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A NOVEL ROLE FOR RASSF1A IN THE REGULATION OF RAS ACTIVATION

By

Desmond Ramón Harrell Stewart
B.S., Xavier University of Louisiana, 2014

A Dissertation
Submitted to the Faculty of the
University of Louisville School of Medicine
In Partial Fulfillment of the Requirements
For the Degree of

Doctor of Philosophy in Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

August 2020

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A Dissertation Approved on

June 5, 2020

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Dr. Geoffrey J. Clark, Dissertation Chair

Dr. Howard Donninger, Committee Member

Dr. Levi Beverly, Committee Member

Dr. Leah J. Siskind, Committee Member

Dr. Brian F. Clem, Committee Member

DEDICATION

“One ever feels his twoness—an American, a Negro; two souls, two thoughts, two unreconciled strivings; two warring ideals in one dark body, whose dogged strength alone keeps it from being torn asunder.” –Dr. W.E.B. Dubois, “The Souls of Black Folk”

Principally, this work is dedicated to my fellow Xavierite, Atatiana Jefferson. We walked the same halls. We shared a common dream, and they deprived us all of the chance to see to what great heights you would climb. In my heart of hearts, I know that the whole of your Xavier family now climbs for you.

This dissertation is also dedicated to Breonna Taylor, Ahmaud Arbery, Botham Jean, Jonathan Ferrell, Renisha McBride, Stephon Clark, Jordan Edwards, Jordan Davis, Alton Sterling, Aiyana Stanley-Jones, Michael Brown, Tamir Rice, Trayvon Martin, Sandra Bland, Philando Castille, Sean Bell, John Crawford III, Corey Jones, Keith Scott, Claude Reese, Eric Garner, Freddie Gray, Walter Scott, Clementa Pinckney, Cynthia Hurd, Susie Jackson, Ethel Lance, Depayne Middleton-Doctor, Tywanza Sanders, Daniel Simmons, Sharonda Coleman-Singleton, Myra Thompson, and countless others lost before their time at the hands of groundless hatred and prodigious cowardice. This nation has always worked to hold us back by any means; your deaths are recent evidence this. However, bruised, bloodied, but never broke, we always march on; this work is proof of that. Rest easy.

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My great grandmother, Alla Pearl Thompson, moved to this city in the thick of Jim Crow with her seven daughters. She never graduated high school—I don't think she ever even started—and provided for her family by cleaning houses in rich neighborhoods. With immeasurable strength, unending love, and fervent prayer, she raised her seven girls into seven phenomenal women who nurtured my family into existence. Big Mama, I still miss you all these years later. You created something wonderful, and I can't thank you enough.

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ABSTRACT

A NOVEL ROLE FOR RASSF1A IN THE REGULATION OF RAS ACTIVATION

Desmond Ramón Harrell Stewart

June 5, 2020

Ras is the most frequently activated oncogene in human cancer. It is not only the most frequently mutated oncogene, but is also rendered hyperactive in the wild-type form by aberrant regulation. Ras drives transformation and contributes to tumor aggressiveness by activating multiple downstream mitogenic effectors. Ras also possesses the paradoxical ability to induce apoptosis and senescence. Ras-induced apoptosis is not well understood, but has been largely attributed to the RASSF tumor suppressors, particularly RASSF1A. RASSF1A mediates Ras-induced apoptosis by activating pro-apoptotic proteins such as the MST kinases and BAX. RASSF1A is among the most frequently inactivated tumor suppressors in human cancer. Loss of RASSF1A expression by promoter hypermethylation uncouples Ras from its pro-apoptotic effectors, thus promoting unrestrained Ras mitogenic signaling. More recently, it has been suggested that RASSF1A may be not only an apoptotic effector for Ras, but also a general inhibitor of Ras activity. Several groups have reported modulation of Ras mitogenic signaling in response to RASSF1A suppression. However, the mechanism by which this occurs has not yet been elucidated. This dissertation establishes a novel, endogenous interaction

between RASSF1A and an important negative regulator of Ras, the RasGAP DAB2IP. We show that RASSF1A expression is an important determinant of DAB2IP protein levels. Loss of RASSF1A dramatically downregulates DAB2IP. This results in increases in Ras-GTP levels, Ras mitogenic pathway activation, and cell proliferation in both wild-type and mutant Ras lung cancer cells. This is the first example of a Ras effector regulating a Ras inhibitor, and the first example of a Ras effector influencing the activation state of Ras.

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CHAPTER I

BACKGROUND AND INTRODUCTION

1.1 Overview

Cancer is the second leading cause of death in the United States, with more than 1.8 million new cases and over 600,000 new deaths expected in 2020 [1]. The past three decades have seen a dramatic reduction in both the incidence and mortality of cancer. This is owed in large part to public health campaigns and advancements in screening and treatment. Indeed, the lung cancer death rate in the United States underwent its most precipitous decline on record in the past five years. Unfortunately, this shining accomplishment is somewhat dulled when put into context, in that the mortality rate of lung cancer had reached the greatest height from which to fall relative to other cancers. Still today, nearly one quarter of all cancer-related deaths are due to lung cancer—more than the next four deadliest cancers (prostate, breast, colorectal, and brain) combined [1].

The three Ras genes—*HRAS*, *KRAS*, and *NRAS*—are probably the best characterized oncogenes in the human genome. Together referred to as simply Ras, they comprise the most frequently activated oncogenes in human cancer, with activating point mutations occurring in roughly one third of tumors across all

tissue types [2]. The frequency of Ras mutations underscores its importance in carcinogenesis. Mutational activation of Ras results in constitutive activation of multiple mitogenic pathways that regulate survival, transformation, and growth [3]. These pathways synergize to mediate transformation [4].

Early experiments involving Ras mutants demonstrated that Ras alone could only transform immortalized cells, and primary cells first required immortalization by cooperating mutations in genes such as p53 [5, 6]. Without immortalization, oncogenic Ras would induce apoptosis and premature senescence [7, 8]. It has since been determined that this paradoxical growth-inhibitory ability of Ras is indeed physiological, and has largely been attributed to the RalGDS/AF-6 Ras association domain family (RASSF) of tumor suppressors [9]. RASSF1A is the best characterized member of the RASSF proteins and is an important mediator of Ras-induced apoptosis and cell cycle restriction [10-12].

Interestingly, induction of apoptosis may not be the only mechanism by which RASSF1A mediates tumor suppression. It has been shown that in addition to induction of apoptosis, RASSF1A can modulate Ras mitogenic effector signaling, suggesting that RASSF1A may directly regulate Ras activation [13-17]. This work focuses on the mechanism by which RASSF1A modulates Ras mitogenic signaling.

While searching for novel binding partners for the RASSF1A tumor suppressor, we identified a direct interaction with another tumor suppressor, disabled homologue 2 interacting protein (DAB2IP), which is an important negative regulator of Ras [18]. Suspecting that this interaction was the basis for the

observed downregulation of Ras mitogenic signaling by RASSF1A, we confirmed this interaction and began to investigate its biological significance. Indeed, we discovered a novel role for RASSF1A. In this work, we present evidence that RASSF1A is a potent positive regulator of DAB2IP. We show that suppression of RASSF1A results in a concomitant and drastic reduction in DAB2IP protein levels in human lung cancer cells. This RASSF1A-mediated suppression of DAB2IP results in a corresponding upregulation of Ras-GTP and Ras mitogenic signaling in both mutant and wild-type Ras lung cancer cells. Furthermore, we show that simultaneous downregulation of RASSF1A and DAB2IP, both frequent events in cancer, enhances Ras signaling and growth in wild-type Ras cancer cells. Thus, we describe the first ever mechanism of Ras regulation by a downstream effector.

1.2. Discovery of Ras

In the 1960s, two murine retroviruses were discovered that possessed the ability to transform infected cells in rodent newborns [19, 20]. Some two decades later, it was revealed that the causative genes in these viruses were in fact rat genes that had been transduced into retroviral genomes [21, 22]. They were called Ha-ras and Ki-ras, after the Harvey and Kirsten rat sarcoma viruses in which they were discovered. Around the same time, the presence of oncogenes in the human genome was discovered, and in 1982 three independent groups realized that the human oncogenes being feverishly investigated were homologues of the rat ras

genes discovered 10 years prior [23, 24]. That same year, a third Ras gene was discovered in a neuroblastoma cell line; it was called *NRAS* [25, 26].

Now, 38 years after their discovery, these once marginally relevant rat genes are known to be the most frequently mutated oncogenes in human cancer [27]. As such, the Ras oncogenes have been the subjects of intense scrutiny in the field of cancer research. Early on, it was revealed that Ras was regulated much the same as other small G proteins, and the mutations found in cancer served to disrupt the normal GTPase cycle [23, 24].

1.2.1. Regulation of Ras

Given the potent ability of Ras to induce transformation, it comes as no surprise that the Ras GTPase cycle is tightly regulated. Indeed, the transition between on and off states is controlled in both directions by two families of regulatory proteins. Guanine nucleotide exchange factors (GEFs) serve as positive regulators, catalyzing exchange of GDP for GTP. GTPase activating proteins (GAPs) negatively regulate Ras by potentially stimulating its intrinsic GTPase activity (figure 1) [28].

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Figure 1: Model for Ras regulation. Ras is primarily localized to the inner leaflet of the plasma membrane. Ras is activated by mitogenic stimuli, such as binding of growth factors to their respective receptors. Receptor activation recruits, through adapter proteins, guanine nucleotide exchange factors (GEFs) to the plasma membrane, which bind inactive Ras and facilitate GDP release, allowing GTP to bind and activate Ras. Ras then recruits and activates mitogenic effectors such as Raf. Ras signaling is silenced by stimulation of its intrinsic GTPase activity by GTPase activating proteins (GAPs).

1.2.1.1. GTPase Activating Proteins

In the mid-1980s, multiple reports demonstrated that Ras mutants commonly found in patient tumors exhibited diminished GTPase activity compared to its wild-type form. However, *in vitro* and *in vivo* data did not appear to correlate. The ability of Ras mutants to induce transformation *in vivo* was striking, but *in vitro* GTPase assays revealed only a modest impedance of GTPase activity in mutants [29]. This discrepancy was resolved in 1987, when it was discovered that a cytoplasmic protein called GTPase Activating Protein (GAP, now called p120GAP or RASA1) was responsible for the dramatic increases in GTPase activity of wild-type Ras observed *in vivo* [30-32]. Three years later, a second RasGAP was discovered and called neurofibromin. This is the protein product of the causative gene in the eponymous heritable tumor syndrome neurofibromatosis type 1 (NF1) [33, 34]. NF1 was identified because of the high degree of homology between its catalytic GAP-Related Domain (GRD) and the GAP domain of p120GAP.

A total of 14 RasGAPs have been described in the literature today [35]. RasGAP proteins are a profoundly heterogeneous group apart from their GAP domains, sharing little to no sequence homology outside of that region and possessing different functional domains that confer unique biological properties [36, 37]. Critical to GAP activity is a highly conserved arginine residue, called an arginine finger. Upon Ras-RasGAP binding, the arginine finger is inserted into the active site wherein it serves two functions. First, the positive charge of the side chain neutralizes charges at the active site in the transition state of GTP hydrolysis.

Second, the arginine residue forms a hydrogen bond with the catalytic glutamine residue in the switch II region of Ras (Gln61), which positions it appropriately in the active site to catalyze GTP hydrolysis [38].

1.2.1.2 Guanine nucleotide Exchange Factors

Just as GTP hydrolysis by Ras requires stimulation from regulatory RasGAPs, GDP-GTP exchange is also a tightly regulated process. The first RasGEF, CDC25, was identified in 1987 in yeast; however, its ability to catalyze nucleotide exchange in Ras was not discovered until 1991 [39-41]. That same year, the RasGEF Son of Sevenless (SOS) was identified as a downstream effector of EGF in *Drosophila*; its homology to yeast CDC25 was reported the following year [42, 43]. These studies led to the discovery of mammalian RasGEFs in 1992 [24].

GEFs do not actively exchange GTP for GDP, rather they bind to Ras-GDP and facilitate GDP dissociation. GTP is present at higher concentrations than GDP in the cell, and therefore predominantly fills the empty nucleotide binding site on Ras [44]. GEF structure is conserved within subfamilies, therefore the mechanisms by which GEFs facilitate GDP dissociation are dependent on the GEF subfamily and the GTPase being activated [28, 45]. They do, however, share a general mechanism of remodeling the switch regions of Ras to lower phosphate binding affinity [28]. RasGEFs are members of the CDC25 family. CDC25 family GEFs contain a highly conserved CDC25 domain and, usually, an N-terminal Ras

Exchanger Motif (REM). The mechanism by which these family members catalyze GDP dissociation is two-fold. First, the GEF inserts a projection (an alpha-helix in the case of the RasGEF SOS1) into the active site of Ras, which distorts switch I away from the active site. Second, it induces a conformational change in switch II, which results in a Mg^{2+} ion that is critical for stabilizing the nucleotide being occluded from the active site by switch II residues [46]. As a result, the alpha and beta phosphate are destabilized in the active site, and GDP is ultimately released. The GEF is then displaced by the binding of a new nucleotide [28].

