylating S338 [99]. In addition to phosphatase activity for attenuation, downstream members of the pathway like ERK1/2, can deactivate C-Raf via phosphorylation to complete negative feedback loops and attenuate Raf signaling [100]. ERK1/2 is responsible for S29, S43, S289, S296, S301, and S642 of which the consequence is disassociation of Raf heterodimers and perturbation of Ras binding [101]. Conversely, these phosphorylation sites need to be removed by PP2A dependent on peptidyl-prolyl-cis-trans isomerase NIMA-interacting 1 (PIN1) to allow for C-Raf to become activated again [100,102]. The duration of the MAPK signaling module is dependent on upstream signals, where the active and inactive states of the signaling modules are often compared to a molecular switch.

1.6.9 Crosstalk with PI3K-mTOR signaling

The MAPK and the phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) cascades are key pathways in the cell that control proliferation, metabolism, motility, and differentiation in response to extracellular signals [103]. These two pathways are interconnected and can crosstalk with each other to regulate cell signaling. The PI3K-mTOR pathway activates downstream targets such as AKT and S6K, which can inhibit the Ras/Raf/MEK/ERK pathway through various mechanisms. On the other hand, the Ras/Raf/MEK/ERK pathway can also negatively regulate the PI3K-mTOR pathway by inhibiting the activation of PI3K. However, under certain conditions, the two pathways can also synergize and promote cell proliferation and survival.

PI3K-mTOR pathway consists of lipid kinase sensor PI3K, which can be activated by growth factor receptors. Following recruitment to the plasma membrane, activated PI3K can

generate phosphatidylinositol 3,4,5 triphosphate (PIP3) which recruits Akt protein kinase. Akt can be activated by 3-phosphoinositide-dependent kinase 1(PDK1) and mTORC2 [104]. MAPK can be negatively regulated via cross inhibition by PI3K-mTOR pathway [105]. For example, protein kinase A (PKA) can phosphorylate C-Raf at S259 residue for 14-3-3 binding and sequestration from MAPK members like Ras and MEK [61]. Akt can also cross inhibit MAPK by phosphorylating inhibitory residues on C-Raf at the regulatory N-terminus [63,106]. In terms of activation, elevated ERK (downstream MAPK) and RSK activity can lead to activated mTORC1 via the TSC2. ERK and RSK also promote phosphorylation of RAPTOR (mTOR's scaffolding protein) which then promotes mTORC1 to phosphorylate 4EB-P [107-109]. ERK, RSK, AKT, and S6K are downstream effectors of both pathways and often act on the same substrates sometimes together to promote various pro survival cellular processes like proliferation, metabolism, and motility [110].

1.6.10 Raf's role in the hallmarks of cancer

Over the past 20 years, Raf has been implicated in oncogenic disease [111]. The somatic mutational landscape of various tumor types shows different mutation frequencies across A, B, and C-Raf, with B-Raf being the most frequently mutated, followed by the rare C-Raf mutations, and finally the even rarer mutations in A-Raf [112]. This is often reflective of the mechanisms of activation for each isoform, with B-Raf being the easiest to activate due to fewer phosphorylation sites required for activation [113]. B-Raf also has stronger affinity for MEK1/2, which are the main substrates for the Raf family [114].

Tumorigenesis

Mariano Barbacid and Manuela Baccarini both independently investigated the unique role of C-Raf in KRAS driven Genetically Engineered Mouse (GEM) cancer models, specifically lung and pancreatic cancer [115,116]. Surprisingly, elimination of C-Raf does not affect MAPK activity in these models as assessed by downstream effector phosphorylation status [117]. C-Raf

elimination resulted in a range of partial to complete tumor regression. This result is striking as it suggests the necessity for C-Raf in tumor growth and progression in this model independent of its role in relaying MAPK pathway activity. Elimination of C-Raf has also been shown to be detrimental to tumor initiation in KRAS-driven mouse models [116,117].

Epithelial to Mesenchymal Transition (EMT) and Mesenchymal to Epithelial Transition (MET)

There have been limited direct studies that implicate Raf's role in metastasis, however the MAPK pathway has many pleiotropic effects which have been shown to regulate various phenotypes conducive of metastasis. Upregulation of the Ras/Raf/MEK/ERK pathway, specifically in Ras driven cancers has been implicated in contributing to the bi-directional epithelial mesenchymal transition (EMT) phenotype [118]. EMT is often recognized as one of the pivotal steps and tumor cell characteristic in early metastasis [119]. The EMT process allows tumor cells the ability to have enhanced plasticity by adoption of epithelial, mesenchymal or a hybrid phenotype [120]. The flexibility of adopting any of these phenotypes can present favorably depending on tumor's cell's process in the metastatic cascade. Studies looking at the main effect of C-Raf on EMT demonstrated that C-Raf overexpression promoted downregulated adherens and tight junctions, and rearranged the actin skeleton aligning with a more EMT like process [121]. Other studies have also shown that the MET process was enhanced in metastatic breast cancer cells following constitutive activation of C-Raf [122].

Migration

C-Raf specifically possesses several MEK/ERK independent roles that contribute to metastatic phenotypes. Much of the focus in scientific studies of C-Raf and its function has been on its role as a signaling module and scaffold in the MAPK pathway. In recent years, these specific additional functions specifically in C-Raf have been appreciated, suggesting that C-Raf's role in various aspects of cancer progression may extend beyond its canonical roles compared to the other Raf paralogs.

C-Raf has been shown to play a crucial role in normal wound healing *in vivo* and *in vitro*, specifically in keratinocyte migration [123]. In addition to its role in wound healing and migration, these functions (based off studies mainly through Dr. Baccarini's group) demonstrated prevalent MAPK independent roles in regulating migration though association with Rho-dependent kinase, Rok-a [124]. These functions are mediated through MAPK-independent mechanisms, with studies from Dr. Baccarini's group demonstrating that C-Raf regulates migration by interacting with Rho-dependent kinase, Rok-a [124].

The association of C-Raf with Rok-a affects its localization and inhibits its activity, resulting in reduced cell motility and migration with a defected contractile appearance in C-Raf ablation models [123]. In the absence of C-Raf regulation, Rok-a becomes hyperactive, preventing the activation of STAT3 and Myc, leading to cell cycle exit and subsequent differentiation. Elegant studies from Niault and colleagues showed that via physical association, C-Raf's N-terminal regulatory domain binds to Rok-a's kinase domain, providing trans-inhibition to Rok-a downstream signaling without phosphorylation [124].

Apoptosis

C-Raf has been shown to play a role in regulating apoptosis through interactions with various proteins. A subset of C-Raf molecules within cells have been demonstrated to localize to the mitochondria through their association with Bcl-2 and BCl-2 associated gene (BAG-1) [125]. P21-activated kinase (PAK) can also promote C-Raf mitochondrial localization through phosphorylation at site S338 [126]. In addition to its recruitment to the mitochondria via association with the above molecules, the Bcl-2 family member Bcl-2 antagonist of cell death (BAD) has been demonstrated to be a substrate of mitochondrial C-Raf [126-128]. This phosphorylation event prevents BAD from localizing to the mitochondria. Walter Kolch's group uncovered additional C-Raf anti-apoptotic roles via interaction and suppression of the MST2 protein in the Hippo pathway [129]. Interestingly, C-Raf prevents dimerization and phosphorylation of MST2's activation

loop in a kinase independent manner [130]. C-Raf also interacts with apoptosis signal-regulating kinase 1 (ASK1) at the mitochondria and results in ASK1 inhibition [131-133].

It is not clear whether this interaction is due to physical association or phosphorylation. Other Non-MAPK effectors of Raf include Rb. C-Raf has been shown to inactivate Rb via phosphorylation leading to cell cycle progression [134]. Disruption of this interaction has been shown to inhibit tumor growth and angiogenesis [135]. Altogether, Raf wears many hats and plays a part in multiple processes in the cell. To explore these concepts further, we first set out to understand the mechanism of C-Raf driven metastasis.

1.6. Understanding the contribution of WT Raf in metastasis

Mutated Raf kinases can have significant impacts on cancer, as mentioned earlier, although not all cancers exhibit these mutations. Our previous study revealed that, in addition to activating mutations, elevated levels of wild-type Raf kinases can drive aggressive metastatic behavior in an animal model [24]. Investigating this mechanism can provide insights into the potential role of Raf in cancers with minimal alterations in the MAPK and other oncogenic pathways.

Continuing our previous investigation, we have narrowed our focus to examine the mechanism of C-Raf-driven metastasis. Among the three Raf kinases, B-Raf is the most frequently mutated due to its lower activation threshold. Therefore, several studies have already highlighted the reliance of mutated B-Raf on wild-type (WT) C-Raf to propagate oncogenic signals [89,117].

This unique relationship is also observed in K-Ras-driven tumor models, where mutated Ras depends on WT Raf for the persistence of advanced lung adenocarcinoma [116]. We have found that an incremental increase in C-Raf protein levels drives metastasis in a dosage-dependent manner.This increased C-Raf protein relies on the kinase activity of the MAPK pathway, exhibited through any of the Raf family kinases, and is not dependent on C-Raf's

inherent kinase activity. Our findings are supported by the work of Shiva Malek and colleagues, who have observed the same phenomenon in K-Ras-driven cancers.

Other researchers, such as Manuela Baccarini's group, also suggest the dispensable nature of C-Raf's inherent kinase activity in various oncogenic and pro-survival behaviors. To further support the importance of kinase activity in the system, but not C-Raf's inherent kinase activity, we have discovered that dimerization is crucial for C-Raf-driven metastasis.

Elimination of heterodimerization partners that cooperate through dimerization significantly dampened the metastatic phenotype, unlike homodimers. Notably, we observed the most significant extension in mouse survival when B-Raf was eliminated. These findings, along with the work of many others, highlight the nuanced role of Raf dimers and their implications for future dimer-specific inhibition in Raf-driven cancers.

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Chapter 2: Wildtype C-Raf gene dosage and dimerization drive prostate cancer metastasis

Title: Wildtype C-Raf gene dosage and dimerization drive prostate cancer metastasis

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Summary

Mutated Ras and Raf kinases are well-known to promote cancer metastasis via flux through the Ras/Raf/MEK/ERK (MAPK) pathway. A role for non-mutated Raf in metastasis is also emerging, but the key mechanisms remain unclear. Elevated expression of any of the three wildtype Raf family members (C, A or B) can drive metastasis. We utilized an *in vivo* model to show that wildtype C-Raf overexpression can promote metastasis of immortalized prostate cells in a gene dosage-dependent manner. Analysis of the transcriptomic and phospho-proteomic landscape indicated that C-Raf driven metastasis is accompanied by upregulated MAPK signaling. Use of C-Raf mutants demonstrated that the dimerization domain, but not its kinase activity is essential for metastasis. Endogenous Raf monomer knockouts revealed that C-Raf's ability to form heterodimers with A-Raf and B-Raf are important for promoting metastasis. These data identify wildtype C-Raf heterodimer signaling as a potential target for treating metastatic disease.
Keywords: Metastasis, Raf, gene dosage, prostate cancer, dimerization, dimer pairs, wildtype, kinase

INTRODUCTION

Metastasis is the primary cause of cancer related deaths and remains a significant clinical challenge [1]. Tumor metastasis involves a multi-step cascade that requires tumor cells to survive a variety of environmental stressors. This process includes tumor cell invasion of the basement membrane, intravasation into the blood stream or lymphatic vessel, survival in the circulation, extravasation and, colonization at a secondary site [136]. The Ras/Raf/MEK/ERK (MAPK) cascade is a well-studied mediator of metastasis. Ras and B-Raf are commonly mutated upstream regulators of MAPK signaling in cancer cells. [12,137]. Targeting activating mutations like BRAFV600E with small molecule inhibitors have been successful and frames kinases as viable targets for therapeutic development [138-140].