1.2.2. Structure and function

Early investigations into the nature of these transforming genes revealed that their oncogenic properties were conferred by an alarmingly simple mechanism: a single missense mutation [47-50]. The ability of Ras proteins to bind GDP and GTP was appreciated early on [24]. It was suspected that Ras may function like the small G proteins known to transduce hormone signals into the cell [51]. The apparent wide tissue distribution and high frequency of mutation of Ras in human tumors underscored the need to understand its role in the molecular basis of cancer [24, 52].

1.2.2.1. Domain overview and isoform homology

In humans, there are three Ras genes—*HRAS*, *KRAS*, and *NRAS*—that encode four 188-189 amino acid proteins (*KRAS* produces two splice variants, K-Ras4A and K-Ras4B) [53]. The Ras proteins share 80-90% primary sequence homology as well as a high degree of structural homology, particularly in the N-terminal half of the sequence (figure 2) [54]. This region, residues 1-86, has been termed the effector lobe and has 100% sequence identity among the Ras proteins. Residues 87-166 constitute the allosteric lobe, which has a sequence identity of 90%. Together, these lobes represent the catalytic G-domain of Ras, which is responsible for GTP binding and hydrolysis as well as effector binding. At the extreme C-terminus lies the hypervariable region (HVR) that contains post-translational modification sites. As the name implies, the HVR exhibits very little sequence identity [55].

1.2.2.2. *3-dimensional structure and mechanistic function*

In 3 dimensions, the G-domain of Ras consists of six beta-sheets and five alpha-helices, forming a hydrophobic core connected by 10 loops (figure 3) [56]. Five of these loops exist on one facet of the G-domain and collectively mediate nucleotide and effector binding; loops 1-3 are in the effector lobe while loops 4 and 5 are in the allosteric lobe. Loop 1 (also called the P-loop or Walker A motif) and loop 4 (containing an N/TKXD motif, which is conserved in GTP binding

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Figure 2: Overview of Ras isoforms. In humans, there are three Ras genes—*KRAS*, *HRAS*, and *NRAS*, that code for four Ras proteins (*KRAS* is alternatively spliced at exon 4 to give rise to K-Ras4A and K-Ras4B, of which K-Ras4B is the major isoform). The effector lobe (residues 1-86 are identical in all 4 protein, and overall the G domains share ~95% sequence homology. At the extreme C-terminus lies the hypervariable region. This region exhibits almost no homology among the Ras isoforms, save for the ending CAAX motif. These last four residues are required for post-translational farnesylation, a modification that is critical to proper function and subcellular localization.

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Figure 3: 3-dimensional structure of Ras. Ras proteins contain a hydrophobic core made of six beta-sheets and five alpha-helices, connected by 10 loops. Five of these loops are on the cytoplasmic-facing facet of the protein and constitute the nucleotide and effector binding domains. The model shows Ras in complex with the catalytic arginine finger of a GTPase activating protein (GAP, purple loop), which coordinates critical residues on Ras—G12, G13, and Q61 (yellow spheres)—to facilitate GTP hydrolysis.

proteins) are responsible for beta/gamma phosphate stabilization and guanine nucleotide specificity, respectively [44, 55]. Loop 5 also contributes to nucleotide binding, mostly indirectly through stabilization of loop 4 [55, 57]. Loops 2 and 3 are major components of two highly mobile regions of Ras, the switch I and switch II regions, respectively.

Switch I and II regions undergo conformational changes in response to GTP binding and hydrolysis due to interactions with the gamma phosphate. The conformational change in these regions is responsible for the “switch” between on and off states of Ras [44, 58]. In the on, GTP-bound state, the switch regions form a platform to which effectors may bind. Different effectors interact with Ras in different (and sometimes multiple) ways, but there is a strong preference for binding to the switch I region [59]. The switch II region contains the catalytic glutamine residue (Gln61) that is responsible for GTP hydrolysis [60].

1.2.2.3. Post-translational modification

It was understood very early that Ras was not a cytosolic protein, but rather was localized to the membrane [61, 62]. The first investigations into what modifications of Ras may confer membrane localization revealed three important characteristics: 1) that H-Ras could be palmitoylated, 2) that the C-terminus—specifically Cys186—was critical to membrane localization, and 3) that membrane localization was necessary for Ras to transform cells [63, 64]. This led to the incorrect assumption that H-Ras must be palmitoylated on Cys186 [24]. It would

be several years before it was discovered that the critical Cys186 residue was part of a motif known in yeast to result in protein prenylation.

The C-terminus contains almost all the sequence and structural variability of the Ras proteins, particularly the last 22-23 residues that make up the HVR (figure 2). However, Cys186 is part of a highly conserved CAAX consensus sequence, first identified in yeast, that is a signal for three post-translational modifications [65]. The CAAX motif—wherein C is cysteine, A is an aliphatic residue, and X is any amino acid—is farnesylated (at Cys186), cleaved, and methylated in all Ras isoforms [66-69]. After farnesylation, the variability present in the HVR dictates which of two divergent paths Ras takes to the plasma membrane. Present in the HVRs of H-Ras, N-Ras, and K-Ras4A are one or two additional cysteine residues that are palmitoylated, resulting in their trafficking through the exocytic pathway to the plasma membrane [70, 71]. The HVR of K-Ras4B lacks additional cysteine residues, but rather contains a poly-lysine sequence that interacts with negatively charged membranes (figure 4). K-Ras4B concentrates at the inner leaflet of the plasma membrane through a complex mechanism. First, upon synthesis, K-Ras4B binds to cytosolic transport protein such as PDE- δ , which shield the poly-lysine HVR from binding to endomembranes [72]. K-Ras4B is unloaded from PDE-delta onto perinuclear membranes, and is ultimately shuttled to the plasma membrane by the recycling endosome [72, 73]. Interestingly, K-Ras4A utilizes both a palmitoylated cysteine and a poly-lysine sequence to achieve membrane localization [74].

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Figure 4. Targeting of Ras to plasma membrane by post-translational modification. All Ras proteins possess a C-terminal CAAX motif, a signal for cysteine farnesylation. H-Ras, N-Ras, and K-Ras4A contain additional cysteine residues in the hypervariable region (HVR) that are palmitoylated by the Golgi and target Ras to the plasma membrane via the exocytic pathway. K-Ras-4B (as well as K-Ras 4A) contain a polybasic sequence in the HVR that targets Ras to the membrane via the recycling endosome system, utilizing chaperone protein such as PDE6-δ.

1.2.3. *Signaling, mutation, and cancer*

The Ras proteins were initially discovered because of their potent ability to transform cells. By the early 1990s, the basic biochemistry of Ras—the GTPase cycle, gross effects of mutation on GTP hydrolysis, and the physical state of the protein necessary for transformation—had been well established. Still unknown were the factors activated by Ras-GTP that confer its oncogenic nature. To identify Ras effector proteins, several groups performed yeast two-hybrid screens. In swift succession, the three canonical Ras effectors were identified.

1.2.3.1. *Canonical mitogenic Ras signaling*

Classical activation of Ras signaling begins at the cell surface with the binding of a ligand, epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) to their respective receptors EGFR and PDGFR [75]. Receptor activation leads to binding of growth factor receptor-bound protein 2 (GRB2) [76], which in turn recruits the RasGEF SOS1 to the plasma membrane where it can activate membrane-bound Ras [77-80]. Activated Ras-GTP is then able to bind and activate its effectors. The first Ras effector identified was the well-known viral oncoprotein Raf [81].

The serine/threonine kinase Raf was identified as a downstream binding partner of Ras-GTP in *Drosophila*, *C. elegans*, and mammalian cells by multiple groups in 1993 [82-86]. Prior knowledge about the oncogenic properties of Raf

combined with more recent evidence of both Ras- and Raf- mediated activation of the mitogen-activated protein kinase (MAPK) cascade led to the rapid elucidation of the first Ras-regulated signaling pathway: Ras-Raf-MEK-ERK [23, 24, 87]. Raf is recruited to and activated at the plasma membrane by Ras-GTP [88]. There are three Raf isoforms: c-Raf/Raf-1, A-Raf, and B-Raf. All three are capable of phosphorylating the first component of the MAPK cascade, MAPK/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2) [89]. MEK1/2 then activate ERK1/2, which regulate transcriptional activity by phosphorylating transcription factors [87], some of which have been implicated in Ras-mediated transformation [90].

In 1991, it was reported that phosphatidylinositol 3-kinase (PI3K) activity increased in response to Ha-ras mediated transformation in epithelial cells [91]. In 1994, the direct, GTP-dependent interaction between Ras and the catalytic p110 subunit of PI3K was reported, thus establishing the second canonical Ras effector [92]. As its name suggests, PI3K catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP₂) to form phosphatidylinositol 3,4,5-triphosphate (PIP₃), which activates the Ser/Thr kinase AKT (also known as protein kinase B (PKB)) [93]. AKT has multiple substrates that confer to it control of cell growth, proliferation, and survival [94, 95]. Specifically, AKT activates the pro-survival/anti-apoptotic transcription factor NFκB, inhibits the pro-apoptotic BAD and Bax proteins, and antagonizes p53-induced apoptosis in order to favor cell survival [96]. PI3K-AKT promotes growth and favors transformation by activating

mammalian target of rapamycin (mTOR) signaling [97], a pathway that is dysregulated in most cancers [98]. PI3K-AKT also regulates the cell cycle [95].

The third canonical Ras effector identified is an activator of two other small GTPases that are structurally related to Ras. The Ras-like (Ral) GTPases, RalA and RalB, were discovered in 1986 and have been implicated in cellular processes ranging from vesicular trafficking to tumor formation and metastasis [99]. As a small GTPase, the Ral GTPase cycle is regulated by GEFs and GAPs. In 1994, the RalGEF Ral guanine nucleotide dissociation stimulator (RalGDS) was identified in a yeast two-hybrid screen as a Ras effector protein [100-102]. RalGDS activates the Ral GTPases, but not structurally similar GTPases such as Ras, Rho, or Rab [99]. The Ras-RalGDS pathway was not given a great deal of attention initially, as it appeared to play only a minor role in Ras-mediated transformation [24]. The Ral GTPases has been shown to be involved in a broad spectrum of biological processes [99]. Indeed, Ral has since been shown to play critical roles in several human cancers, and has even been deemed necessary for tumor development in an H-Ras-driven mouse model of skin cancer [103, 104].

1.2.3.2. Mutation in cancer

The importance of Ras mutations to carcinogenesis was apparent at the inception of the Ras field, as it was the transforming power of Ras mutants that initially garnered the attention of researchers and led to its characterization. Numerous reports of mutations in codon 12, normally a glycine, in cancer cell lines

were published in the early 1980s [47, 48, 50]. Two 1984 publications demonstrating the presence of mutant Ras in tumor cells, yet the absence thereof in normal cells from the same patient, definitively proved that the link between Ras point mutation and malignancy was a naturally-occurring phenomenon, not just an artifact of serial passaging in cell culture [105, 106].

Ras is predominantly mutated at codon 12 in human cancers, followed by codon 13 [107]. Mutations at codon 61 are observed in certain cancers, such as melanoma [35]. In fact, 97-99% of Ras mutations in all three isoforms are found in these three codons [24]. The most common mutations render Ras oncogenic by inhibiting GTP hydrolysis; however, these effects are more attributable to the inhibition of GAP activity than the intrinsic GTPase activity of Ras [29]. Targeted mutation of codon 12 demonstrated that every amino acid except glycine—present in the wild-type protein—and proline rendered Ras oncogenic [108]. The initial report concluded that an alpha-helical secondary structure must be required for Ras GTPase activity. However, analysis of the crystal structure of Ras bound to the p120GAP catalytic domain revealed that introduction of the arginine finger into the active site of Ras creates steric constraints therein such that even alanine is incapable of fitting in that space [38]. Interestingly, Gly12 mutants are still able to bind to RasGAPs, suggesting that glycine is more important for transition state formation as opposed to ground state [109].

The same can largely be said for codon 13 mutations. Gly13 is positioned further away from the RasGAP arginine residue [38]. It thus appears that mutations here are slightly more forgiving, in that alanine and valine mutants are able to form

the transition state whereas larger amino acids cannot [38, 110]. Gln61 is the catalytic residue of Ras that activates a water molecule for GTP hydrolysis. Therefore, mutations at this codon do technically disrupt the intrinsic GTPase activity of Ras. However, differences in Ras GTPase activity are only appreciable upon stimulation with GAPs [28, 29]. As with the other mutation hotspots, extremely few amino acid substitutions—in this case, proline and glutamate—are non-transforming at position 61 [111].