Though much focus has been on mutated Raf, we and others have shown that wildtype (WT) Raf plays an important role in promoting cancer and metastasis [24,117,141,142]. Using a functional screen, we previously found that all three wildtype Raf kinases (C, A and B-Raf) can promote metastasis of immortalized prostate cells in an *in vivo* metastatic model [24]. In concert with the screen, interrogation of human tissue microarray samples demonstrated enrichment of Raf kinase proteins in metastatic samples compared to localized tumors and normal tissue [24]. Among the three kinases, C-Raf drove the most penetrant metastatic phenotype. Whether C-Raf drives metastasis via it's gatekeeping role in the MAPK pathway or via other interactions remained unknown.

Raf kinase activation is a complex process that requires a series of dephosphorylation and phosphorylation events that prime Raf molecules for dimerization with other Raf monomers [143] (review). This homo or heterodimerization with other Raf kinase competent monomers is critical for propagating MAPK signaling. Specific dimer combinations can determine the aggressiveness of cancer phenotypes. Recent work pointed to wildtype A-Raf:C-Raf heterodimers as key regulators in K-Ras driven tumor growth [144]. Venkatanarayan and colleagues found that K-Ras mutant cells contained more A-Raf:C-Raf than B-Raf:C-Raf dimers. Further work done by this group showed that C-Raf's dimerization function, but not its kinase activity is important in driving malignant phenotypes in an *in vitro* setting. Their study extensively explored C-Raf dimerization in the context of Ras driven disease. However, the relevance of WT C-Raf's role has not been explored in a non-mutant Ras context. Additionally, many of the key findings were modeled in a soft agar assay, necessitating further testing of C-Raf's dimerization and kinase functions in promoting metastasis in an *in vivo* setting.

We performed a systematic investigation of wildtype C-Raf's functions in driving cancer metastasis in a wildtype K-Ras context. We chose to interrogate C-Raf's function as an archetype of the Raf kinases due to its more potent metastatic phenotype in our *in vivo* models [24]. To model metastasis, an intracardiac injection mouse model was used, which captures

multiple steps of the metastatic cascade, including resistance to anoikis and cell death, seeding ability in distant tissues, and most importantly bypasses sequestration of disseminated cells in the lungs [145] (review). Utilizing orthogonal mass spectrometry, transcriptomic analyses, various mutants targeting specific Raf functions, and select Raf monomer genetic deletions, we show that even subtle increases in C-Raf protein expression can drive metastasis. Exogenously added C-Raf driven metastasis is dependent on its dimerization domain. Overexpressed C-Raf dimerizes with endogenous A-Raf and B-Raf to drive metastasis. This work highlights the importance of C-Raf heterodimerization and gene dosage in driving metastatic cancer and points to the potential of perturbing these interactions for better therapeutic outcomes.

RESULTS

C-Raf overexpression enables transformation and metastasis of two immortalized prostate cell lines.

To better understand the mechanism by which non-mutated C-Raf promotes metastasis, we tested C-Raf heightened expression in two immortalized prostate epithelial cell lines, BPH-1, and RWPE-1. These lines are immortalized by SV40 and HP18 respectively. Neither line forms metastasis *in vivo*. C-Raf overexpressing human cells were administered to immune defective NOD scid gamma (NSG) mice via intracardiac injection to monitor metastatic dissemination (Figure 1A). Both cell lines were engineered to express firefly and gaussia luciferase to track the spatial distribution of metastatic tumors [146] and to quantify whole body tumor burden, respectively (Figure 1A) [147]. C-Raf overexpression did not result in significant changes in MEK1/2 and ERK1/2 phosphorylation levels (Figure 1B). This observation corroborates other studies that interrogated wildtype B-Raf and A-Raf overexpression [148,149]. Naïve RWPE-1 and BPH-1 cells did not form tumors upon intracardiac injection for the duration of the study (100 days). In contrast, C-Raf overexpression caused both cell lines to develop robust metastasis in all injected mice (Figure 1C and 1D). C-Raf drove metastasis to multiple visceral organs and

bone in both RWPE-1 and BPH-1 models (Figure 1E & 1F). The most common site of metastasis in prostate cancer patients is bone [150]. Bone was consistently was one of the most common metastatic sites in our models, with greater than 60% of animals harboring metastatic tumors in this location in RWPE-1 and greater than 70% in BPH-1 model (Figure 1E & 1F). Whereas control mice survived for the duration of the study (100 days), Mice harboring RWPE-1 C-Raf cells became moribund on average 42 days post injection (Figure 1G) and mice with BPH-1 C-Raf died on average 30 days post injection (Figure 1H). In summary, elevated C-Raf protein reproducibly drives metastasis in mice in two prostate cell lines to sites that resemble prostate cancer metastasis locations.

C-Raf drives metastasis and mortality in a gene dosage dependent manner

C-Raf protein can accumulate above normal levels via genetic amplification or dysregulation at the mRNA and protein levels [137,139,151]. We chose to proceed with the BPH-1 cell line for our mechanistic studies due to the expedited *in vivo* growth kinetics. To determine the threshold of C-Raf protein necessary to drive metastasis, C-Raf gene dosage was exogenously manipulated via various strength promoters in BPH-1 cells. C-Raf protein was expressed at approximate levels of ~2-fold, 3-fold and 4-fold above endogenous levels driven by EFS, PGK and Ub promoters, respectively (Figure 2A) (quantification in supplemental Figure 3A). Increasing C-Raf protein expression did not increase downstream phospho-MEK1/2 and phospho-ERK1/2 levels as well as their corresponding total MEK1/2 and ERK1/2 (Figure 2A). This may be because MEK1/2 and ERK1/2 phosphorylation levels are likely constrained by negative feedback regulation [14].

We performed cell cycle analysis by flow cytometry to check whether C-Raf overexpression enhanced cell proliferation to promote metastasis. C-Raf promoted accumulation of cells in the G1 phase of the cell cycle in a gene dosage-dependent manner under adherent conditions. Although dose dependent increase of C-Raf did not result in

significant growth differences under adherent conditions *in vitro* (Figure 2B & 2C), it significantly enhanced proliferation in an anchorage-independent environment (Figure 2D). To verify this dose-dependent effect *in vivo*, we injected mice and quantified metastatic tumor burden using gaussian blood measurements and bioluminescence imaging (BLI) (Figure 2E and 2F). Increasing dosage of C-Raf resulted in a dose-dependent increase in metastatic burden (Figure 2E and 2F) and a concomitant decrease in survival (Figure 2G). Only two-fold higher expression above endogenous was sufficient to drive metastasis. Further increase in C-Raf levels conferred an additional advantage to cells and promoted more aggressive metastasis. These results indicate that even modest increases in C-Raf protein expression can promote malignancy and metastasis.

C-Raf overexpression increases MAPK pathway flux and is associated with metastasis.

C-Raf is a serine/threonine protein kinase and canonically functions via altering the phosphorylation status of downstream substrates and their physical association with other proteins [52] (review). To explore the activity and downstream effects of C-Raf overexpression, we analyzed the phosphoproteome of parental C-Raf overexpressing cells compared to vector control using tandem mass tag (TMT) isobaric labeling and phospho-proteomic analysis [152]. TMT labeling coupled with phosphorylation enrichment and mass spectrometry allows quantification of the relative abundance of phosphorylated peptides [153] (Supplemental Figure 1A). C-Raf induced drastic changes to the phosphoproteome as expected. The PC1 spread in principal component analysis (PCA) captured 57.7% of the variance that primarily separates C-Raf and vector groups (Supplemental Figure 1B). 48,029 distinct phospho-peptides were detected in C-Raf cells compared to control when adjusted to their corresponding protein abundance, indicating a robust restructuring of the phosphoproteome (Supplemental Table 1).

We performed inferred kinase activity (IKA) analysis [154] to identify kinases that have upregulated activity based on the quantification of known phosphorylated substrates

(Supplemental Figure 1C). IKA analysis showed significant upregulation of C-Raf, CDK1 and CDK2, which are involved in cell division and proliferation. CDK2 has been shown to be regulated by MAPK activation [155-157] (Supplemental Figure 1D). VRK1 and VRK2, two serine/threonine kinases in the same family that regulate chromatin remodeling and cell cycle progression are also significantly enriched [158]. Though neither kinase has been shown to have a strong relationship with C-Raf signaling, increased mTOR signaling via MAPK and PI3K pathway crosstalk has been shown to regulate VRK1 and VRK2 activity [159]. IKA analysis also provided information on overall pathway/complex upregulation. C-Raf samples demonstrated significant upregulation of Ras/Raf signaling compared to vector control (Supplemental Figure 1E). Other statistically significant pathways include several chromatin and histone modifying complexes, the DNA damage binding complex, and the ubiquitin E3 ligase complex (Supplemental Figure 1E). Increased flux through chromatin remodeling pathways coincides with increased VRK1 and VRK2 activity. Taken together, these data highlight that overexpression of C-Raf increases MAPK flux and downstream crosstalk with PI3K pathway effectors.

Transcriptional targets of the MAPK pathway are upregulated in metastatic C-Raf driven tumors

C-Raf signals through MEK and ERK to drive widespread transcriptional change [17,52] (review). mRNA sequencing was performed in vector control, parental C-Raf overexpressing cells, and C-Raf metastasis derived cell lines to understand the transcriptomic changes induced by our C-Raf model (Supplemental Figure 2A). Macroscopic tumors were resected from the bone, lymph node, spine, and thymus and cultured over a period of a week to establish metastasis derived lines. Differential expression analysis revealed that C-Raf drives a distinct transcriptional program, with over 650 differentially abundant genes compared to parental cells (Supplemental Figure 2B). Comparisons performed between pooled metastasis derived cell lines and their C-

Raf-overexpressing parental lines identified over 600 differentially regulated genes. Combined analysis of vector control, parental C-Raf, and C-Raf metastasis derived lines demonstrated that 46 genes were perturbed in the same direction (increasing or decreasing) in a stepwise fashion from vector control to parental C-Raf, and to C-Raf metastasis derived lines (Supplemental Figure 2C). Out of the 18 genes that demonstrated such step wise increase in expression, 9 were known targets of Ras/Raf signaling (Supplemental Figure 2C). In concert with the phospho-proteomic data, transcriptional analysis of C-Raf overexpression demonstrated increased MAPK pathway activation.

Each cell line was scored for key cancer hallmark signature gene sets to evaluate which biological processes are changed in C-Raf metastatic lines compared to the C-Raf parental and vector control lines [160,161]. K-Ras signaling was upregulated in both the C-Raf parental and metastatic lines as compared to the vector control (Supplemental Figure 2D). TGFβ signaling, a known downstream target of MAPK signaling [162], was also upregulated in both C-Raf parental and C-Raf derived metastasis lines (Supplemental Figure 2D). Notch signaling is significantly suppressed with C-Raf overexpression in C-Raf parental and metastasis derived lines [163]. There was no clear evidence of MAPK independent C-Raf processes, like transcriptomic upregulation of anti-apoptotic pathways with MST2 or ASK1. Overexpression of C-Raf predominantly drives a MAPK transcriptional program associated with tumor metastasis.

Mutation in the dimerization domain ablates C-Raf's metastatic-promoting effects

Dimerization is a crucial step in C-Raf's activation sequence and is necessary for wildtype Ras dependent Raf kinase activation as reviewed in Lavoie et al. 2015 [143]. To test whether dimerization is necessary for C-Raf-induced metastasis, a dimerization null mutant, R401H was introduced into BPH-1 cells. R401H mutation is in the RKTR motif within the αC-helix region of the dimerization interface [164]. Mutation of arginine to histidine has been demonstrated to significantly diminish kinase activity and inhibit dimerization with other Raf

monomers [78,165,166]. R401H expression resulted in decreased phospho-MEK1/2 and phospho-ERK1/2 (Figure 3A). Importantly, cells expressing the C-Raf R401H mutant did not produce metastasis *in vivo* (Figure 3B). Mice harboring C-Raf R401H cells did not exhibit any metastatic burden at 10 weeks post injection (Figure 3C). Cell titration was performed *in vitro* using D-luciferin substrate to ensure that the cells had adequate reporter gene output *in vivo*. All cells were positive for expression of firefly luciferase (Figure 3D). These results indicate that protein-protein interactions at the dimerization interface are required for C-Raf's ability to drive metastasis.