Analysis of the pan-tumor mutation spectrum of Ras reveals patterns in the frequency and types of mutations in the three Ras genes that are dependent on the tissue type. This suggests that Ras isoforms may vary in their predisposition for activation from cell type to cell type [35]. Across all tissues, *KRAS* is the most frequently activated Ras gene by far, with mutations occurring in 22% of samples in the COSMIC database, followed by *NRAS* at 7%, then *HRAS* at 3%.

KRAS mutations are predominant in cancers of the pancreas, colon, and lung [112-114]. *KRAS* mutations affect codon 12 in 80% of cancer cases of all types. G12D and G12V are the most common substitutions, representing in 36% and 24% of all *KRAS* mutations, respectively. Interestingly, G12C mutations, which account for just under 15% of *KRAS* mutations overall, are overwhelmingly represented in lung cancer, where its frequency surpasses that of G12D and G12V mutations combined [35]. It was recently discovered that K-RAS^{G12C} retains the ability to complete the GTPase cycle [115, 116]. The implications of this are threefold: K-RAS^{G12C} 1) is susceptible to activation by upstream signals, 2) may be

rendered hyperactivated by upstream deregulation, and 3) may confer susceptibility to upstream targeted therapies such as tyrosine kinase inhibitors.

Despite representing less than 10% of Ras mutations overall, *NRAS* is the predominant isoform mutated in melanoma and acute myelogenous leukemia (AML) [117, 118]. *NRAS* is mutated in 30% of melanomas, making it the second most common oncogenic driver in melanoma behind *BRAF* [119]. *NRAS* is mutated primarily at codon 61 [35]. *HRAS* is the least frequently mutated isoform, found in 3% of tumors. *HRAS* is associated with squamous cell cancers of the head and neck, and is mutated at codons 12 and 61 at roughly equal rates [35, 54]. Whatever the specific substitution, mutational activation of Ras leads to sustained signaling through the canonical mitogenic effectors, which can synergize to mediate transformation. However, Ras can paradoxically stimulate apoptosis and senescence in response to mutational activation as a protective mechanism against transformation.

1.3. RASSF family of tumor suppressors

1997 reports demonstrating the ability of Ras to induce senescence and apoptosis naturally led to the hypothesis that additional effector proteins for Ras must exist that regulate these effects (figure 5). In a span of three years, this hypothesis was confirmed with the discovery of NORE1A (also known as RASSF5) and RASSF1A, the founding RASSF members [120-122].

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Figure 5. Ras regulates mitogenic and growth-inhibitory pathways. Ras, in both wild-type and oncogenic forms, possess the paradoxical ability to stimulate both growth-promoting and growth-inhibiting signaling pathways. Canonical Ras effectors—Raf, PI3K, and RalGDS—predominantly favor growth. Conversely, several Ras effectors, particularly RASSF proteins, induce apoptosis and senescence in response to Ras stimulation. Loss of RASSF expression uncouples Ras from growth inhibition, thus favoring transformation.

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Figure 6. The classical RASSF proteins. The 10 RASSF proteins can be divided into two subfamilies, classical/C-RASSF and N-RASSF. Classical RASSF proteins have been extensively implicated in tumor suppression. They contain a conserved C-terminal Salvador-RASSF-Hippo (SARAH) domain and adjacent Ras association (RA) domain. RASSF1A and NORE1A/RASSF5, the major members of the family, also contain a C1/Diacylglycerol binding domain. Both RASSF1A and NORE1A are alternatively spliced to give rise to multiple isoform. RASSF1 has 8 isoforms, RASSF1A-H, of which RASSF1C is the major variant.

NORE1A and RASSF1A were identified because of their RaIGDS/AF-6 Ras Association (RA) domains. There are 10 RASSF proteins, which can be divided into two groups based on the location of the RA domain: C-terminal or N-terminal [123]. RASSF1-RASSF6 are in the C-terminal group and are considered the classical RASSF proteins (figure 6). They contain a conserved Salvador/Ras/Hippo (SARAH) domain adjacent to the RA domain that facilitates homo- and heterodimerization as well as connection to the Hippo kinases MST1 and 2 [124]. The classical RASSF proteins are downregulated in human cancers, and most have been characterized as tumor suppressors [9, 123]. The N-terminal RASSF proteins, RASSF7-RASSF10, do not contain SARAH domains and their role in human disease is much less understood [125].

1.3.1. The RASSF1A tumor suppressor

RASSF1A is perhaps the best characterized member of the RASSF proteins. Numerous studies indicated that the 3p21.3 locus underwent loss of heterozygosity frequently and very early in lung cancer development, suggesting that a tumor suppressor gene was located there [126]. In 2000, the *RASSF1* gene was discovered at that locus, and the resultant protein was shown to induce apoptosis in a Ras-dependent manner [121, 122]. RASSF1A has since been identified as a key tumor suppressor and mediator of cellular homeostasis [127-129].

The *RASSF1* gene utilizes two promoters and eight exons that are alternatively spliced to give rise to eight isoforms, RASSF1A-H [127]. RASSF1A and RASSF1C are the predominant isoforms and are ubiquitously expressed. Their expression is driven by different promoters located in different CpG islands, and therefore may be differentially regulated by promoter methylation [130]. Indeed, hypermethylation of the *RASSF1A* promoter is a frequent and early event in many human cancers [127, 131]. Somatic mutations in RASSF1A have also been reported [132]; however, this is not considered a major mechanism of RASSF1A inactivation. Early studies in mice, wherein *rassf1a*^{-/-} animals were more susceptible to spontaneous tumor formation than wild-type littermates, established the role of RASSF1A in cancer as a *bona fide* tumor suppressor [133].

1.3.1.1. *RASSF1A* in RAS-mediated apoptosis

When the *RASSF1* gene was first identified, it was immediately noted that it possessed the ability to suppress *in vitro* and *in vivo* growth of lung cancer cells and induce apoptosis in a GTP-dependent manner [121, 122]. It has since been demonstrated that RASSF1A utilizes several pathways to mediate this apoptotic effect. Perhaps the best characterized mechanism of RASSF1A-mediated apoptosis is activation of the Hippo pathway [134]. The Hippo pathway is a highly conserved kinase cascade, first identified in *Drosophila* as a regulator of tissue growth, that has since emerged as a critical mediator of malignancy and metastasis [135, 136].

The Hippo pathway begins at the MST kinases (MST 1/2). In response to Ras activation, RASSF1A binds MST1/2 and stabilizes their active state by preventing dephosphorylation by PP2A domain [137-140]. When activated, MST1/2 phosphorylate the kinases LATS1/2. LATS1/2 in turn phosphorylate their targets YAP and TAZ. This leads to cytoplasmic retention and subsequent degradation of YAP and TAZ, and a switch in YAP affinity from pro-proliferative TEAD and SMAD family transcription factors to pro-apoptotic p73 [138, 141, 142]. LATS1 also binds to and inhibits the ubiquitin ligase MDM2, a negative regulator of the p53 tumor suppressor, thereby stabilizing p53 and inducing apoptosis [137]. RASSF1A can also directly bind MDM2 after phosphorylation following DNA damage [143]. This leads to MDM2 auto-ubiquitination and degradation [144].

RASSF1A can also induce mitochondrial apoptosis by activating BAX. In response to TNF or mutant K-RAS, RASSF1A binds to modulator of apoptosis (MOAP-1), opening the conformation of MOAP-1 to facilitate binding to the pro-apoptotic protein BAX [11, 145]. The MOAP-1-BAX interaction induces conformational change of BAX, insertion into the mitochondrial membrane, and cytoplasmic release of cytochrome c [146].

1.3.1.2. Other tumor suppressive effects of RASSF1A

In the cell, RASSF1A does far more than serve as a kill switch for Ras mutants. Since its discovery, roles for RASSF1A have been found in cell motility, invasion, and metastasis [12, 147]; cell cycle progression [10]; DNA damage repair

[148]; organelle trafficking and cell-cell communication [149]; and inflammation [9, 150]. Interestingly, much of RASSF1A activity, including its regulation of cell proliferation and apoptosis, is mediated through its interaction with and stabilization of microtubules [129].

RASSF1A binds to microtubule associated proteins (MAPs), which mediate its interaction with microtubule filaments [151, 152]. This interaction prevents deacetylation of microtubules by histone deacetylase 6 (HDAC6), resulting in the accumulation of hyper-stable circular microtubule bundles [152, 153]. RASSF1A-mediated stabilization of microtubules is also associated with mitotic arrest and inhibition of DNA synthesis [151, 152]. Mitotic arrest is mediated by regulation of centrosomes by RASSF1A. Centrosomes are critical regulators of mitotic spindle assembly [154]. Whereas knockdown of RASSF1A results in multipolar spindle formation, overexpression of RASSF1A inhibits centrosome separation, providing an explanation for why RASSF1A promotes G2/M arrest specifically [151, 155, 156].

RASSF1A appears to regulate cell migration and cell cycle progression by multiple parallel mechanisms. Increases in invasion and metastasis observed upon RASSF1A depletion have also been attributed to disruption of cell-cell adhesions [12, 157]. RASSF1A negatively regulates Janus kinase (JNK) activity and subsequent cyclin D1 accumulation, thereby inhibiting G1-S transition in addition to G2-M [158].

More recently, the ability of RASSF1A to regulate inflammation has been reported. Chronic inflammation is an important driver of tumor development and

progression [159, 160]. RASSF1A-deficient animals and tumors exhibit elevated IL-6 production [14, 150]; tumors also exhibit increased macrophage invasion [14]. Part of the oncogenic program of YAP is upregulation of IL-6 production[161]. Moreover, IL-6 has been shown induce epithelial-to-mesenchymal-transition (EMT). Thus, control of inflammation and apoptosis by RASSF1A may be linked [129].

1.3.2. A role for RASSF1A in RAS regulation

A final and curious aspect of RASSF1A activity is its ability to modulate mitogenic effectors of Ras. Modulation of the three canonical Ras mitogenic pathways—PI3K-AKT, Raf-MEK-ERK, and RalGEF-Ral—in response to RASSF1A depletion have been reported [13, 14]. Some of this may be explained by simple stoichiometry. Less RASSF1A-bound Ras will inevitably lead to more PI3K, RAF, and RalGDS-bound Ras. However, RASSF1A has been shown to directly modulate both AKT and RAF activity through the MST kinases [9, 162, 163]. MST2 binds Raf and inhibits its activation by Ras [16]. RASSF1A competes with Raf for MST2 binding [138], and therefore titrates the degree of Raf inhibition by MST2. These interactions suggest that RASSF1A should serve to positively regulate Raf [9]. However, MST2 is also regulated by AKT, which phosphorylates MST2 and increases its affinity for RASSF1A [15]. This should theoretically result in simultaneous activation of RASSF1A-Hippo and Raf-MEK-ERK pathways, thus

the ultimate outcome of these interactions is unclear. Others have shown that RASSF1A loss activates Raf [14, 17].

As AKT regulates MST2, RASSF1A1 regulates AKT activity, in part through MST1 [13, 162, 163]. MST1 directly binds AKT1 and both MST2 kinases suppress activating phosphorylation of AKT at T308 and S473 [163]. In addition to preventing dephosphorylation, RASSF1A also stabilizes MST1/2 protein levels through an unknown mechanism [140, 162]. RASSF1A suppression depletes MST1 protein and results in increased AKT phosphorylation at T308 [162].

While the ability of RASSF1A to inhibit PI3K-AKT activity is established, reports describing the interactions between RASSF1A, Raf, and the MST kinases do not provide a concise mechanism of RASSF1A-mediated regulation of Raf. Moreover, they do not correlate with observed increases in Raf activity upon RASSF1A depletion. The recent report of RASSF1A negatively regulating Ral activation underscores the potential for a simpler, universal mechanism by which RASSF1A restrains Ras mitogenic signaling. The Clark group recently identified and confirmed a novel binding partner for RASSF1A that explains its control over Ras activity: the RasGAP DAB2IP (Harrell Stewart et al., *in press*).

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Figure 7. overview of DAB2IP signaling. DAB2IP is a RasGAP and *bona fide* tumor suppressor with multiple Ras-independent functions. DAB2IP can directly modulate Ras activation through its GAP domain. DAB2IP can bind to activated VEGFR2 and inhibit its induction of angiogenesis. Finally, DAB2IP is a critical mediator of TNF-induced apoptosis. Upon TNFR activation, DAB2IP is phosphorylated, which allow for simultaneous activation of pro-apoptotic ASK-JNK signaling, direct inhibition of the pro-survival PI3K-AKT complex, and suppression of TRAF2-induced activation of NFκB, an important regulator of survival, inflammation, and transformation.

1.4. DAB2IP: RasGAP, tumor suppressor, TNF transducer

Disabled homologue 2(DAB2) interacting protein (DAB2IP) is one of fourteen RasGAPs in the human genome and is among the best characterized [36]. While its GAP activity is required for full expression of its tumor suppressive power, additional functional domains of DAB2IP allow it modulate numerous biological processes involved in cancer, including angiogenesis, inflammation, proliferation, and apoptosis [164-167].