Knock-out of Raf paralogs diminishes C-Raf driven metastasis

To determine which dimer pairs are necessary and sufficient to contribute to C-Raf driven metastasis, we knocked out multiple combinations of endogenous Raf kinases. We found that triple knockout of A, B & C-Raf was lethal to BPH-1 cells. As a follow up strategy, two combinations of knockouts were selected based on prior literature highlighting the importance of B-Raf:C-Raf and A-Raf:C-Raf heterodimers. B-Raf and C-Raf have been shown to be the most preferential dimer pair, while A-Raf and C-Raf dimerization has been shown to mediate K-Ras driven malignancy [72,76,144]. Two combinations with knock-out of B-Raf were made. A clonal B-Raf knockout line was first generated using CRISPR/Cas9 [167] and sequentially modified by either A-Raf or C-Raf knock-out (Figure 4A & 4B). The resulting endogenous A-Raf$^{-/-}$; B-Raf$^{-/-}$ and B-Raf$^{-/-}$; C-Raf$^{-/-}$ cell lines are subsequently referred to as AB KO and BC KO respectively. Exogenous C-Raf overexpression in AB KO and BC KO cell lines decreased phospho-MEK1/2 and phospho-ERK1/2 levels (Figure 4B), indicating robust negative feedback relative to vector control. While AB KO cells proliferated at a slower rate than naïve BPH-1 cells, BC KO showed no growth difference in *in vitro* anchorage independent conditions (Figure 4C). Consistent with our previous results (Figure 2D), C-Raf overexpression significantly increased proliferation in ultra-low attachment conditions, which was not reduced by BA or BC knockout (Figure 4D).

Although BA and BC KO did not reduce C-Raf induced heightened proliferation *in vitro*, mice administered with either C-Raf AB KO or C-Raf BC KO cells exhibited drastically reduced metastatic burden compared to C-Raf overexpression only, as reflected in their survival (Figure 4E). AB KO in C-Raf overexpressing cells, significantly extended survival by ~50 days compared to the C-Raf overexpression only group. BC KO in C-Raf overexpressing cells also extended survival compared to C-Raf overexpression only group by ~110 days (Figure 4E). Based on the combinatorial KO's, in C-Raf BC KO cells, the only available monomers to form dimers are endogenous A-Raf and exogenous C-Raf. In the C-Raf AB KO cells, the only available monomers are endogenous and exogenous C-Raf. The metastatic phenotypes above suggest the ability to form either A-Raf:C-Raf heterodimers and/or C-Raf homodimers is sufficient to drive metastasis albeit with longer latency. The ablation of B-Raf in both KO groups severely dampened C-Raf driven metastasis. Therefore, these data demonstrate that the ability to form B-Raf:C-Raf dimers promotes the most aggressive metastatic disease in our model.

Mutation of the DFG motif and the ATP binding site in C-Raf's kinase domain result in different metastatic phenotypes

To determine whether C-Raf's kinase activity is required for the metastatic phenotype, cell lines expressing C-Raf, and two types of C-Raf kinase dead mutants (D486A and K375M) were generated. C-Raf D486A mutant alters a key aspartate residue responsible for coordinating Mg2+ for ATP binding in the activation segment/DFG motif of the kinase domain [168]. C-Raf K375M mutant targets a catalytic lysine in the kinase domain responsible for mediating ATP catalysis [77,141] (Figure 5A). Both mutations severely dampen C-Raf's kinase activity [165,168-170]. Surprisingly, C-Raf D486A did not significantly affect phospho-MEK1/2 and phospho-ERK1/2 levels compared to WT C-Raf (Figure 5B), while expression of C-Raf K375M diminished phospho-ERK1/2, but not phospho-MEK1/2 levels (Figure 5C). *In vivo* administration of C-Raf D486A resulted in robust metastasis, which significantly reduced survival to levels

comparable to WT C-Raf (Figure 5D & 5E). In contrast, injection with C-Raf K375M expressing cells resulted in significantly delayed metastatic latency compared to WT C-Raf, with some animals surviving until the end of the study (Figure 6E). These differing results for the two C-Raf kinase deficient mutants indicate functional nuances in C-Raf's kinase domain that may be independent of its kinase activity.

Overexpression of C-Raf DFG kinase dead mutant requires endogenous A-Raf and B-Raf to drive metastasis

Mutation of the catalytic lysine K375 is within the region of reported high affinity for B-Raf binding, while the DFG motif is reported to have medium binding affinity [77]. Since dimerization precedes kinase activation, the differences in metastatic phenotypes between these two mutants may be due to their differential effect on C-Raf's ability to heterodimerize with B-Raf. Studies have shown that A and B-Raf kinase mutants can interact with endogenous kinase competent C-Raf monomers to drive aberrant signaling in cancer [75,114,171]. We hypothesize that the C-Raf D486A mutation ablates kinase activity but does not detrimentally affect B-Raf binding. Endogenous B-Raf may dimerize with C-Raf D486A and compensate for its compromised kinase activity. To test this hypothesis, C-Raf D486A was expressed in BC KO cells and evaluated for its effect on metastatic output (Figure 6A). Mice harboring C-Raf D486A BC KO cells had significantly delayed metastatic growth and survived ~110 days longer relative to C-Raf D486A (Figure 6B & 6C). While ablation of endogenous B-Raf and C-Raf significantly increased metastatic latency, the presence of endogenous A-Raf may cooperate with C-Raf D486A for weak metastatic output via the MAPK pathway. We expressed C-Raf D486A in AB KO cells to test the additional role of A-Raf in C-Raf D486A driven metastasis. Unexpectedly, when the only available endogenous Raf monomer is C-Raf in the AB KO background, mice harboring C-Raf D486A had no metastatic lesions and survived to the conclusion of the study with no measurable tumors as assessed by BLI (Figure 6B & 6C) This experiment was repeated

twice to ensure accuracy of results. The presence of endogenous A-Raf in BC KO cells can compensate for C-Raf D486A's loss of kinase activity, presumably via dimerization. However, endogenous C-Raf was not sufficient to rescue C-Raf D486A's low kinase activity in AB KO cells, resulting in no metastasis (Figure 6C). A model of how C-Raf dimerization and interactions with other Raf monomers contributes to metastasis is described in Figure 6D. These data suggest that C-Raf's inherent kinase activity is dispensable in driving metastasis because C-Raf can heterodimerize with kinase competent A-Raf or B-Raf.

DISCUSSION

Cancer cells frequently exhibit increased output through the MAPK pathway, mediated by activating mutations and alterations in Raf function. [12,88,172]. However, the role of wild-type (WT) Raf in metastasis is still poorly understood. In this study, we investigated the critical components of elevated C-Raf signaling in driving metastasis.

We found that non-mutated C-Raf's ability to cause metastasis depends on elevated expression compared to endogenous levels. Various mechanisms can lead to elevated expression and pathway flux, independent of genomic amplification or activating mutations. Dysregulation may involve negative feedback mechanisms, such as hyperphosphorylated C-Raf via ERK1/2 feedback can return to a signaling-competent state through the action of phosphatase PP2A and prolyl isomerase Pin1 [70]. Changes in PP2A activity can result in accumulated active C-Raf. In a parallel example, PTP1B elevation in renal cell carcinoma sensitizes it to SRC inhibition [173]. Genomic changes in non-coding gene regulatory regions can also affect protein expression. Recent whole genome sequencing efforts have identified structural alterations in non-coding cis-regulatory elements of multiple genes that correspond to elevated expression [174].

We demonstrate that C-Raf's dimerization function is crucial for metastasis. The difference in metastatic phenotype between two different C-Raf kinase domain mutations (K375M and D486A) may be explained by their effect on dimerization ability with B-Raf. Rushworth et al.'s molecular studies highlight the significance of distinct binding affinity regions in C-Raf's kinase domain to B-Raf [77]. D486 falls into a medium affinity region for B-Raf binding while K375 is shown to be in the high binding affinity region for B-Raf. Since dimerization precedes kinase activation, mutation of K375 may compromise and impede dimerization with kinase competent B-Raf. This may truncate MAPK flux and consequently cell fitness, resulting in decreased metastases. Therefore, C-Raf's intrinsic kinase activity is dispensable for metastasis if it can compensate by dimerizing with other kinase competent monomers.

Substantiating this study, Venkatanarayan and colleagues report that expression of C-Raf K375M mutant results in drastically reduced B-Raf heterodimer formation. Despite reduced heterodimer formation, this mutant can still drive malignant phenotypes in K-Ras driven cancer, presumably through constitutive activity of mutant K-Ras [144]. They also show that C-Raf K375M has preferential dimerization with A-Raf, which has the weakest kinase activity out of the three Raf kinases [111]. This collection of evidence indicates that C-Raf’s intrinsic kinase activity is dispensable for metastasis if it can compensate by dimerizing with other kinase competent monomers.

Heterodimerization is essential for C-Raf to drive metastasis, as C-Raf D486A expression in the absence of endogenous B-Raf or A-Raf significantly reduced or completely ablated metastatic ability (Figure 4E). This survival advantage provided by genetic deletion of A-Raf and B-Raf suggests that C-Raf D486A homodimerization with endogenous WT C-Raf is not sufficient to facilitate metastasis. Concordant results were reported by Sanclemente et al. 2021, in which a kinase dead C-Raf was able to rescue tumor regression from C-Raf ablation in K-Ras mutant lung adenocarcinomas. Similar results were also highlighted by Venkatanarayan and

colleagues who pointed to the specific role of A-Raf:C-Raf dimer pairs in propagating mutant K-Ras signal *in vitro*. This data suggests non-mutated C-Raf contributes to the metastatic phenotype through a C-Raf kinase-independent, dimerization-dependent signaling via the MAPK pathway.

C-Raf has been shown to possess MAPK independent roles that highlight its scaffolding functions. C-Raf's MAPK independent roles are supported by a body of work that extensively interrogates its role in apoptosis, cell contractility and migration via interactions with various proteins like Bad, Bcl-2, ASK1, MST2 and Rok-α [123,126,128,175,176]. Further investigation of these interactions in reference to metastasis can be explored in future studies.

The next generation allosteric type II Raf inhibitors or "paradox breakers," prevent pathway reactivation via higher affinity for B-Raf mutants and specific targeting of the DFG-out/αC-helix-in conformation [112]. Many of them are currently in development with some in early-stage clinical trials [177-180]. Phase I clinical trials for pan-Raf inhibitor LY3009120 showed toxicity and no change in Phospho-ERK inhibition despite sufficient drug blood plasma levels [180]. While pan-Raf inhibition is important, our study and others highlight the importance of heterodimerization of Raf molecules over homodimerization [72,77,144,181,182]. Higher specificity of future Raf inhibitor development may be required to halt Raf mediated metastatic phenotypes.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.T and O.N.W; Methodology, L.T.; Software, B.L.T; Validation, L.T.; Formal Analysis, L.T., B.L.T, W.D. and W.T.; Investigation, L.T., W.D., J.S.; Writing, L.T. Review & Editing, L.T.; G.V.; Visualization, L.T.; W.T.; Supervision, J.R.C, J.A.W. and O.N.W; Project Administration, O.N.W.; Funding Acquisition, O.N.W,

DECLARATION OF POTENTIAL CONFLICTS OF INTERESTS

O.N.W. currently has consulting, equity, and/or board relationships with Trethera Corporation, Kronos Biosciences, Sofie Biosciences, Breakthrough Properties, Vida Ventures, Nammi Therapeutics, Two River, Iconovir, Appia BioSciences, Neogene Therapeutics, 76Bio, and Allogene Therapeutics. None of these companies contributed to or directed any of the research reported in this article.

MATERIALS & METHODS

Cell Culture and Reagents

BPH-1 cells were propagated in RPMI supplemented with 10% (vol/vol) FBS (Gibco) and glutamine (1 mM). RWPE-1 cells were purchased from ATCC and cultured in keratinocyte serum-free medium (K-SFM) (Gibco) supplemented with 0.05 mg/mL bovine pituitary extract (Gibco), 5 ng/mL EGF (Gibco), penicillin (100 U/mL), and streptomycin (100 µg/mL). 293t cells used for lentiviral production were cultured in DMEM supplemented with 10% (vol/vol) FBS and glutamine (1 mM).

Cell titer glo experiments

Proliferation experiments were performed following 5 days incubation in ultra-low attachment plates (Corning cat. CLS3471-24EA) and read using Promega Cell Viability assay (Promega cat. G7570).

Cloning

For cloning of the Ub-C-Raf overexpression vectors (C-Raf WT, C-Raf R401H and C-Raf D486A) C-Raf in C-Raf-ILYW (Falteirmeier et al. 2013) was mutated using site direct mutagenesis kit (Agilent 200523). Ub reporter was swapped out for EFS and PGK promoters via Gibson cloning. LentiCRISPR v2 CRISPR/cas9 system all-in-one dox inducible system was used to express cas9 and sgRNA targeting A-Raf, B-Raf and C-Raf genes. TLCV2 (Addgene #87360) was generated by insertion of A-Raf, B-Raf and C-Raf guide RNA (sgA-Raf – ACAATTTTGTGAGTGCAGGG, sgB-Raf – TTGAAGGCTTGTAACTGCTG, and sgC-Raf – GACCATGTGGACATTAGGTG) into TLCV2 vector. All cloning was sequence verified.

Virus production

Third-generation lentiviruses were prepared by calcium phosphate precipitation transfection of 293t cells with plasmids expressing kinases with firefly luciferase reporter gene (FU-ILYW), Cas9 and guide RNAs (TLCV2), and gaussian luciferase plasmid (CMV-Gluc-IRES-GFP) (Targeting systems cat. GL-001). The lentiviruses were prepared as described [183]. Viruses were tittered using serial transduction protocol of naïve 293t and assessed via flow cytometry using YFP and GFP.

Clonal knock out using CRISPR/Cas9

BPH-1 cells were infected with B-Raf-TLCV2 virus at an MOI of 10 for 48 hours. Infected cells were then treated with puromycin for selection of plasmid positive cells at 1 ug/mL for 72 hours. After puromycin selection, cells were then treated with 1 ug/mL of doxycycline for 72 hours to induce Cas9 expression and cutting. Cells were then expanded and then plated into 96 wells at once cell/well. Clones were then individually grown out, and screened via western blot for changes in protein levels. Clones that exhibited diminished or non-existent specific protein

bands were additionally DNA sequenced at the site of the CRISPR edit to determine heterozygous or homozygous frame shift.

Western blot

Whole-cell lysates were prepared in Urea lysis buffer (8M Urea, 4% CHAPS, cOmplete™ Protease Inhibitor Cocktail from Roche with phosphatase inhibitor). Equal amounts of protein were separated by 4–12% (mass/vol) Bolt™ 4 to 12%, Bis-Tris SDS/PAGE (Thermo Fisher), followed by immunoblotting analysis with the indicated antibodies. The following antibodies were used to detect the corresponding proteins V5 (Invitrogen R960-25; 1:2,500); BRAF (Cell Signaling 55C6; 1:1,000); ARAF (Cell Signaling 4432S; 1:1000); C-Raf (Sigma HPA002640; 1:1000); Phospho-p44/42 MAPK (Erk1/2) (Cell Signaling 4370S; 1:1000); Phospho-MEK1/2 (Cell signaling 9154S; 1:1000); MEK1/2 (Cell Signaling 8727S; 1:1000); p44/42 MAPK (Erk1/2) (L34F12) (Cell Signaling 4696S; 1:1000); GAPDH (Biolegend 607903; 1:2,500)

Animal studies and tumor models

All animal experiments were performed according to the protocol approved by the Division of Laboratory Medicine at the University of California, Los Angeles. NOD-scid gamma mice were purchased from Jackson Laboratories. For all experiments, male mice between 6 and 8 weeks of age were used. Mice were anesthetized at 2% (vol/vol) Isoflurane prior to intracardiac injection. A single cell preparation was prepared at 0.250e6/injection of 100 uL in 1x PBS and injected into the left ventricle of the mouse heart. Gaussia luciferase measurements were conducted weekly and were performed as described [147].

Bioluminescence imaging

Bioluminescence Imaging (BLI) was conducted using an IVIS Lumina II (PerkinElmer). D-luciferin (150 mg/kg) was injected intraperitoneally. After 15 min, anesthetized mice (using 2% (vol/vol) isoflurane) were imaged. BLI analysis was performed using Living Image software, version 4.0 (PerkinElmer).

Phospho-proteomics

Cells were grown at 70% confluency (10e6) harvested and washed 2x with ice cold PBS. Cells were subsequently scaped off using a cell scraper, pelleted and washed 2x with ice cold PBS. Cell pellets were lysed in Urea lysis buffer (8M Urea, 100mM Tris pH8.5, AEBSF, phosphatase inhibitor, benzonase and 1mM DTT) and incubated at RT for 0.5 hour. Lysates were cleared by centrifuging at 12000 g for 15 minutes. Supernatants from samples were transferred to a set of new tubes and concentrations were determined by absorbance at 280nm. 150ug of protein was taken from each sample and proceeded to reduction (5mM TCEP) and alkylation (10mM iodoacetamide). Reduced and alkylated protein samples were cleaned up by SP3 method [184], then 0.2ug of Lys-C and 2ug of Trypsin proteases were added to each sample and digestion were conducted for overnight. Digested peptide samples were labeled by 0.3ug of TMT isobaric tagging reagent for 1 hour and quenched by adding hydroxylamine to 0.5%. Equal amount of labeled peptide from each sample was pooled. The pooled mixture was used to perform phospho-peptide enrichment using Thermo High-Select Fe-NTA phospho-peptide enrichment kit (A32992). Finally, enriched TMT-labeled phospho-peptides were fractionated by CIF method [152] to 6 fractions.

LC-MS acquisition

A 75 µm x 25 cm homemade C18 column was connected to a nano-flow Dionex Ultimate 3000 UHPLC system. The 70-minute gradient of increasing acetonitrile (ACN) was delivered at a 200nl/min flow rate as follows: 1% ACN phase from minutes 0 – 6, 6 - 25% ACN from minutes 6 – 55, 25 - 32% ACN from minutes 55 - 63.5, 32 - 80% ACN from minutes 63.5 – 67, and then 1% ACN from minutes 68 - 70. An Orbitrap Fusion Lumos Tri-brid mass spectrometer was used for data acquisition in TMT-SPS-MS3 [185] mode. Full MS scans were acquired at 120K resolution with the AGC target set to standard and a maximum injection time set to 50 ms. MS/MS scans were collected in linear ion trap in Turbo mode after isolating precursors with an isolation window of 0.7 m/z and CID-based fragmentation using 35% collision energy. Synchronized precursor selection was performed and 10 precursors were fragmented with 75% energy of

HCD and sent for MS3 scans in Orbitrap with 50K resolution. were collected. For data dependent acquisition, a 3-second cycle time was used to acquire MS/MS and MS3 spectra corresponding to peptide targets from the preceding full MS scan. Dynamic exclusion was set to 30 seconds.

Mass spectrometry data analysis

MS/MS database search was performed using MaxQuant (1.6.10.43) against the human reference proteome from EMBL (UP000005640_9606 HUMAN Homo sapiens, 20874 entries). The search included carbamidomethylation on cysteine, TMT isobaric tag on lysine and peptide N-terminus as a fixed modification. Serine, threonine and tyrosine phosphorylation, methionine oxidation and N-terminal acetylation were set as variable modifications. The digestion mode was set to trypsin and allowed a maximum of 2 missed cleavages. The precursor mass tolerances were to 20 and 4.5 ppm for the first and second searches, respectively. Datasets were filtered at 1% FDR at the PSM level. Peptide quantitation was performed using MaxQuant's multiplexing TMT 10plex MS3 mode. Site level t-test was performed using summarized phosphosites quantitation output from MaxQuant. Differentially (p-value<=0.05) quantified peptides from each pair of comparison were sent to PhosFate [154] to infer kinase activity change.

Transcriptional Profiling of C-Raf cell lines

RNA-Sequencing

Transcriptomic profiling was performed using the TOIL pipeline. The transcriptomic dataset was filtered for coding genes with low additional filtering of variance and low abundance. We used ComBat-seq [186] to adjust for batch effects attributed to tumors coming from two different cell lines/models. Expected counts were log2 transformed. After processing, the final transcriptomic dataset consisted of 10,958 genes. Raw and processed RNA-seq data is available in GEO accession number XXX"

Differential RNA abundance

DESeq2 [187] was used to perform differential RNA abundance analysis. Comparisons were performed between parental C-Raf vs vector control, which identified 667 differentially abundant genes, and C-Raf metastasis derived line vs parental C-Raf, which identified 634 differentially abundant genes. Statistical significance was determined using FDR-adjusted p-value < 0.05. Using these sets differentially abundant genes, we identified 46 genes that were perturbed in a stepwise fashion. That is, these genes were altered in the same direction for both comparisons.

Hallmarks signature scoring

To evaluate well-characterized biological processes, we scored each sample for the 50 Hallmarks gene sets [188]. The transcriptomic dataset was first normalized by z-scoring across samples and across genes, then scored by taking each gene in the Hallmarks gene sets and median dichotomizing the samples. Samples with RNA abundance levels greater than the median were assigned a score of +1 for the gene, and samples with RNA abundance levels lesser than the median were assigned a score of -1 for the gene. This was repeated for every gene in the gene set, and the scores were summed for each sample to give the sample's signature score.

The volcano plot, heatmap and boxplots were generated using the BoutrosLab.plotting.general R package [189].

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FIGURE LEGENDS

Figure 1: C-Raf overexpression drives metastasis of two immortalized prostate cell lines. **A)** Schematic of model system and workflow used to study C-Raf overexpression *in vivo*. C-Raf and reporter genes were expressed in immortalized prostate lines and injected into mice via intracardiac injection followed by serial blood measurements and bioluminescence imaging (BLI). Firefly luciferase reporter system was used to assess spatial metastatic activity via BLI. Gaussia luciferase reporter system was used to assess whole body tumor burden measurement via real-time blood measurements. **B)** Western blot analysis demonstrating C-Raf-v5 overexpression and subsequent downstream effectors including P-MEK1/2 and P-ERK1/2 in RWPE-1 and BPH-1 cell lines. **C)** BLI imaging at 28 days post RWPE-1 injection. **D)** BLI imaging at 14 days post injection of BPH-1. **E)** Distribution of macroscopic tumors across 12 mice with C-Raf overexpression and vector control in RWPE-1 cells. **F)** Distribution of macroscopic tumors across 30 mice with C-Raf overexpression and vector control in BPH-1 cells. **G)** Kaplan-Meier curve of mice harboring RWPE-1 vector control vs C-Raf overexpression cells (n=10/group, Log-rank (Mantel Cox) test p = 0.0001). **H)** Kaplan-Meier curve of mice harboring BPH-1 vector control vs C-Raf overexpression cells (n=10/group, Log-rank (Mantel Cox) test, p = 0.0001).

Figure 2: C-Raf drives metastasis and mortality in a gene dosage dependent manner **A)** Western blot depicting increasing C-Raf protein expression driven by promoters of varying strength in BPH-1 cells. **B)** Cell cycle analysis using propodium iodide across cell lines with increasing C-Raf expression in adherent culture conditions. Vector (G1: 49.7%, S: 17.5%, G2: 29%) PGK (G2: 56.4%, S: 17.0%, G2: 20.9) EFS (G1: 58.5%, S: 14.4%, G2: 21.5%) Ub (G1:

64%, S: 10.3%, G2: 22.1%). **C)** Proliferation as measured by trypan blue staining over 10 days in adherent conditions. **D)** Proliferation of cells measured by cell titer glo assay of C-Raf increasing expression cell lines in anchorage independent conditions at 5 days (unpaired t-test, p= 0.0002, 0.001, 0.0001 from left to right). **E)** Gaussia luciferase blood measurements as a surrogate marker of tumor burden over 17 days. **F)** BLI imaging of C-Raf dosage lines at 28 days post intracardiac injection. **G)** Kaplan-Meier curve of mice harboring cells with increasing C-Raf expression (n=4/group, Log-rank (Mantel-Cox) test p=0.0014, all groups significantly different).