1.4.1. Discovery of DAB2IP

Disabled homologue 2 (DAB2) interacting protein (DAB2IP) was first identified in a 2002 yeast two-hybrid screen as an N-terminal binding partner for the tumor suppressor DAB2, originally called differentially expressed in ovarian carcinoma-2 (DOC2) [18]. Its original characterization established several important aspects of the function of DAB2IP and its role in human cancer [18]. DAB2IP was found to possess several putative functional domains, including RasGAP homology (GAP), proline rich (PR), leucine zipper dimerization (LZ), and Src homology 3 (SH3). The RasGAP activity of DAB2IP was confirmed *in vitro* and in prostate cancer cells, with corresponding suppression of Raf-MEK-ERK signaling and 2D growth when overexpressed. It was found to be expressed in wide variety of normal tissues including prostate, as well as in primary prostate tumor cell lines, but was markedly downregulated in metastatic cell lines. This led

to the correct speculation that DAB2IP was involved in prostate cancer progression[18, 168].

1.4.2. DAB2IP in cancer – regulation and signaling

DAB2IP activity is frequently lost multiple cancers, including prostate, breast, and lung [169]. One report mentions mutational inactivation of DAB2IP in breast cancer; otherwise, mutation of *DAB2IP* appears to be a rare event [170]. Instead, tumors utilize a variety of mechanisms to subvert DAB2IP-mediated tumor suppression [171]. Aberrant histone deacetylation and promoter methylation has been reported in prostate cancer [168, 172]. Aberrant promoter methylation has also been reported in breast, lung, gastrointestinal, and brain cancers [173-176]. DAB2IP activity can also be suppressed by microRNA-mediated downregulation, ubiquitin-mediated degradation, inhibitory phosphorylation, and sequestration by mutant p53 [171, 177-179]. The multifaceted approach employed to abrogate DAB2IP activity in cancer implies both that loss of DAB2IP confers significant survival and/or proliferative advantage to tumor cells, and that DAB2IP must be a powerful tumor suppressor (figure 7).

1.4.2.1. DAB2IP favors apoptosis over survival

A second group identified DAB2IP in a yeast two-hybrid screen as a binding partner of apoptosis signal-regulating kinase 1 (ASK1) and named it ASK1

interacting protein (AIP1) [180]. ASK1 is an upstream activator of c-Jun N-terminal kinase (JNK) and p38 MAPK which regulate survival and apoptosis [181]. In a series of reports, these two groups elucidated a mechanism in which DAB2IP serves as a critical determinant in the decision between survival and apoptosis in response to TNF-TNFR signaling [180, 182-184].

In resting cells, DAB2IP is in a closed conformation in complex with TNFR [180, 182]. The PR domain of DAB2IP is responsible for maintaining this closed conformation [182]. Upon TNFR activation by TNF, TRADD is recruited to the receptor and serves as a platform for the formation of a large complex involving TRAF2, DAB2IP, ASK1, RIPK1, IKK, and MAP3K [184-187]. TRAF2 is an important transducer of TNFR signaling, capable of stimulating pro-apoptotic ASK-JNK and pro-survival NF κ B pathways [188]. This complex is released into the cytoplasm, where DAB2IP adopts an open conformation and is phosphorylated at S604 by either RIPK1 or ASK1, a required modification for activation of ASK1 [184]. ASK1 is then dephosphorylated by PP2A and 14-3-3, the inhibitor of ASK1, is transferred to phosphorylated DAB2IP, allowing for ASK1-mediated activation of JNK and subsequent induction of apoptosis [180, 184, 189]. The interaction between DAB2IP and ASK1 is significantly enhanced by TRAF2 [180]. DAB2IP simultaneously inhibits TNF-induced activation of NF κ B, an important pathway in cell survival [182, 190].

DAB2IP further tips the scales in favor of apoptosis over survival by inhibiting the PI3K-AKT pathway [165]. AKT negatively regulates ASK1 by phosphorylation at S83 [191]. PI3K-AKT is also an important positive regulator of

NFκB [192]. DAB2IP inhibits AKT by preventing its phosphorylation at S473[165]. This is mediated by inhibitory interactions between DAB2IP and both AKT and the p85 regulatory subunit of PI3K [165, 167].

1.4.2.2. *DAB2IP restrains EMT and metastasis*

The initial characterization of DAB2IP revealed that its overexpression in metastatic prostate cancer cells could suppress their growth in culture [18]. At that time, it was speculated that DAB2IP played a role in metastatic prostate cancer. It has since been firmly established that DAB2IP is an important regulator of EMT and metastasis in multiple cancers [166, 193-197].

Regulation of EMT and metastasis by DAB2IP occurs through simultaneous inhibition of Ras-Raf-MEK-ERK and PI3K-AKT-NFκB signaling pathways [167]. Using a combination of *in vitro* and *in vivo* techniques, it was demonstrated that DAB2IP-mediated suppression of EMT and tumor initiation was a result of GTPase activity, whereas inhibition of metastasis was caused by NFκB suppression [167]. NFκB suppression by DAB2IP here was mediated by the same mechanisms described in 1.4.2.1.

DAB2IP also inhibits metastasis through suppression of Wnt/β-catenin signaling. Wnt is frequently activated in cancer [198]. Canonical Wnt signaling involves stabilization of the transcriptional activator β-catenin [199]. DAB2IP suppresses this pathway by recruiting PP2A to the kinase GSK-3β [200].

Dephosphorylation of GSK-3 β at S9 is activating, allowing it to phosphorylate β -catenin and target it for degradation [201].

1.4.2.3. DAB2IP suppresses inflammatory angiogenesis

DAB2IP knockout mice were generated in 2008 to investigate the function of the protein *in vivo*. It was discovered that DAB2IP was key regulator of inflammatory angiogenesis [164]. The initial report demonstrated suppression of VEGFR and AKT activation caused by DAB2IP, and that GAP activity on Ras was not responsible for AKT inhibition. Instead, DAB2IP was found to bind directly to the VEGFR2-PI3K complex and prevent VEGFR2-mediated activation of PI3K. However, Ras does still activate PI3K, and Ras and NF κ B signaling are linked [202]. Thus it appears, that the regulatory roles of DAB2IP are tightly interweaved, and the importance of DAB2IP in disease is due to concomitant deregulation of all of these pathways [203].

CHAPTER II

Materials and Methods

2.1. *Plasmids*

pCDNA3-HA-RASSF1A – pcDNA3-HA-RASSF1A was described previously [152]. pcDNATM3.1 mammalian expression vector was purchased from Invitrogen (Cat. #V79020). This vector was modified to express a fused hemagglutinin (HA) epitope tag by Dr. Geoffrey Clark. The full length RASSF1A coding sequence was cloned from pcDNA3.1-RASSF1A into pcDNA3-HA as a BamHI/NotI restriction fragment.

KATE-RASSF1A – pmKate2-C-RASSF1A has been described previously [10]. The BamHI/EcoR1 restriction fragment from pcDNA3-HA-RASSF1A was cloned in to the pmKate2-C vector (Evrogen, Moscow, Russia, Cat. #FP181).

K-RAS^{G12V} – pCGN-KRASG12V was described previously [204].

GFP-DAB2IP – pEGFP-DAB2IP was a gift from Dr. Karen Cichowski (Harvard).

shRASSF1A – pBRS-shRASSF1A was generated by cloning the shRNA sequence from pRS-RASSF1A 777 (Origene, Cat. #TR307696) into the pGFP-BRS vector (Origene, Cat. #TR30018).

shDAB2IP – pGIPZ-shDAB2IP was a generous gift from Jer-Tsong Hsieh (UT Southwestern) [205].

2.2. Cell lines

HEK-293T – Human Embryonic Kidney 293 T (HEK-293T) cells were purchased from the ATCC. These cells are modified with the SV40 Large T-antigen, allowing for enhanced protein expression. This cell line is easy to transfect and is therefore useful in overexpressed co-immunoprecipitation studies. HEK-293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep).

COS-7 – COS-7 (CV-1 in Origin with SV40 genes) cells were purchased from the ATCC. These fibroblast-like cells are derived from the African green monkey (*Cercopithecus aethiops*) kidney cells. Morphologically, these cells are large and flat, making them particularly useful for imaging fluorescently tagged proteins. COS-7 cells were maintained in DMEM supplemented with 10% FBS and 1% pen/strep.

NCI-H1299 – These cells were purchased from the ATCC. H1299 is a non-small cell lung carcinoma line derived from the lymph node of a 43-year-old Caucasian male. This line harbors a Q61K mutation in the *NRAS* gene. It is also p53-null, resulting from a homozygous partial deletion of *TP53*. Additionally, these cells have lost endogenous expression of *RASSF1A* [206]. H1299 cells were maintained in RPMI 1640 medium (RPMI) supplemented with 10% FBS and 1% pen/strep.

NCI-H1792 – NCI-H1792 cells were purchased from the ATCC. This is a stage 4 non-small cell lung adenocarcinoma cell line derived from the pleural effusion of a 50-year-old Caucasian male. It harbors a G12C mutation in the *KRAS* gene [115]. NCI-H1792 cells were maintained in RPMI supplemented with 10% FBS and 1% pen/strep

NCI-H1437 – H1437 cells were purchased from the ATCC. This is a stage 1 non-small cell lung adenocarcinoma cell line derived from the pleural effusion of a 60-year-old Caucasian male. H1437 cells were maintained in RPMI supplemented with 10% FBS and 1% pen/strep. H1437 cells retain wild-type Ras expression [207].

MCF-10A – MCF-10A breast epithelial cells were purchased from the ATCC. This is an immortalized, non-transformed epithelial cell line derived from the mammary gland of a 36-year-old Caucasian female [208]. As a non-tumorigenic line, MCF-

10A cells have not undergone hypermethylation of the *RASSF1A* promoter, making them a useful tool for RASSF1A co-immunoprecipitation studies. MCF-10A cells were maintained in DMEM/F12 50:50 mix supplemented with 5% horse serum, 20 ng/mL epidermal growth factor (EGF), 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, and 1% pen/strep.

2.3. Cell culture, treatment, and genetic manipulation

2.3.1. Cell culture

Growth Media – Cell lines used in this work were maintained in conditions recommended by the ATCC. All growth media were purchased from Corning. DMEM (Cat. #10-013-CV) and contains L-glutamine, sodium pyruvate, and 4.5 g/L glucose. DMEM was supplemented with 10% FBS (Corning, Cat. #35-010-CV) and 1% penicillin/streptomycin (Corning, Cat. #30-002-CI). RPMI 1640 (Cat. #10-040-CV) contains L-glutamine and was supplemented with 10% FBS and 1% pen/strep. DMEM/F12 50:50 Mix (Cat. #15-090-CV) is a 50:50 mixture of DMEM and Ham's F12 medium. It contains L-glutamine and was supplemented with 5% horse serum (Gibco, Cat. #26050-07), 20 ng/mL EGF (Gibco, Cat. #PHG0311), 0.5 µg/mL hydrocortisone (Sigma, Cat. #H0888), 10 µg/mL insulin (Sigma, Cat. #I92780-5ML), and 1% pen/strep.

Passaging – Trypsin-EDTA (0.25%) was purchased from Corning (Cat. #25-053-CI) and was used to remove adherent cells from culture vessels. Culture medium was aspirated and cells were washed once with sterile 1X phosphate-buffered saline (PBS) (Corning, Cat. #21-040-CM). The PBS was aspirated, then trypsin-EDTA was applied to the cells and incubated at 37°C for several minutes until cells were detached from the culture vessel. Trypsin was neutralized with complete medium (containing FBS) and cells were collected via centrifugation at 1500 rpm for 5 minutes. The resulting supernatant was aspirated and the cells were resuspended in complete medium and plated as needed.

2.3.2. Chemicals for cell treatment

To elucidate mechanisms of protein stabilization we treated cells with various inhibitors. MG-132 (Selleckchem, Cat. #S2619) is a reversible proteasome inhibitor. MG-132 powder was reconstituted in DMSO (Sigma, Cat. #D2438) at a stock concentration of 50 mM and diluted in culture media to a final concentration of 50 μ M. In these experiments, cells were treated with MG-132 for 4 hours prior to lysis. Chloroquine (Sigma, Cat. #C6628) is an anti-malarial medication that is used in cell culture to inhibit the lysosome. Chloroquine powder was dissolved in DMSO to a stock concentration of 50 mM and diluted in complete media to a final concentration of 50 μ M. In these experiments, cells were treated with chloroquine for 24 hours prior to lysis. Cycloheximide (Sigma, Cat. #7698) is an inhibitor of protein synthesis. Cycloheximide powder was dissolved in ethanol (Sigma, Cat.

#E7023) to a stock concentration of 20 mg/mL and diluted in complete media to a final concentration of 20 µg/mL. Cells were treated for 0, 2, 4, and 8 hours prior to lysis.