Figure 3 Mutation of C-Raf dimerization domain ablates metastatic ability

A) Western blot analysis of C-Raf dimerization mutant overexpression compared to WT C-Raf in BPH-1 cells. **B)** BLI 28 days post injection of WT C-Raf compared to C-Raf dimerization null (R401H) and vector control. **C)** Kaplan-Meier curve of mice harboring WT C-Raf and mutant cells (n=5/group, Log-rank (Mantel-Cox) test p = 0.0064, all groups significantly different). **D)** *In vitro* cell titration detected using D-luciferin substrate to confirm firefly surrogate tumor measurements *in vivo.*

Figure 4: Knock-out of Raf family members diminishes C-Raf driven metastasis

A) Schematic of double knock-out cell line generation starting from viral transduction, inducible Cas9 induction via doxycycline at 1 ug/mL, single cell cloning, and subsequent confirmation and sequential generation of double knock-out line. **B)** Protein level confirmation of endogenous KO combinations A-Raf and B-Raf (AB) or B-Raf and C-Raf (BC) and their corresponding C-Raf overexpression lines. **C)** Proliferation of double knock-out cell lines compared to parental control in anchorage independent conditions (n=3/group, unpaired t-test, p = 0.04). **D)** Proliferation in anchorage independent conditions with C-Raf overexpression (unpaired t-test from left to right, p = 0.0004, 0.0027, 0.0001). **E)** Combined survival curve of mice harboring cells with C-Raf

overexpression in BPH-1 cells and BPH01 AB & BC KO cell lines (n=8/group Log-rank (Mantel-Cox) test, p = 0.0001, all groups significantly different).

Figure 5: Mutation of the DFG motif and the ATP binding site in C-Raf's kinase domain result in different metastatic phenotypes

A) Schematic of C-Raf structure indicating location of mutations relative to the rest of the protein structure. **B)** Western blot depicting expression of C-Raf and C-Raf D486A mutant and downstream signaling. **C)** Western blot depicting expression of C-Raf and C-Raf K375M and downstream signaling. **D)** BLI imaging of both C-Raf kinase dead mutants at 28 days post intracardiac injection compared to controls. **E)** Kaplan-Meier curve of mice harboring cells with vector control, C-Raf and C-Raf D486A and C-Raf K375M, (n=4/group Log-rank (Mantel-Cox) test, p = 0.0001, all groups significantly different).

Figure 6: Overexpression of C-Raf DFG kinase dead mutant requires endogenous A-Raf and B-Raf to drive metastasis

A) Western blot depicting C-Raf D386A mutant expressed in wildtype or combination AB KO or BC KO and downstream signaling. **B)** BLI imaging at 70 days post intracardiac injection of mice expressing vector control, C-Raf and C-Raf D486A in AB KO or BC KO backgrounds. **C)** Kaplan-Meier curve of groups depicted in B) (n=8/group Log-rank (Mantel-Cox) test, p = 0.0001, all groups significantly different). **D)** Cartoon model depicting the implied role of hetero and homodimerization in C-Raf driven metastatic phenotype.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: C-Raf overexpression increases MAPK pathway flux and is associated with metastasis.

A) Phospho-proteomic workflow using tandem mass tag (TMT) isobaric labeling and mass spectrometry for multiplexing. Cells are lysed using proteolytic digestion and subsequently labeled with TMT isobaric labeling to allow for sample multiplexing. Samples simultaneously underwent Immobilized metal affinity chromatography (IMAC) phospho-enrichment and subsequent MS/MS analysis to determine the phosphoproteome relative to each other. **B)** Principal component analysis of phospho-proteomic samples captured a total of 84.74% of variance in PC1 and PC2. **C)** Schematic of inferred kinase activity (IKA) analysis. **D)** List of top 15 upregulated kinases in C-Raf samples compared to vector control. **E)** Top 10 pathways upregulated via pathway flux analysis derived from inferred kinase hits with p value < 0.05.

Supplemental Figure 2: Transcriptional targets of the MAPK pathway are upregulated in metastatic C-Raf driven tumors

A) Schematic of C-Raf metastasis derived cell line generation and subsequent mRNA sequencing analysis compared to parental cell lines. Cell lines were generated from bone, lymph, thymus, spine, and liver metastases. **B)** Volcano plots of genes altered upon C-Raf addition compared to vector control and C-Raf metastasis cell lines compared to C-Raf parental line. Statistical significance was determined using FDR <0.05. **C)** 46 differentially expressed genes that were perturbed in a stepwise fashion across parental and metastasis derived lines in BPH-1 and RWPE-1 (increasing and decreasing). Bold italicized genes indicate genes that are part of the Ras/Raf signaling pathway that increased from vector to C-Raf parental to C-Raf metastasis derived cell line. **D)** Significantly altered cancer hallmark pathway analysis using GSEA across sample groups (unpaired t - test, $p = 0.005$).

Supplemental Figure 3: C-Raf gene dosage western quantification

A) Densitometric quantification of C-Raf gene dosage protein expression and downstream effectors.

Figure 1

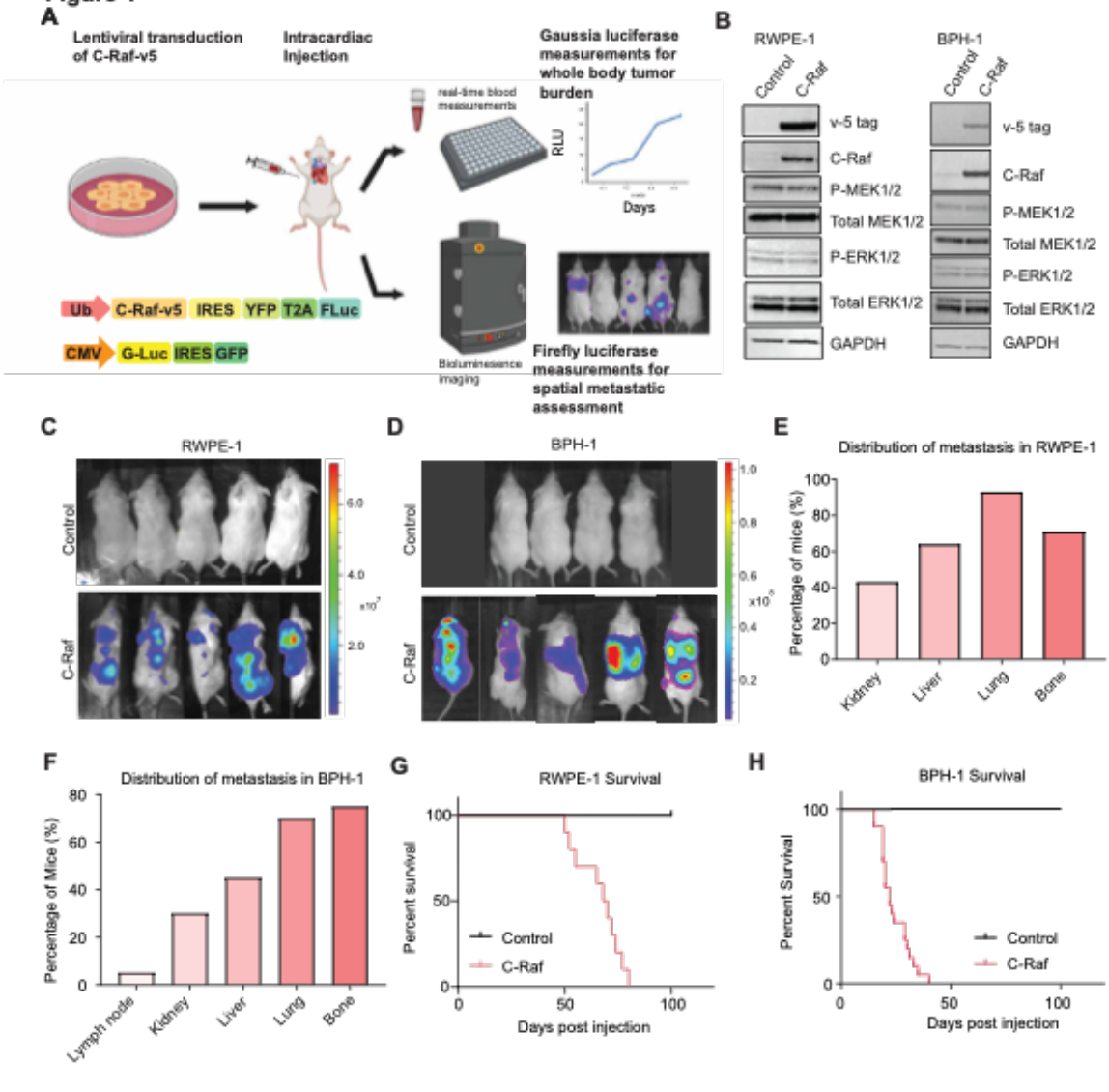

A
Lentiviral transduction of C-Raf-v5
Intracardiac Injection
Gaussia luciferase measurements for whole body tumor burden
real-time blood measurements
RLU
Days
Ub
C-Raf-v5
IRES
YFP
T2A
FLuc
CMV
G-Luc
IRES
GFP
Bioluminescence imaging
Firefly luciferase measurements for spatial metastatic assessment
B
RWPE-1
BPH-1
Control
C-Raf
v-5 tag
C-Raf
P-MEK1/2
Total MEK1/2
P-ERK1/2
Total ERK1/2
GAPDH
C
RWPE-1
Control
C-Raf
D
BPH-1
Control
C-Raf
E
Distribution of metastasis in RWPE-1
Percentage of mice (%)
Kidney
Liver
Lung
Bone
F
Distribution of metastasis in BPH-1
Percentage of Mice (%)
Lymph node
Kidney
Liver
Lung
Bone
G
RWPE-1 Survival
Percent survival
Control
C-Raf
Days post injection
H
BPH-1 Survival
Percent Survival
Control
C-Raf
Days post injection