2.3.3. Transfection reagents

jetPRIME® – The jetPRIME® DNA/siRNA transfection reagent is manufactured by Polyplus Transfection (Illkirch, France, Cat. #114-07) and can be purchased from VWR (Cat. #89129-922). jetPRIME® was primarily used for transient transfections. 1 µg of each plasmid DNA construct to be transfected was diluted in 200 µL of jetPRIME® transfection buffer, provided by the manufacturer, and vortexed briefly. To this, 2 µL of jetPRIME® was added per microgram of DNA. The mixture was then vortexed for 10 seconds, spun down for 10 seconds, then incubated at room temperature for 10 minutes. After incubation, the mixture was added dropwise to cells in a 60 mm culture dish, evenly dispersed by gentle rocking, and incubated for 16 hours at 37°C. This reaction can be scaled for culture vessels of different sizes. Also, if cytotoxicity is observed, the 37°C incubation can be performed for 6-8 hours, followed by overnight incubation in fresh complete medium.

Lipofectamine™ 3000 – Lipofectamine™ 3000 (Invitrogen, Cat. #L3000008) is a lipid nanoparticle-based transfection reagent designed for cells that are difficult to transfect using other transfection reagents such as jetPRIME®. NCI-H1792, NCI-H1437, and NCI-H1299 cells were transfected using Lipofectamine 3000®

according to the manufacturer's protocol for generation of stable knockdown cell lines. For these transfections, 2-5 µg of plasmid DNA and 2 µL of P3000™ reagent (provided by the manufacturer) were diluted in 125 µL Opti-MEM reduced serum media (Gibco, Cat. #31985070), briefly vortexed, and spun down for 2-3 seconds. 7.5 µL of Lipofectamine™ 3000 reagent was diluted in another 125 µL of Opti-MEM, briefly vortexed, and spun down. 125 µL of each mixture was combined and incubated at room temperature for 10 minutes. The mixture was then added dropwise to one well of cells in a 6-well plate and incubated at 37°C for 24 hours. Fresh media was then applied and the cells were incubated for an additional 24 hours prior to antibiotic selection.

2.3.4. Lysis buffers

Co-immunoprecipitation reactions require cell lysis to be performed in buffers that can disrupt the plasma membrane while leaving intact cytoplasmic protein-protein interactions. For this purpose, a modified RPIA buffer (NP-40 buffer) was used. This buffer consists of 50 mM Tris-HCl pH 7.5 200 mM NaCl, and 1% Tergitol™ Type NP-40 (Sigma, Cat. #NP40). Cell lysates that did not require preservation of protein-protein interactions were prepared in RIPA buffer (Sigma, Cat. #R0278), which contains 150 mM NaCl, 1.0% IGEPAL® CA630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris pH 8.0. Lysates for Active Ras pulldown assays were prepared in Active Ras Pulldown Cell Lysis Buffer provided by the manufacturer (Cytoskeleton, Cat.

#BK008). It contains 50 mM Tris pH 7.5, 10 mM MgCl₂, 0.5 M NaCl, and 2% IGEPAL® CA630. All lysis buffers were prepared fresh at time of cell lysis and supplemented with Protease Inhibitor Cocktail (Sigma, Cat. #P8340), 1 mM Na₃VO₄, and 1 mM NaF.

2.4. *Molecular Biology*

During the course of this work, multiple plasmid constructs were used to confirm a novel protein-protein interaction and characterize its function. Overexpressed co-immunoprecipitations, fluorescent co-localization studies, and shRNA knockdown of endogenous proteins all employ the use of plasmid DNA constructs that can be propagated and purified from competent *E. coli*.

2.4.1. *Bacterial transformation*

Chemically competent DH5 α cells were purchased from Invitrogen (Cat. #18261-017). For bacterial transformation, approximately 100 ng of whole plasmid was mixed with 30 μ L DH5 α bacteria and incubated on ice for 30 minutes. The bacteria were subjected to a 42°C heat shock for 45 seconds, allowing DNA to enter through pores in on the bacterial surface. This was followed by another 2-minute incubation on ice. 200 μ L of SOC media (Corning, Cat. #46-003-CR) was added to the mixture and incubated at 37°C for 1 hour. Following this, bacteria were gently resuspended and 100 μ L of this suspension was streaked on to LB

agar plates [2% LB Base (Invitrogen, Cat. #12780052), 1% Select Agar (Invitrogen, Cat. #30391023)] containing the appropriate antibiotic based on the bacterial resistance marker of the plasmid. For these experiments, the antibiotic used was either Ampicillin (Sigma, Cat. #A9518) at a concentration of 100 µg/mL, or Kanamycin sulfate (Calbiochem, Cat. #420331) at a concentration of 50 µg/mL. Plates were incubated, inverted, overnight at 37°C.

2.4.2. Plasmid purification

Following transformation, individual colonies were picked from the LB agar plates and grown in 5 mL a sterilized 2% solution of LB Base in water containing the appropriate antibiotic, using a shaking incubator at 37°C, 300 RPM. 5 mL cultures were grown overnight and immediately used for DNA Plasmid Mini-Prep (Qiagen, Cat. #27106), or used to inoculate 100 mL cultures for Midi-Prep (Qiagen, Cat. #12145; Sigma, Cat. #NA0200-1KT). Both DNA preparations were performed according to the manufacturer's protocol. Briefly, they involve lysis of the bacterial membrane followed by precipitation and pelleting of bacterial genomic DNA. Plasmid DNA is bound to a column, proteins and RNA are washed away, and plasmid DNA is finally eluted from the column. DNA concentration was determined using a NanoDrop™ 8000 Spectrophotometer (Thermo Scientific, Cat. #ND-8000-GL).

2.4.3. Generation of stable RASSF1A and DAB2IP knockdown lines

In order to characterize the interaction between RASSF1A and DAB2IP, we generated matched sets of NCI-H1792 and NCI-H1437 cells that were either wild-type or stably knocked down for DAB2IP and/or RASSF1A. We also generated a matched set of NCI-H1299 cells in which DAB2IP was knocked down and RASSF1A was overexpressed.

Knockdown of RASSF1A in NCI-H1792 cells was described previously [11]. Briefly, an shRNA against RASSF1A was cloned into the pSM2 vector and transfected into NCI-H1792 cells. Cells were then selected in puromycin. pGIPZ and pSM2 both contain puromycin resistance markers, so lentiviral infection was used by Dr. Howard Donninger to knockdown DAB2IP these cells. To generate DAB2IP knockdown cells, 293FT cells were transfected with pGIPZ-shDAB2IP (see section 2.1) and viral packaging plasmids pLP1, pLP2, and pLP/VSVG (Invitrogen) at a ratio of 1:3 using jetPRIME® transfection reagent. 48-72 hours after transfection, growth medium containing viral particles was harvested and clarified by centrifugation at 2 krpm for 5 minutes. 2 mL of the clarified viral supernatant supplemented with 5 µg/mL polybrene was applied to NCI-H1792 cells overnight at 37°C. Fresh medium was applied to the 293FT cells and the viral transduction was performed two additional times. After transduction, GFP-positive cells (which express GFP from stable incorporation of the DAB2IP shRNA) were selected using a MoFlo XDP Cell Sorter (Beckman Coulter).

Stable knockdown of DAB2IP and RASSF1A in NCI-H1437 cells was achieved by Lipofectamine™ 3000 transfection followed by antibiotic selection.

pGIPZ-shDAB2IP was transfected into NCI-H1437 cells. 48 hours after transfection, cells were selected in 1.5 µg/mL puromycin (Sigma, Cat. #8833-10MG). Stable transfectants were expanded and assayed/frozen as an early passage pooled population of cells. H1437-shDAB2IP and control cells were then transfected with pBRS-shRASSF1A 777 in a similar fashion, and selected in 6 µg/mL blasticidin (Sigma, Cat. #15205-25MG).

Stable re-expression of HA-RASSF1A in NCI-H1299 cells was described previously [148]. Briefly, 1 µg of pZip-neoHA-RASSF1A was transfected into H1299 cells using Lipofectamine™ 2000, a precursor to Lipofectamine™ 3000. Stable transfectants were selected in 500 µg/mL G4138 sulfate. Knockdown of DAB2IP in these cells was achieved in the same manner as in H1437 cells (see above). For each stably expressed shRNA or tagged expression construct, a stable line expressing an empty vector was generated to create a matched set of four lines that are +/+, +/-, -/+, and -/- for RASSF1A/DAB2IP expression, respectively, in each lung cancer cell line.

2.5. *Biological assays*

Confirming and characterizing a novel protein binding interaction requires the use of many biological techniques. Some of the assays employed in this work to test the hypotheses established herein are technically involved and are performed over the course of several days or weeks. *In vitro* and *in vivo* growth

assays and Western blot analysis of signaling pathways were utilized in this work. A detailed account of how these assays were performed is provided below.

2.5.1. *Growth assays*

A classical method of measuring the tumor suppressive ability of a protein is to assay its ability to restrict the growth of cultured cancer cells. There are multiple growth assays that will provide differing degrees of physiological relevance. Generally, the degree of relevance of a particular result is dependent on the amount of stress applied to the cell in the course of the assay. For example, enhanced *in vitro* 2D growth in response to RASSF1A loss implicates RASSF1A as a suppressor of growth. Enhanced anchorage-independent growth or, better, enhanced xenograft growth in response to RASSF1A loss more clearly demonstrates the importance of RASSF1A in tumor suppression as it highlights the heartiness of RASSF1A-deficient cells and their ability to survive unfavorable conditions. In this investigation, two growth assays were used: a 2D growth curve and a subcutaneous xenograft growth curve.

2.5.1.1. *2D growth curves*

2D growth curves were performed in triplicate in 6-well plates. For each cell line, 10^4 cells in log phase growth were plated in 9 wells of 6-well plates. 24 hours after plating, 3 wells were trypsinized, pelleted, resuspended in complete growth

medium, and counted on a hemocytometer. 3 wells were again counted on days 3 and 7.

2.5.1.2. *Xenograft growth curves*

Immunodeficient mice can be used to measure the *in vivo* effects of a protein on tumor formation. To identify differences in *in vivo* growth of the matched sets of knockdown cells, each cell line was seeded at low density in several T75 flasks and allowed to propagate for 3 days to reach log phase growth. Cells were trypsinized, pelleted, and washed twice in 1X PBS by resuspension and re-pelleting. Cells were finally resuspended at a concentration of 10×10^6 cells/mL in 1X PBS and placed on ice. For each cell line 6 NRG mice were anesthetized with isoflurane and 100 μ L of cell suspension containing 1×10^6 cells was injected subcutaneously into the left flank. Animals were monitored and tumor length and width measurements were taken with calipers. Tumor volume was calculated using the formula $V = \frac{L \times W^2}{2}$. When the first tumors reached a diameter at 1.5 cm, all animals were sacrificed and tumors were harvested and snap frozen in liquid nitrogen.

2.5.2. *Western blot analysis*

Western blotting was performed under reducing conditions. For this, 450 μ L 4X LDS sample buffer (Invitrogen, Cat. #NP0007) was mixed with 50 μ L of 2-

mercaptoethanol and stored in an amber microcentrifuge tube. Washed IP beads resuspended in RIPA buffer and whole cell lysates were combined with prepared LDS buffer and vortexed thoroughly to mix. Tubes were boiled at 95°C for 10 minutes to denature proteins, then spun down briefly to collect condensation on the tube lids. Samples were loaded onto a precast NuPAGE™ 4-12% Bis-Tris polyacrylamide gel (Invitrogen, Cat. #NP0321BOX, NP0322BOX, NP0336BOX). Electrophoresis was performed at 120V in 1X NuPAGE™ MOPS SDS Running buffer (Invitrogen, Cat. #NP0001) until the dye front reached the bottom of the gel.

Resolved proteins were transferred to a 0.2 µm pore nitrocellulose membrane (Invitrogen, Cat. #LC2009) using complete immersion (wet) transfer in 1X Transfer Buffer (5% NuPAGE™ Transfer Buffer (20X) (Invitrogen, Cat. #NP00061), 20% methanol, 75% DI water). Transfer buffer was cooled prior to use. Transfers were performed using the Mini Blot Module (Life Technologies, Cat. #B1000) at 10V for at least 3 hours.

After transferring, membranes were blocked for 1 hour in a 5% solution of non-fat dry milk in TBS-T. Membranes would then be washed twice for 2 minutes in TBS-T, then incubated with primary and secondary antibody as described in section 2.5.2.1. After each antibody incubation, membranes were washed 3 times for 5 minutes in TBS-T before continuing to the next incubation. After the secondary incubation, a fourth wash of 10 minutes in TBS-T was performed to ensure minimal background detection. Blots were then immersed in West Pico PLUS solution for 5 minutes. Bands were detected using chemi-luminescent detection film (MidSci™, Cat. #EBA45).