Figure 2

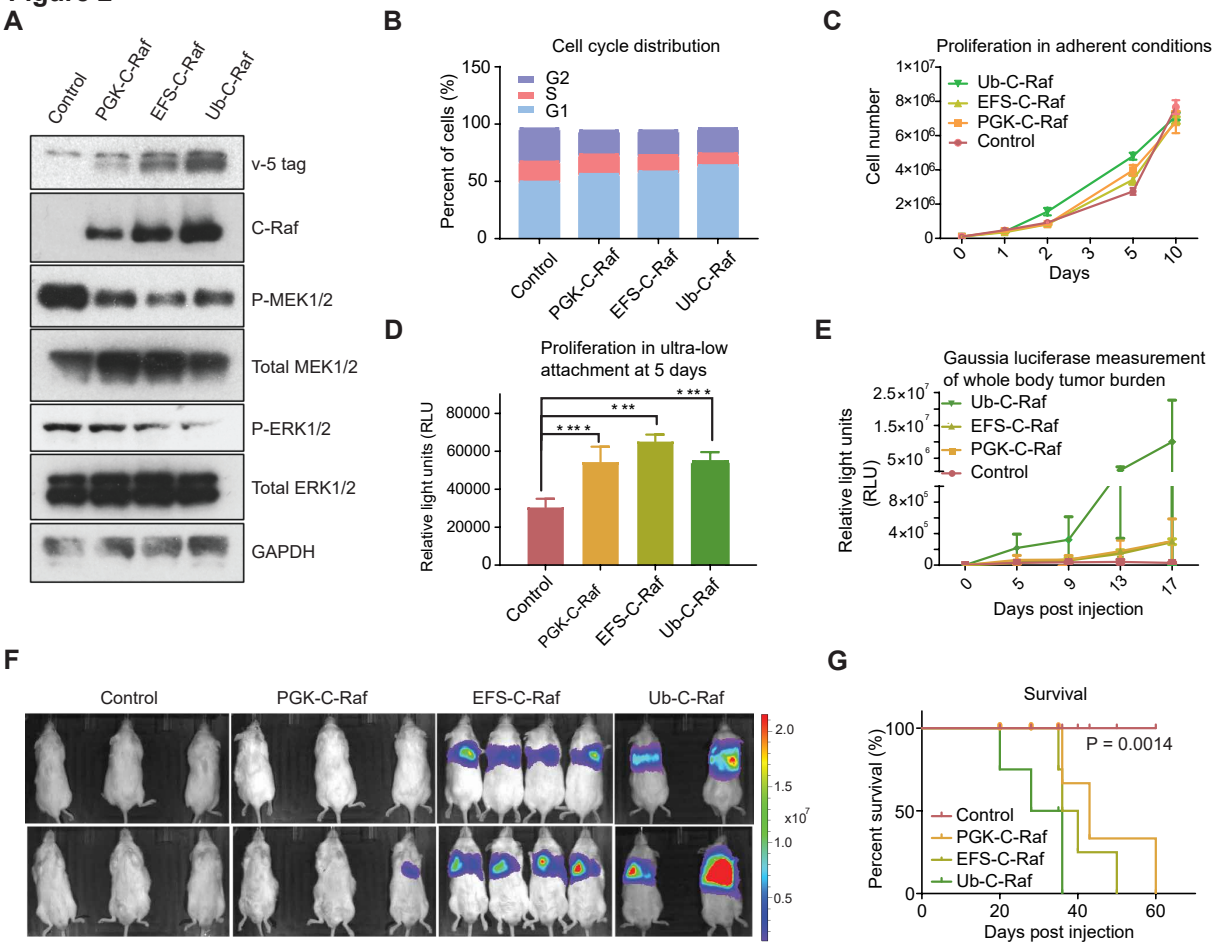

A
Control
PGK-C-Raf
EFS-C-Raf
Ub-C-Raf
v-5 tag
C-Raf
P-MEK1/2
Total MEK1/2
P-ERK1/2
Total ERK1/2
GAPDH
B
Cell cycle distribution
Percent of cells (%)
G2
S
G1
C
Proliferation in adherent conditions
Cell number
Ub-C-Raf
EFS-C-Raf
PGK-C-Raf
Control
Days
D
Proliferation in ultra-low attachment at 5 days
Relative light units (RLU
E
Gaussia luciferase measurement of whole body tumor burden
Relative light units (RLU)
Days post injection
F
Control
PGK-C-Raf
EFS-C-Raf
Ub-C-Raf
G
Survival
P = 0.0014
Percent survival (%)
Days post injection

Figure 3

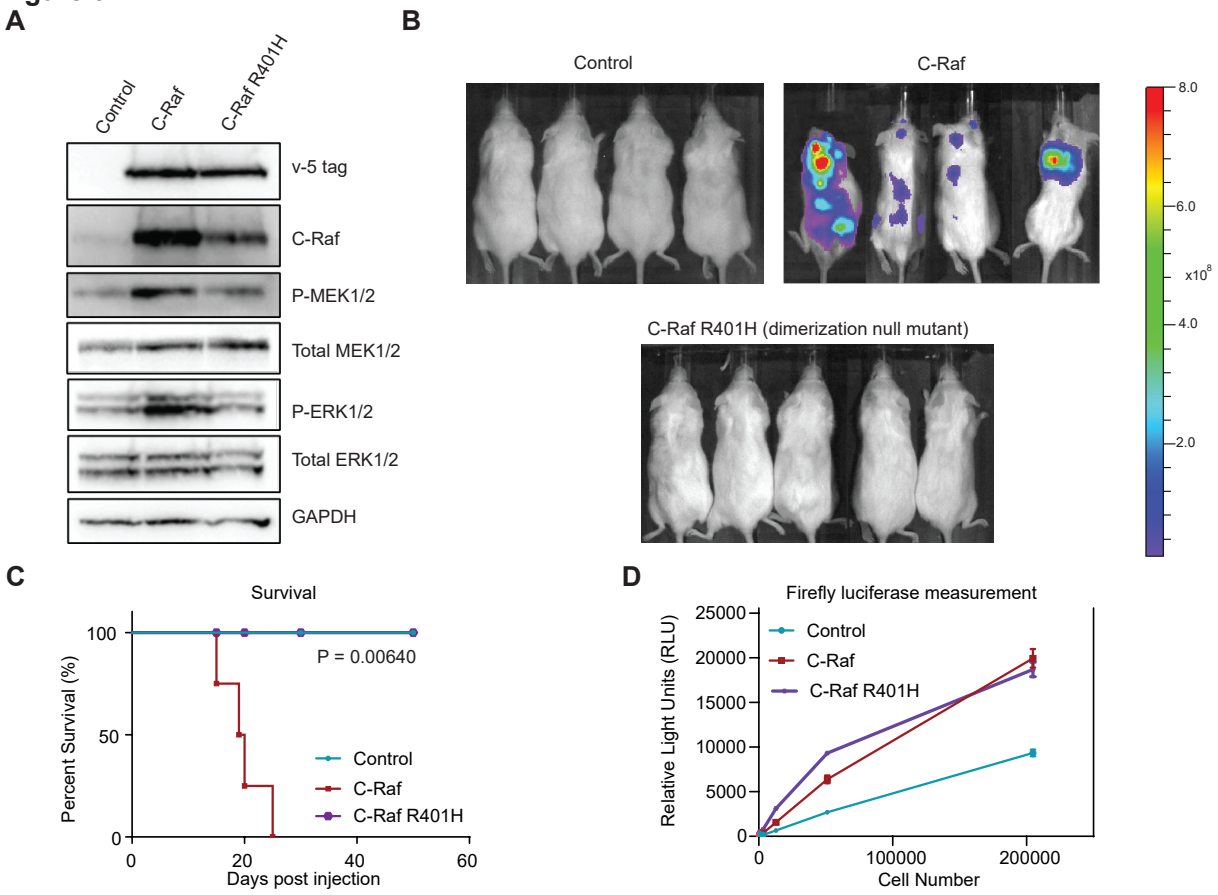

A
Control
C-Raf
C-Raf R401H
v-5 tag
C-Raf
P-MEK1/2
Total MEK1/2
P-ERK1/2
Total ERK1/2
GAPDH
B
Control
C-Raf
C-Raf R401H (dimerization null mutant)
8.0
6.0
x10^8
4.0
2.0
C
Survival
Percent Survival (%)
100
50
0
P = 0.00640
Control
C-Raf
C-Raf R401H
0
20
40
60
Days post injection
D
Firefly luciferase measurement
Relative Light Units (RLU)
25000
20000
15000
10000
5000
0
Control
C-Raf
C-Raf R401H
0
100000
200000
Cell Number

Figure 4

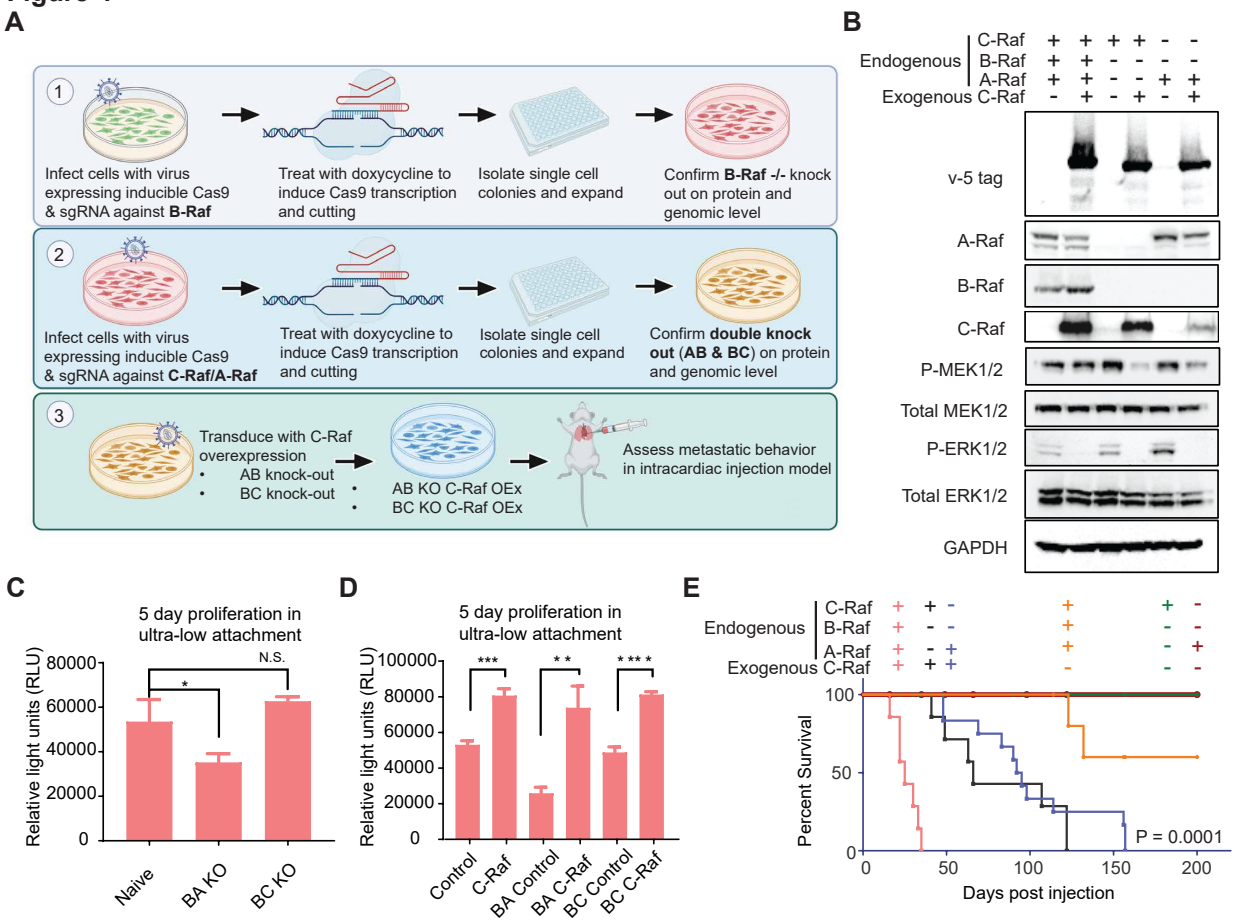

A
1
Infect cells with virus expressing inducible Cas9 & sgRNA against B-Raf
Treat with doxycycline to induce Cas9 transcription and cutting
Isolate single cell colonies and expand
Confirm B-Raf -/- knock out on protein and genomic level
2
Infect cells with virus expressing inducible Cas9 & sgRNA against C-Raf/A-Raf
Treat with doxycycline to induce Cas9 transcription and cutting
Isolate single cell colonies and expand
Confirm double knock out (AB & BC) on protein and genomic level
3
Transduce with C-Raf overexpression
AB knock-out
BC knock-out
AB KO C-Raf OEx
BC KO C-Raf OEx
Assess metastatic behavior in intracardiac injection model
B
Endogenous
C-Raf
B-Raf
A-Raf
Exogenous C-Raf
v-5 tag
A-Raf
B-Raf
C-Raf
P-MEK1/2
Total MEK1/2
P-ERK1/2
Total ERK1/2
GAPDH
C
5 day proliferation in ultra-low attachment
Relative light units (RLU)
80000
60000
40000
20000
0
N.S.
*
Naive
BA KO
BC KO
D
5 day proliferation in ultra-low attachment
Relative light units (RLU)
100000
80000
60000
40000
20000
0
***
**
****
Control
C-Raf
BA Control
BA C-Raf
BC Control
BC C-Raf
E
Endogenous
C-Raf
B-Raf
A-Raf
Exogenous C-Raf
Percent Survival
100
50
0
P = 0.0001
0
50
100
150
200
Days post injection