2.5.2.1. Antibodies

Hemagglutinin (HA) – Mouse α -HA monoclonal antibody was purchased from Covance (Cat. #MMS-101P). For detection of HA-tagged proteins, the antibody was diluted at 1:10,000 in a 5% solution of non-fat dry milk in Tris-buffered saline with Tween-20 (TBS-T: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20). Membranes were incubated in primary antibody overnight at 4°C with constant agitation, then in HRP-linked α -mouse secondary antibody (Cell Signaling Technology, Cat. #7076S) at a dilution of 1:10,000 in TBS-T for 1 hour at room temperature with constant agitation. Detection was performed using SuperSignal™ West Pico PLUS Chemi-Luminescence substrate (Thermo Scientific, Cat. #34579).

Green fluorescent protein (GFP) – Mouse α -GFP (B2) monoclonal antibody was purchased from Santa Cruz Biotechnology (Cat. #sc-9996). For detection of GFP-tagged proteins, the antibody was diluted at 1:200 in a 5% solution of non-fat dry milk in TBS-T. Membranes were incubated in primary antibody overnight at 4°C with constant agitation, then in HRP-linked α -mouse secondary antibody at a dilution of 1:10,000 in TBS-T for 1 hour at room temperature with constant agitation. Detection was performed using West Pico PLUS.

Phospho-S473 AKT – The Ser/Thr kinase AKT is phosphorylated at S473 in response to activation by Ras. Phosphorylation of AKT at S473 serves as a measure of Ras activity. Endogenous phosphorylated AKT was detected using a rabbit α -P-S473AKT polyclonal antibody (Cell Signaling Technology, Cat. #4060S). The antibody was diluted at 1:1,000 in a 5% solution of BSA in TBS-T. Membranes were incubated overnight at 4°C with constant agitation, then in HRP-linked α -rabbit secondary antibody at a dilution of 1:10,000 in TBS-T for 1 hour at room temperature with constant agitation. Detection was performed using West Pico PLUS.

AKT - Endogenous AKT was detected using a rabbit α -P-S536 NF κ B p65 polyclonal antibody (Cell Signaling Technology, Cat. #9272S). The antibody was diluted at 1:1,000 in a 5% solution of BSA in TBS-T. Membranes were incubated overnight at 4°C with constant agitation, then in HRP-linked α -rabbit secondary antibody at a dilution of 1:10,000 in TBS-T for 1 hour at room temperature with constant agitation. Detection was performed using West Pico PLUS.

Phospho-ERK – Extracellular signal-regulated kinase 1/2 (ERK 1/2) is the terminal effector of the Ras-Raf-MEK-ERK pathway. Upon activation, ERK 1/2 are phosphorylated on T202 and Y204, respectively. Endogenous phosphorylated ERK 1/2 were detected using a rabbit α -P-T202/Y204 ERK antibody (Cell Signaling Technology, Cat. #9101S). The antibody was diluted at 1:1,000 in a 5% solution of BSA in TBS-T. Membranes were incubated overnight at 4°C with

constant agitation, then in HRP-linked α -rabbit secondary antibody at a dilution of 1:10,000 in TBS-T for 1 hour at room temperature with constant agitation. Detection was performed using West Pico PLUS.

ERK – Endogenous ERK 1/2 was detected using a rabbit α -ERK 1/2 polyclonal antibody (Cell Signaling Technology, Cat. #9102S). The antibody was diluted at 1:1,000 in a 5% solution of BSA in TBS-T. Membranes were incubated overnight at 4°C with constant agitation, then in HRP-linked α -rabbit secondary antibody at a dilution of 1:10,000 in TBS-T for 1 hour at room temperature with constant agitation. Detection was performed using West Pico PLUS.

DAB2IP – Endogenous DAB2IP was detected using a mouse α -DAB2IP monoclonal antibody (Aviva Systems Biology, Cat. #OAAL00940). The antibody was diluted at 1:1,000 in a 5% solution of non-fat dry milk TBS-T. Membranes were incubated overnight at 4°C with constant agitation, then in HRP-linked α -mouse secondary antibody at a dilution of 1:10,000 in TBS-T for 1 hour at room temperature with constant agitation. Detection was performed using West Pico PLUS.

RASSF1A – Endogenous RASSF1A was detected using a mouse α -RASSF1 (3F3) monoclonal antibody (Abcam, Cat. #ab23950). The antibody was diluted at 1:200 in a 5% solution of non-fat dry milk in TBS-T. Membranes were incubated overnight at 4°C with constant agitation, then in HRP-linked α -mouse secondary

antibody at a dilution of 1:2000 in TBS-T for 1 hour at room temperature with constant agitation. Detection was performed using West Pico PLUS. Expression of *RASSF1A* is often suppressed in cancer, making endogenous RASSF1A protein difficult to detect in some cell lines. To increase sensitivity, West Pico PLUS was supplemented with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, Cat. #34094) at concentration of 10-15%, as needed.

TrueBlot® Mouse Secondary – For co-immunoprecipitation experiments, the primary antibodies used to detect endogenous protein were of the same species as conjugated antibody used in the agarose immunoprecipitation beads. This leads to detection of IgG heavy and light chain bands that may obscure bands of interest on Western blots. To avoid this, a TrueBlot® mouse secondary was used (Rockland Inc., Cat. #18-8817-33), which does not detect IgG chains. TrueBlot mouse secondary was diluted at 1:1,000 in a 5% solution of non-fat dry milk. Incubation and detection were performed as with the standard mouse secondary antibody.

2.6. *Protein biochemistry*

2.6.1. *Co-immunoprecipitations*

Co-immunoprecipitation (Co-IP) is a widely-used technique for investigating protein-protein interactions in cells. In this technique a protein of interest is bound

by an antibody, and the protein-antibody complex is precipitated using beads, typically made of agarose, that bind to the Fc region of the antibody. In the case of exogenous tagged proteins, antibody pre-conjugated to agarose is commercially available to simplify the protocol. Whatever the case, the use of mild detergents in the lysis buffer is critical, as strong detergents like SDS readily disrupt protein-protein interactions. This assay is straightforward in principle; however, the length of the assay and the multiple binding interactions that must be maintained *ex-vivo* for multiple days makes successful co-immunoprecipitation quite difficult to achieve, particularly for endogenous proteins that are expressed in low abundance.

2.6.1.1. Immunoprecipitation of overexpressed proteins

In this work, HEK-293T cells were transfected in 60 mm dishes 24 hours prior to lysis. Cells were scraped in an appropriate volume of NP-40 buffer, typically 350 μ L, and transferred to a microcentrifuge tube. Cell suspensions were placed on a rotator and incubated at 4°C for 4 hours. The lysates were passed through a 21 ga needle five times, then centrifuged at ≥ 14 krpm for 5 minutes at 4°C. The supernatants were reserved and immediately placed on ice; the pellets were discarded. The protein concentrations of the lysates were determined using the Bio-Rad protein quantification assay (Cat. #5000006). 1 mg of whole cell lysate was used per immunoprecipitation (500 μ g if there was insufficient volume for 1 mg) and brought to a volume of 1 mL with NP-40 buffer in microcentrifuge tubes.

5 μ L of GFP-nAb™ agarose (Allele Biotechnology, Cat. #ABP-nAb-GFPA025) was added to each tube. Samples were placed on a rotator and incubated at 4°C overnight. The next day, the beads were pelleted by centrifugation at 3 krpm, 4°C for 3 minutes. The supernatant was aspirated and the beads were washed with 500 μ L of NP-40 buffer. This was repeated two additional times. After the third wash was aspirated, beads were resuspended in 10 μ L RIPA buffer and 4 μ L NuPAGE™ LDS Sample Buffer (Invitrogen, Cat. #NP0007). IPs and 30 μ g of whole cell lysate were resolved on SDS-PAGE gels and analyzed as described in section 2.5.2.

2.6.1.2. *Immunoprecipitation of endogenous proteins*

Endogenous proteins are immunoprecipitated using antibodies that recognize three-dimensional epitopes on the surface of the protein. Therefore, not all antibodies that recognize a protein on a Western blot, where the protein has been denatured, are appropriate for immunoprecipitation. In this particular experiment, the precipitated protein, RASSF1A, is endogenously expressed at low levels, and therefore large quantities of protein are needed for immunoprecipitation. This can be challenging using mild detergents like NP-40; however, such detergents are required for this assay and thus other steps were taken to maximize protein yield. Multiple 10 cm dishes were grown to confluence and scraped into a small volume of NP-40, typically 500 μ L for 2 dishes. The cell suspension was placed on a rotator and incubated at 4°C for 8 hours. The

lysate was then passed through a 21 ga needle 5 times and centrifuged at ≥ 14 krpm, 4°C for 10 minutes. The supernatant was transferred to a new microcentrifuge tube and centrifuged again if needed to obtain a clear lysate. Protein concentration was again determined using the Bio-Rad protein quantification assay. 8 mg of whole cell lysate was aliquoted into two microcentrifuge tubes (4 mg per tube) and each was diluted to a final volume of 1 mL. 1 mL of NP-40 buffer was added to a third tube. The buffer tube and one of the lysate tubes served as negative controls. To one lysate-containing tube and the buffer only tube, the immunoprecipitation antibody was added. NP-40 buffer was added to remaining tube as a blank. All tubes were placed on a rotator and incubated at 4°C overnight. The next morning, 20 μ L of TrueBlot® IP beads (Rockland Inc., Cat. #00-8800-25) were added to each tube, and tubes were rotated at 4°C for 2 hours. The beads were washed three times with NP-40 buffer and resuspended as described in section 2.6.1.1.

2.6.2. Active Ras pulldown assay

Analysis of downstream signaling activity is a useful method of determining the activation state of a particular protein if downstream targets are known and reagents to detect their activation are available. In cancer, however, such a method can often lead to inconclusive results. First, the complex networks involved in cell signaling result in effector proteins usually having multiple upstream regulators, and so the activity of an effector may not be readily attributable to the regulator of

interest. Indeed, this is the case in homeostatic tissue as well. Additionally, a hallmark of cancer is dysregulation of cell signaling. Mutations or other disruption in different parts of a particular pathway may obscure the activity of the protein of interest. Thus, a method of directly measuring the activation state of a protein is favorable when possible.

Fortunately, since Ras is a small GTPase whose binding affinity depends on its activation state, the degree of activation of Ras can be determined directly by precipitating the active GTP-bound form of Ras using an exogenously expressed Ras Binding Domain (RBD) conjugated to agarose beads. The Active Ras Pulldown Kit (Cytoskeleton, Cat. #BK008) was used to perform this assay according to the manufacturer's protocol. 10 cm dishes of each cell line were seeded at 5×10^5 cells per dish and incubated for 72 hours to achieve log phase growth. Cells were then washed once with ice-cold 1X PBS and scraped, on ice, into Cell Lysis Buffer provided by the manufacturer (see section 2.4.4). Cell suspensions were transferred to microcentrifuge tubes and were passed through a 21 ga needle five times, then centrifuged at 10 krpm for 10 minutes. After centrifugation, the supernatants were transferred to clean microcentrifuge tubes. 30 μ L of each lysate was aliquoted for protein quantification using the Pierce™ BCA Protein Assay (Thermo Scientific, Cat. #23225). The remaining lysates were snap frozen in liquid nitrogen to prevent GTP hydrolysis. After quantification, lysates were thawed in a room temperature water bath and immediately placed on ice. 500 μ g of whole cell lysate was diluted to a final volume of 500 μ L, and 10 μ L Raf-RBD agarose beads were added to each sample. Samples were placed on a

rotator and incubated at 4°C for 1 hour. Beads were pelleted by centrifugation at 3,000 x g, 4°C for 1 minute. The -supernatants were aspirated, then beads were washed once with 500 µL of Wash Buffer provided by the manufacturer. Beads were again pelleted by centrifugation at 5,000 x g, 4°C for 3 minutes. The Wash Buffer was aspirated and the beads were resuspended in 10 µL RIPA buffer and 4 µL LDS Sample Buffer. IPs and 30 µg whole cell lysate were resolved on SDS-PAGE gels and analyzed as described in section 2.6.2.

CHAPTER III

RASSF1A BINDS THE RASGAP DAB2IP

3.1. *Introduction*

Ras is the most frequently activated oncogene in human cancer, with point mutations occurring in approximately 30% of all tumors [27]. Experimentally, mutant Ras has been shown repeatedly to rapidly transform cultured cells [209-211]. Upon activation, Ras binds multiple downstream mitogenic effectors, whose activities synergize to confer potent transforming abilities to Ras. The three best characterized mitogenic effectors are Raf, PI3K, and RalGDS [212]. For decades, attempts have been made to target the Ras proteins therapeutically as a treatment for cancer, and for decades those attempts have failed [213]. That is, until very recently. Several reports in the past five years have revealed a previously unknown characteristic of a particular Ras mutant. In the course of developing covalent inhibitors of K-Ras^{G12C}, it was demonstrated that this mutant actively undergoes GTP-GDP cycling in tumors, and these inhibitors work by trapping the Ras mutant in the GDP-bound state [116].

While Ras can transform immortalized cells, it invokes apoptosis and premature senescence in primary cells [8, 214]. Much about this paradoxical

growth inhibitory nature of Ras remains to be discovered; however, the RASSF family of tumor suppressors has been implicated in mediating these effects, particularly RASSF1A [9, 127]. In order to induce transformation, Ras requires cooperating mutations, such as deletion of p53 or activation of MYC [215-217]. This aids Ras-mediated transformation in part by blocking apoptosis/senescence in response to Ras hyperactivation [218, 219]. Similarly, loss of *RASSF1A* expression, a frequent event in human cancer primarily caused by promoter hypermethylation, has also been shown to contribute to Ras-mediated transformation [14, 127, 131]. RASSF1A binds Ras directly and induces apoptosis in a GTP-dependent manner [11, 122]. Mechanisms of RASSF1A-induced growth arrest are not fully described, but among the best characterized RASSF1A effectors are the Hippo kinases MST1/2 and Bax, an inducer of mitochondrial apoptosis.

RASSF1A lacks apparent enzymatic activity, instead functioning as a scaffold. Upon Ras activation, RASSF1A binds Ras-GTP and connects it to modulator of apoptosis-1 (MOAP-1) [11], or the MST kinases [220], activating them and subsequently inducing apoptosis through Bax or the transcriptional activator YAP, respectively [11, 138, 141, 145, 146, 220, 221]. Thus, the frequent loss of RASSF1A in cancer, considered an early/premalignant lesion [130, 222, 223], permits Ras-mediated transformation by uncoupling Ras from its apoptotic effector pathways. However, this is not the full extent of deregulation caused by loss of RASSF1A expression. Indeed, multiple groups have reported upregulation of Ras mitogenic signaling, including Raf-Mek-Erk, PI3K-AKT, and RalGDS-Ral, upon

downregulation of RASSF1A *in vitro* and *in vivo* [13, 14, 17]. Collectively, this work lends itself to the tantalizing possibility of a feedback inhibition activity of RASSF1A on Ras, an exceedingly common mechanism of protein inhibition generally, but one that has not yet been described for Ras [37].

RASSF1A has been shown previously to modulate the activation on AKT and Raf; however, the ultimate effect of these interactions is unclear. Raf can bind to the Hippo kinase MST2 and inhibit its activation [224]. However, this interaction also inhibits Raf by preventing its activation by Ras [16]. RASSF1A and Raf compete for binding with MST2, and disruption of the Raf-MST2 complex is required for RASSF1A-MST2-induced apoptosis [138]. This suggests that the RASSF1A-MST2 interaction would serve to counterintuitively activate Ras-Raf-Mek-Erk [9]. Raf activity is regulated by phosphorylation, and S259 is an inhibitory residue. Phosphorylation of Raf at S259 is mediated by AKT and enhances its reciprocally inhibitory association with MST2 [225]. AKT also phosphorylates MST2, which enhances its interaction with Raf [15]. RASSF1A inhibits these phosphorylation events by blocking the activating phosphorylation of AKT on S473 [13]. The direct mechanism of AKT inhibition by RASSF1A has not been reported. S473 phosphorylation of AKT is regulated by the mammalian target of rapamycin complex 2 (mTORC2) [13, 226], which in turn is activated by Ras [227]. Thus, control of AKT by RASSF1A may not be direct, but instead mediated through control of RAS activation.

Because of its apoptotic nature and frequent inactivation in premalignant tissue, it was long suspected that RASSF1A suppression was an important step in

Ras-mediated tumorigenesis. In 2018, the definitive experiment proving this hypothesis was performed [14]. Using *rassf1* transgenic mice that also harbored a doxycycline-inducible *kras*^{G12V} mutation specifically in type II pneumocytes, it was shown that loss of one allele of *rassf1* was sufficient to significantly enhance Ras-mediated tumor initiation and growth. These tumors exhibited simultaneous activation of the three canonical Ras effector pathways. Moreover, loss of one allele of *rassf1a* alone—without induction of mutant *kras*—was sufficient to enhance Ras mitogenic signaling in normal lung tissue [14]. This suggested that perhaps RASSF1A loss can stimulate RAS. In the course of studying the tumor suppressive activities of RASSF1A, this lab commissioned a yeast two-hybrid screen, using RASSF1A as bait, to identify novel binding partners (Myriad Genetics, Salt Lake City, UT). The RasGAP DAB2IP was identified as a binding partner for the 37-120 aa region of RASSF1A, which contains the cysteine-rich domain CRD.

3.2. Results

We hypothesized that RASSF1A may regulate Ras by enhancing the Ras-RasGAP interaction. Thus, we began our investigation by confirming the interaction between RASSF1A and DAB2IP.

3.2.1. DAB2IP co-localizes with RASSF1A at the microtubules

RASSF1A is an important regulator of microtubule dynamics, and as such prominently localizes to the microtubules when overexpressed [151, 152, 228]. The subcellular localization of DAB2IP is more complex. DAB2IP plays an important role in regulating neuron and dendrite growth in the developing brain [229-231]. Thus, its localization in these specialized cells is well characterized. Its localization in other tissues has not been well characterized. DAB2IP has been detected at the plasma membrane, in a complex with TNFR in resting cells [182]. As DAB2IP is a RasGAP, it is undoubtedly recruited to the plasma membrane in response to Ras activation. We determined whether RASSF1A and DAB2IP co-localized in mammalian cells using transient transfection of fluorescent-tagged expression constructs in COS-7 cells (figure 8). GFP and Katushka (KATE) expression constructs were used as negative controls. Katushka is a far-red fluorescent protein derived from *E. quadricolor* [232, 233]. As expected, the negative controls nonspecifically filled the cytoplasm, whereas RASSF1A was localized to microtubules. When expressed alone, DAB2IP filled the cytoplasm; however, co-expression of RASSF1A and DAB2IP results in recruitment of DAB2IP to the microtubules with RASSF1A.

Figure 8. RASSF1A and DAB2IP colocalize in COS-7 cells. COS-7 cells were transiently transfected for 24 hours with GFP-tagged DAB2IP and KATE-tagged DAB2IP either together or with the empty expression vector of the other protein. RASSF1A prominently localizes at the microtubules when overexpressed alone, whereas DAB2IP fills the cytoplasm. When together, RASSF1a recruits DAB2IP to the microtubules. Representative images were captured using an IX50 inverted system microscope (Olympus) and a SPOT camera (Diagnostic Instruments, Inc.)

3.2.2. *RASSF1A associates with DAB2IP independent of Ras*

To ensure the co-localization of DAB2IP and RASSF1A observed in COS-7 cells was due to direct association of the two proteins, we performed transient overexpression of epitope-tagged constructs followed by co-immunoprecipitation assays (figure 9). We hypothesized that RASSF1A mediated its suppression of Ras through scaffolding activated Ras to its RasGAP inhibitor. Thus, we expected the RASSF1A-DAB2IP interaction to be dependent on Ras activation and so included a constitutively activate K-Ras^{G12V} mutant in our study. To our surprise, we found that inclusion of the Ras mutant did not enhance the association between HA-RASSF1A and GFP-DAB2IP.

Finally, we sought to detect the endogenous association of RASSF1A and DAB2IP in human cells (figure 10). RASSF1A is a difficult protein to detect endogenously. It is a tumor suppressor and inhibitor of cell cycle progression, and thus is expressed at low levels in normal tissues. Its expression is commonly suppressed in human tumors and cancer cell lines, compounding the issue. Antibodies against RASSF1A that are suitable for immunoprecipitation are not commercially available. Moreover, α -RASSF1A antibodies for Western blotting are not very sensitive in our hands, and detecting endogenous RASSF1A requires resolving large amounts of protein on gels. To provide the most favorable conditions possible, we performed endogenous co-immunoprecipitation in MCF-10A breast epithelial cells. This is a non-transformed epithelial cell line. While not a lung cancer cell line, we chose to use MCF-10A cells for this assay

Figure 9: RASSF1A and DAB2IP co-immunoprecipitate when overexpressed.

HEK-293T cells were co-transfected with GFP-DAB2IP, HA-RASSF1A and oncogenic K-Ras^{G12V} for 24 hours. Cells were lysed and lysates were immunoprecipitated (IP) with GFP-nAb™ agarose beads and immunoblotted (IB) for the indicated epitope tags.

Figure 10: RASSF1A and DAB2IP form an endogenous complex. Whole cell lysates were prepared from MCF-10A cells. Lysates were immunoprecipitated (IP) with a rabbit polyclonal RASSF1A antibody (Prosci, Poway, CA) and anti-rabbit IgG immunoprecipitation beads for 16 hours and immunoblotted (IB) for DAB2IP. Whole cell lysates served as a positive control. Anti-RASSF1A/IgG beads incubated in lysis buffer and IgG beads incubated in whole cell lysate were used as negative controls.

because they can be easily grown in large quantities and express relatively high amounts of RASSF1A compared to cancer cell lines. Using a rabbit polyclonal antibody against RASSF1A (generated by Prosci using the peptide ELRELAPAGRAGKGRTRLER, [14]) we successfully detected the endogenous interaction between DAB2IP and RASSF1A.

3.2.4. RASSF1A regulates DAB2IP protein levels

To identify a biological role for the novel RASSF1A-DAB2IP tumor suppressor complex, we chose to disrupt the interaction using shRNA-mediated knockdown of each protein alone and in combination. The use of shRNAs is not as clean or cutting-edge as newer techniques such as CRISPR-Cas9-mediated knockout. However, we chose this method of gene suppression because it more accurately mimics the method of RASSF1A and DAB2IP suppression in lung tumors of real patients. The expression and activities of RASSF1A and DAB2IP are both suppressed frequently in lung cancer; however, neither is usually lost entirely. Mutations in these genes are rare, therefore functional ablation of their signaling activities is not a physiological mechanism of suppression in this case. The major mechanism of RASSF1A and DAB2IP suppression in cancer is promoter hypermethylation [171, 234]. Thus, expression is reduced, sometimes substantially, but rarely completely absent. Indeed, the suppressed-but-not-absent state of Ras effectors in cancer may be of particular importance in Ras-driven tumors, as mutant Ras may be supported by the remaining wild-type alleles [137].

In the mouse study, effects of RASSF1A on Ras mitogenic signaling were observed in mutant K-Ras -induced and -uninduced tissues [14]. Thus, for these experiments we chose to employ the use of NCI-1437 NSCLC cells, which retain wild-type Ras expression, and NCI-H1792 NSCLC cells, which harbor a heterozygous mutation in *KRAS*, resulting in a G12C substitution [207, 235, 236]. When we confirmed the knockdown of RASSF1A and DAB2IP in these cells, we found that expression of the shRNA-mediated knockdown of RASSF1A caused a striking reduction in DAB2IP protein levels (figure 11). This was not a nonspecific effect of the shRNA as NCI-H1792 and NCI-H1473 were transfected with different shRNA constructs and selected using different resistance markers. Moreover, stable re-expression of RASSF1A in NCI-H1299 cells, which lack endogenous RASSF1A expression, increased DAB2IP protein levels (figure 12).

RASSF1A has been shown to regulate the stability of other cellular proteins, in part through regulating proteasomal degradation [144, 148, 237, 238]. DAB2IP has been shown to contain phosphodegron sequences that target it for degradation by FBW7-SCF complexes [171]. We hypothesized that RASSF1A may regulate DAB2IP stability by inhibiting its proteasomal degradation. To test this, we treated NCI-H1792 cells with the proteasome inhibitor MG-132. MG-132 treatment had no significant effect on DAB2IP protein levels in these cells (Figure 13a). We also inhibited lysosomal activity by treating

Figure 11: Confirmation of shRNA knockdown of RASSF1A/DAB2IP in NCI-H1792 and NCI-H1437 cells. NCI-H1792 cells expressing pSM1-shRASSF1A or empty vector [11], were transfected for 24 hours with pGIPZ-shDAB2IP or empty vector. pGIPZ contains a GFP marker, and GFP-positive cells were isolated using a MoFlo XDP cell sorter (Beckman Coulter), and then selected in 2.5 µg/mL puromycin. NCI-1437 cells were sequentially transfected and selected with pGIPZ-shDAB2IP/pBRS-shRASSf1A and 1.5 µg/mL puromycin/6 µg/mL blasticidin, respectively. Cells were maintained as an early-passage pooled population and knockdown was confirmed via Western blot. Band density was quantified using ImageJ and DAB2IP fold change was calculated by normalizing to vector-expressing cells.

Figure 12: Exogenous re-expression of RASSF1A upregulates DAB2IP in lung cancer cells. NCI-H1299 cells stably expressing pZip-HA-RASSF1A or empty vector [11] were transfected for 24 hours with pGIPZ-shDAB2IP or empty vector and selected in 1 μ g/mL puromycin. Cells were maintained as an early-passage pooled population of cells, and DAB2IP knockdown was confirmed via Western blot. Band density was quantified in ImageJ and DAB2IP fold change was calculated by normalizing to vector-expressing cells (lane 3).