Figure 5

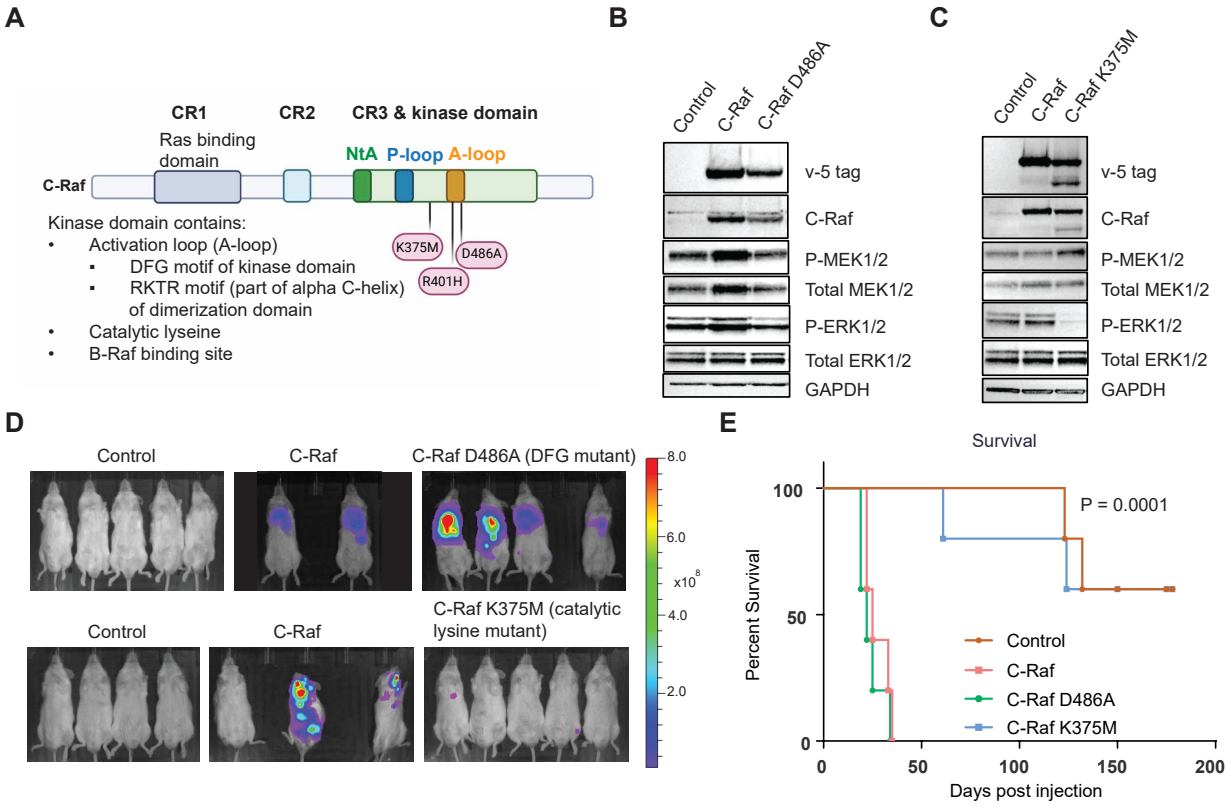
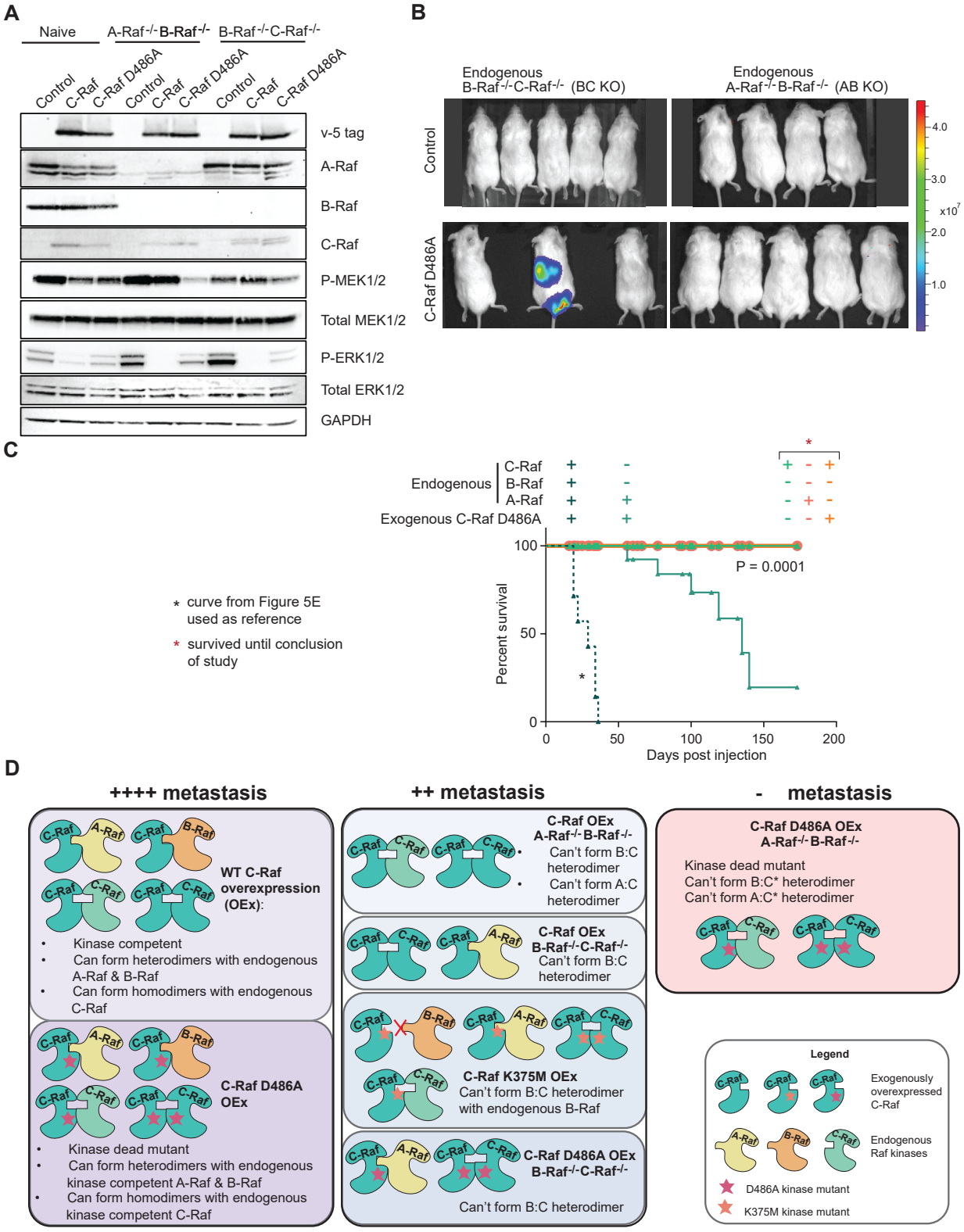
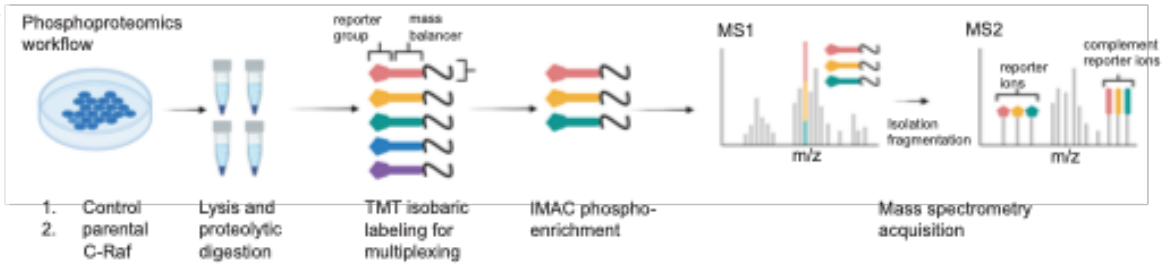

A
CR1
CR2
CR3 & kinase domain
Ras binding domain
NtA
P-loop
A-loop
C-Raf
K375M
D486A
R401H
Kinase domain contains:
Activation loop (A-loop)
DFG motif of kinase domain
RKTR motif (part of alpha C-helix) of dimerization domain
Catalytic lyseine
B-Raf binding site
B
Control
C-Raf
C-Raf D486A
v-5 tag
C-Raf
P-MEK1/2
Total MEK1/2
P-ERK1/2
Total ERK1/2
GAPDH
C
Control
C-Raf
C-Raf K375M
v-5 tag
C-Raf
P-MEK1/2
Total MEK1/2
P-ERK1/2
Total ERK1/2
GAPDH
D
Control
C-Raf
C-Raf D486A (DFG mutant)
Control
C-Raf
C-Raf K375M (catalytic lysine mutant)
8.0
6.0
x10^8
4.0
2.0
E
Survival
P = 0.0001
Percent Survival
100
50
0
0
50
100
150
200
Days post injection
Control
C-Raf
C-Raf D486A
C-Raf K375M

Figure 6

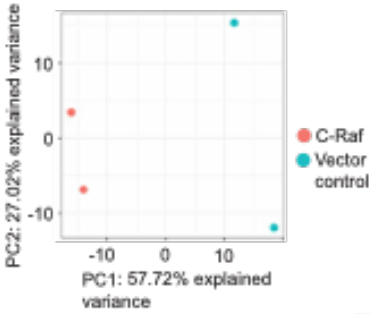

A
Naive
A-Raf-/- B-Raf-/-
B-Raf-/- C-Raf-/-
Control
C-Raf
C-Raf D486A
v-5 tag
A-Raf
B-Raf
C-Raf
P-MEK1/2
Total MEK1/2
P-ERK1/2
Total ERK1/2
GAPDH
B
Endogenous
B-Raf-/- C-Raf-/- (BC KO)
Endogenous
A-Raf-/- B-Raf-/- (AB KO)
Control
C-Raf D486A
4.0
3.0
x10^7
2.0
1.0
C
Endogenous
C-Raf
B-Raf
A-Raf
Exogenous C-Raf D486A
P = 0.0001
Percent survival
Days post injection
* curve from Figure 5E used as reference
* survived until conclusion of study
D
++++ metastasis
++ metastasis
- metastasis
WT C-Raf overexpression (OEx):
Kinase competent
Can form heterodimers with endogenous A-Raf & B-Raf
Can form homodimers with endogenous C-Raf
C-Raf D486A OEx
Kinase dead mutant
Can form heterodimers with endogenous kinase competent A-Raf & B-Raf
Can form homodimers with endogenous kinase competent C-Raf
C-Raf OEx A-Raf-/- B-Raf-/-
Can't form B:C heterodimer
Can't form A:C heterodimer
C-Raf OEx B-Raf-/- C-Raf-/-
Can't form B:C heterodimer
C-Raf K375M OEx
Can't form B:C heterodimer with endogenous B-Raf
C-Raf D486A OEx B-Raf-/- C-Raf-/-
Can't form B:C heterodimer
C-Raf D486A OEx A-Raf-/- B-Raf-/-
Kinase dead mutant
Can't form B:C* heterodimer
Can't form A:C* heterodimer
Legend
Exogenously overexpressed C-Raf
Endogenous Raf kinases
D486A kinase mutant
K375M kinase mutant

Supplemental 1

A



B



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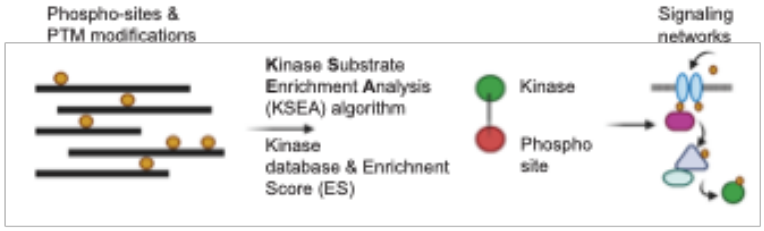


D

Upregulated kinases in C-Raf vs Vector control

Upregulated kinase activities	Identified Substrates in dataset
CDK2	28
VRK1	3
VRK2	3
HPK2	1
PRKG1	3
CAMK2A	1
CAMK2B	1
CDK1	21
CAMK4	1
RAF1	3
SGK1	1
LATS1	1
MAPKAPK2	3
PLK1	1
PRKDC	1
AKT2	1

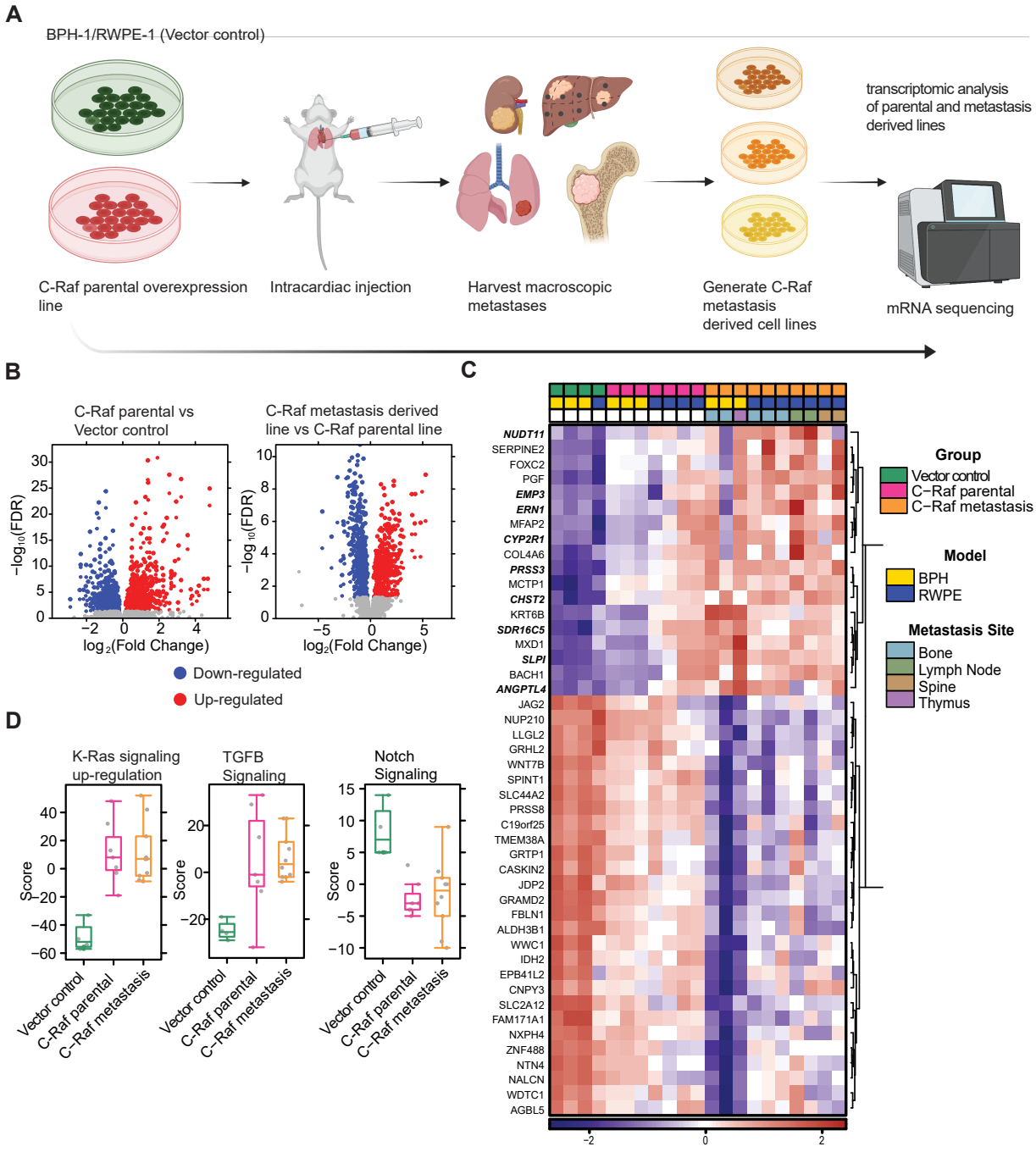
0 10 20 30 40 60

$p < 0.05$

E

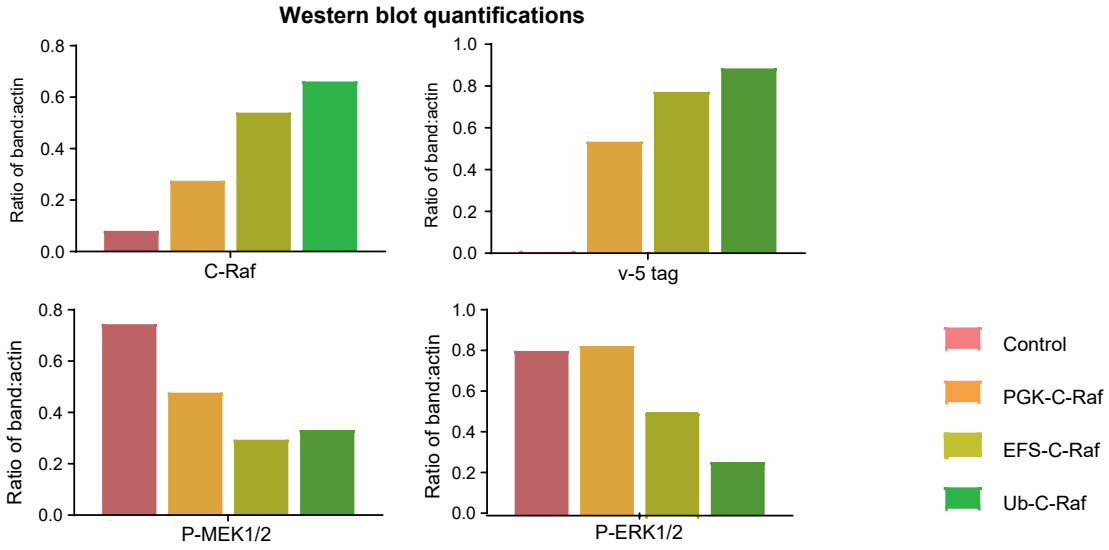
pvalue	Pathway	quantSites	proteins
0.001	RAF1-RAS complex, EGF induced	5	4
0.002	P-TEFb-BRD4-TRAP220 complex	6	4
0.002	DDB complex	2	2
0.003	Ubiquitin E3 ligase (DDB1, DDB2, CUL4A, CUL4B, RBX1)	2	5
0.007	PTIP-HMT complex	1	10
0.017	Chromatin remodeling complex (TACC2, TACC3, PCAF)	1	3
0.017	Menin-associated histone methyltransferase complex	1	7
0.018	ITAGV-ITGB3-F11R complex	2	3
0.026	NFAT-JUN-FOS DNA-protein complex	2	3
0.03	ITGA2B-ITGB3-F11R complex	2	3
0.038	GR-hnRNP U complex	1	2

Supplmental 2



Supplemental 3

A



Chapter 3: Discussion and Future Directions

Raf's role in MAPK signaling in the context of cancer has been recognized for the past 30 years. Despite the prevalence of alterations in this pathway across various cancer types, effectively targeting it remains a challenge. While current therapies show promise in extending patients' progression-free survival, the emergence of resistance mutations remains a persistent issue. Clinical trials have explored the use of combination therapies involving multi-target inhibition. In metastatic melanoma, for instance, the combination of mutant B-Raf inhibition with MEK inhibition has shown some efficacy [190]. However, the combined effect of dual MAPK inhibition often leads to significant toxicity, with some patients experiencing adverse effects [191]. To address this problem, various approaches can be considered, such as the continuous development of inhibitors targeting resistance mechanisms, drawing inspiration from natural interactions between plants and viruses. Another innovative strategy involves exploring oncogenic dependencies, vulnerabilities, and genes that control crucial aspects of the malignant process. This is where studying the impact of wild-type genes, particularly kinases, becomes relevant.

In this thesis, our objective is to comprehend the nature of WT C-Raf-driven metastasis by examining different functional domains of C-Raf. Through our research, we demonstrate that even subtle elevation of C-Raf protein levels can instigate cancer metastasis in an intracardiac mouse tumor model. Our findings emphasize the critical role of the dimerization interface in the kinase domain for C-Raf-driven metastatic behavior, while the inherent kinase activity itself is not essential. This conclusion is further supported by the impact of eliminating other Raf isoforms on mice survival. These results highlight the importance of delineating the dimerization domain independently from the kinase domain. By understanding how these structural domains regulate various Raf processes, we can gain insights for more effective therapeutic development targeting the Raf kinases. Given that this thesis establishes the significance of

kinase activity in driving metastatic activity, it would be valuable to discuss the current landscape of Raf inhibitors and their interactions with Raf kinases.

There are currently three categories of Raf inhibitors: Type I, Type II, and Type III, along with some inhibitors that do not fit neatly into these categories [192]. These inhibitors are classified based on their interaction with the DFG motif (Asp-Phe-Gly) and the Alpha C helix. The DFG motif is a conserved sequence found in the activation loop of protein kinases, including Raf kinases, and plays a crucial role in regulating kinase activity through conformational changes [193]. The Alpha C helix, located adjacent to the DFG motif, also contributes to kinase regulation by influencing the accessibility of the ATP-binding site [81].

1. Type I Raf inhibitors/ATP-competitive inhibitors: Type I inhibitors interact with the DFG-in alpha C helix inward conformation of Raf kinases. They bind to the ATP-binding site and stabilize the inactive state of Raf, preventing its activation. Sorafenib and vemurafenib are examples of type I inhibitors that have demonstrated clinical efficacy in Raf-mutant cancers.
2. Type II Raf inhibitors/Allosteric inhibitors: Type II inhibitors target the DFG-out and alpha C helix outward conformation of Raf kinases. They bind to an allosteric pocket adjacent to the ATP-binding site and induce conformational changes that disrupt Raf kinase activity. By modulating the conformation, these inhibitors prevent downstream signaling activation. Some type II inhibitors, such as PLX8394 and LY3009120, exhibit improved selectivity and potency compared to type I inhibitors [180,194].
3. Type III Raf inhibitors/active conformation stabilizers: Type III inhibitors specifically target the alpha C helix of Raf kinases. They interact with a unique pocket formed by the alpha C helix and adjacent residues, resulting in conformational changes that inhibit kinase activity. This interaction stabilizes Raf proteins in their active conformation, effectively blocking downstream signaling by preventing their transition to an inactive state. BGB-

283 and CCT196969 are examples of active conformation stabilizers that have shown promise in preclinical studies [195,196].

4. Dimerization-dependent inhibitors: Dimerization-dependent inhibitors disrupt the interaction between Raf monomers, preventing their activation. Promising dimerization inhibitors like RRD-251 and CHIR-265/RAF265 have demonstrated efficacy in preclinical models and have shown positive results in phase I and phase II clinical trials [197,198].

These different types of Raf inhibitors target key aspects of Raf kinase function, such as conformational changes, ATP-binding site interactions, and dimerization. Understanding the mechanisms of action of these inhibitors provides valuable insights for the development of effective therapeutic strategies against Raf-driven cancers.

Dimerization-dependent inhibitors may seem to hold the key to addressing the complexities of Raf biology. The development of pan-Raf inhibitors, such as KIN-2787 (Exarafenib) and BGB-3245, which target both pan-Raf and dimerization, has shown promising early phase I trial data [199,200]. These diverse inhibitors, with their ability to target different Raf conformations, have deepened our understanding of Raf kinase biology and opened avenues for personalized treatment approaches. However, challenges related to drug resistance and selectivity persist. This thesis underscores the significance of dimerization selectivity and its role in metastatic disease. Further research is needed to optimize the potency, selectivity, and pharmacokinetic properties of Raf inhibitors. Additionally, exploring strategies to overcome resistance mechanisms and enhance the clinical efficacy of these inhibitors is crucial.