Figure 13. Effects of chloroquine and MG-132 treatment on DAB2IP protein levels in NCI-H1792 cells. NCI-H1792 cells were treated with either 50 μ M chloroquine for 24 hours or 50 μ M MG-132 for 4 hours. Cells were lysed in RIPA buffer and DAB2IP levels were measured via Western blot. Experiments were performed in duplicate and quantified using ImageJ.

the cells with chloroquine [239]; however, lysosomal inhibition also had no effect on DAB2IP protein levels (figure 13b).

3.3. Discussion

The RASSF1A tumor suppressor is an important mediator of Ras-induced apoptosis [11, 122, 138, 238, 240]. *RASSF1A* expression is frequently suppressed in human tumors by promoter hypermethylation [223, 241-245]. Loss of RASSF1A activity uncouples oncogenic Ras from pro-apoptotic signaling and facilitates unrestrained Ras mitogenic activity. Furthermore, reports of RASSF1A modulating Ras mitogenic activity suggest that RASSF1A may also directly regulate Ras activation [13, 14, 16, 17].

In our studies, we identified a novel binding interaction between RASSF1A and another tumor suppressor, DAB2IP, that is an important GAP for RAS [18, 37, 180]. Thus, we hypothesized and confirmed that RASSF1A and DAB2IP form an endogenous complex in human cells. However, while we expected this interaction to be regulated by Ras activation, we found that RASSF1A associated with DAB2IP irrespective of Ras activation. Perhaps our most important observation was that shRNA-mediated knockdown of RASSF1A significantly downregulated DAB2IP protein levels.

These results clearly demonstrate a physiological interaction between RASSF1A and DAB2IP, but the exact function of the interaction remains elusive. The function of RASSF1A as a scaffolding protein for Ras guided our initial

investigation in to the RASSF1A-DAB2IP complex. Ras activation has been shown previously as a prerequisite for interactions between RASSF proteins and their effectors [11, 137, 246, 247]. However, in our co-immunoprecipitation studies we observed no such dependence on Ras activity of the interaction between DAB2IP and RASSF1A. What we did find was that RASSF1A upregulates DAB2IP proteins levels in human NSCLC cells.

Ras is rendered constitutively active by point mutation in many tumors [35]. However, this is not the only mechanism by which Ras can be rendered hyperactive. Aberrant activity of Ras-regulating proteins, GEFs and GAPs, can also give rise to Ras-driven tumors. Such is the case in the RASopathy Noonan syndrome, a familial cancer syndrome frequently caused by germline activating mutations in the Ras/MAPK pathway [248]. While germline mutations in Ras genes themselves are to blame for some cases, in others, mutations in upstream activators of Ras such as SOS1 have been reported [249, 250]. Another RASopathy, neurofibromatosis type 1 (NF1), is caused by deleterious mutation in the RasGAP gene *NF1* [251]. In addition, deletions in *NF1* may occur in up to 25% of breast tumors [252]. Multiple RasGAPs, including DAB2iP have been found to be dysregulated in human cancer [169]. This dysregulation can be critical tumor progression. Indeed, suppression of DAB2IP is a frequent event and primary driver in prostate cancer [167, 200]. Thus, the loss of DAB2IP upon RASSF1A suppression is likely a significant contributor to the phenotype observed in RASSF1A-deficient cells.

DAB2IP is regulated at the levels of transcription, translation, and post-translation. Phosphorylation is the predominant post-translational modification of DAB2IP, resulting in its activation, inhibition, or targeting for proteasomal degradation depending on the phosphorylated residue [178, 184]. Interestingly, DAB2IP is inhibited by AKT via phosphorylation at S847, which targets DAB2IP for proteasomal degradation [178]. RASSF1A inhibits AKT [13]. Thus, RASSF1A may stabilize DAB2IP by inhibiting its AKT-mediated degradation. However, this mechanism involves the proteasome, and proteasomal inhibition with MG-132 did not significantly affect DAB2IP protein levels (figure 4). Antibodies to detect phosphorylated residues of DAB2IP are not commercially available, therefore the effect of RASSF1A on DAB2IP phosphorylation cannot be determined by Western blot. DAB2IP was not previously identified as being under the transcriptional control of RASSF1A [253]. However, comparison of RASSF1A and DAB2IP expression in lung adenocarcinoma tumors in The Cancer Genome Atlas (TCGA), reveals a strong correlation (figure 14), suggesting that the control of DAB2IP by RASSF1A may be transcriptional in nature after all. Additionally, DAB2IP expression is also regulated by microRNAs [177, 194, 254, 255]. Thus, it is possible that RASSF1A may regulate the transcription of a microRNA that regulates DAB2IP.

Figure 14: Correlation of RASSF1A and DAB2IP expression in patient lung adenocarcinoma tumors. RNA-seq expression analyses of *RASSF1* and *DAB2IP* genes in human lung adenocarcinoma tumor and normal tissue samples in The Cancer Genome Atlas (TCGA) were accessed through GEPIA [256]. Log 2 of transcripts per kilobase million (TMP) of *DAB2IP* was plotted against that of *RASSF1*. Correlation was calculated using Pearson's correlation coefficient.

CHAPTER IV

EFFECTS OF RASSF1A AND DAB2IP ON GROWTH AND RAS SIGNALING

4.1 *Introduction*

While deregulated Ras is a potent oncoprotein, properly regulated Ras signaling plays an important, and sometimes indispensable role in embryogenesis and tissue homeostasis [257-259]. Normal activation of Ras involves a multi-protein complex consisting of Ras, receptor tyrosine kinases, adaptor molecules, and GEFs [24]. A classical example is EGFR-GRB2-SOS1-Ras. All of these proteins can be overexpressed or mutationally activated in cancer, leading to enhanced Ras-MAPK signaling [260-262]. Less is known about how the inactivation of Ras is regulated. However, the Ras-RasGAP interaction is direct, and the most common mutations in Ras render it constitutively active by preventing the catalytic subunit of RasGAPs from being properly positioned in the Ras active site [38]. Mutation of the RasGAP NF1 is both the basis of a familial cancer syndrome and detectable in sporadic human tumors [33, 34, 263]. Other RasGAPs, such as DAB2IP, are not commonly mutated in cancer. Rather, their expression is suppressed by epigenetic mechanisms [169, 171]. These alternative mechanisms of Ras dysregulation play important roles in disease. For example,

Ras mutations occur in less than 5% of breast tumors [35]. However, elevated Ras-MAPK signaling is detected in approximately 50% of breast tumors, particularly in aggressive subtypes, due to dysregulation upstream and downstream of Ras [170, 197, 264-266].

DAB2IP is downregulated in breast, lung, prostate, and gastrointestinal cancer [171]. The GTPase activity of DAB2IP was identified when it was first discovered [18]. GAP activity against Ras is an important mechanism of DAB2IP-mediated tumor suppression, but it is only partially responsible for the full tumor suppressive phenotype of DAB2IP [37]. DAB2IP is a multi-faceted tumor suppressor, that also acts independently of Ras to regulate multiple cellular processes critical to cancer progression, including EMT, proliferation, and apoptosis/survival [36].

DAB2IP utilizes independent domains to simultaneously suppress tumor initiation and metastasis [167]. Through the GAP domain, DAB2IP suppresses Ras-MAPK signaling and inhibits tumor initiation/growth. DAB2IP also inhibits metastasis by inhibiting NF κ B activation. Ras stimulates NF κ B [267, 268]. Therefore, DAB2IP-mediated inhibition of Ras may indirectly suppress NF κ B to some extent; however, GAP activity is dispensable for DAB2IP-mediated suppression of NF κ B [167]. Indeed, suppression of NF κ B is part of the pro-apoptotic transduction of TNFR signaling by DAB2IP, wherein phosphorylated DAB2IP simultaneously inhibits pro-survival signaling through NF κ B and activates the pro-apoptotic ASK/JNK pathway [180, 182, 184, 189]. Interestingly, Raf inhibits

ASK1 activation [269, 270]. Thus again, GAP activity may play an indirect role in potentiating a GAP-independent process of DAB2IP.

A third convergence of GAP-dependent and -independent activities of DAB2IP involves the PI3K-AKT pathway. As a canonical Ras effector, GAP activity obviously suppresses PI3K activation. However, it has been shown that DAB2IP can directly bind the p85 regulatory subunit of PI3K, sequestering the p85-p110 complex and preventing its activation of AKT [165]. This also requires TNF-dependent phosphorylation of DAB2IP, and is therefore part of its anti-survival/pro-apoptotic activity.

DAB2IP is a complex tumor suppressor that is tightly interwoven with Ras signaling. DAB2IP restrains Ras-mediated tumorigenesis by inhibiting Ras itself and its effectors, canonical or otherwise, by distinct Ras-dependent and -independent mechanisms. Thus, loss of DAB2IP expression in cancer substantially disrupts homeostatic signaling and favors tumor growth and metastasis [37, 169, 171]. We have found that DAB2IP protein levels are regulated by RASSF1A and that suppression of RASSF1A, an exceedingly common event in cancer [127, 131], markedly downregulates DAB2IP (see section 3.2). We suspected that this phenomenon may underlie the effects on Ras signaling observed upon suppression of RASSF1A, and thus analyzed the growth and signaling patterns in our matched sets of NSCLC cells.

4.2 *Results*

We hypothesized that RASSF1A knockdown cells would exhibit enhanced growth and activation of Ras mitogenic pathways due to elevated basal levels of Ras-GTP. As epigenetic suppression of DAB2IP is itself a frequent occurrence in lung cancer, we further hypothesized that co-suppression of RASSF1A and DAB2IP would synergistically enhance Ras activation.

4.2.1. RASSF1A and DAB2IP cooperatively regulate Ras activation

GAP activity is critical to DAB2IP-mediated suppression of tumor initiation and growth [167]. We have previously shown in a mouse model of K-Ras-driven lung cancer that loss of one allele of *rassf1a* stimulated signaling through canonical Ras mitogenic effectors Raf, PI3K, and RalGDS in mutant- and wild-type-Ras tissues, suggesting that RASSF1A may directly regulate Ras [14]. We wanted to determine if the loss of DAB2IP observed upon shRNA knockdown of RASSF1A resulted in a corresponding increase in GTP loading onto Ras. For this, we performed active Ras pulldown assays and compared the amount of Ras-GTP precipitated to the total amount of Ras in standardized aliquots of whole cell lysate (figure 15). Consistent with our hypothesis and previous results, elevated levels of Ras-GTP were detected in RASSF1A knockdown cells for both NCI-H1792 and NCI-H1437 cell lines. Moreover, in both cells, co-suppression of RASSF1A and DAB2IP potentiated this effect.

We chose to assess the activation state of a canonical Ras mitogenic pathway to confirm our results from the active Ras pulldown assay. Thus, we

analyzed both cell lines for PI3K activity during log phase growth via Western blot, using P-S473 AKT as a readout (figure 16). We found in both cell lines that P-S473 AKT was undetectable in control and DAB2IP-knockdown cells. RASSF1A knockdown elevated AKT activation, and addition of the DAB2IP shRNA to RASSF1A knockdown cells potentiated this effect.

4.2.2. Effects of RASSF1A and DAB2IP on NSCLC growth

In the mouse model, heterozygosity at the *rassf1a* locus significantly increased lung tumor burden upon induction of the K-Ras mutant compared to wild-type littermates. Moreover, loss of one allele of *rassf1a* in normal, wild-type Ras tissue significantly increased Ras mitogenic signaling [14]. We wanted to determine if such a role could be established for RASSF1A in our knockdown cells. To that end, we measured *in vitro* growth in our mutant K-Ras cells (figure 17a). Knockdown of DAB2IP alone modestly but insignificantly enhanced 2D growth, whereas RASSF1A significantly enhanced growth. Co-suppression of DAB2IP/RASSF1A had little effect above that of RASSF1A knockdown alone.

We also assessed *in vitro* 2D growth in our wild-type Ras NCI-H1437 matched set (figure 17b). Interestingly, here we observed synergy between RASSF1A and DAB2IP. Only co-suppression of both proteins significantly increased 2D growth. This synergistic effect fit well with the Ras and AKT activation observed in these cells. So, we chose to further examine this cell line to determine if RASSF1A and DAB2IP would synergize *in vivo*. We injected our NCI-H1437

matched set subcutaneously into NRG mice, and measured the volume of the resultant xenograft tumors over the course of several weeks (figure 18). Knockdown of DAB2IP alone had no effect on tumor growth, whereas RASSF1A knockdown modestly increased tumor growth. Similar to *in vitro* growth, co-suppression of RASSF1A and DAB2IP significantly enhanced *in vivo* growth of NCI-H1437 cells.

Finally, we examined Ras pathway activation in tumor lysates via Western blot (figure 19). In the dual knockdown tumors, we observed consistent, statistically significant upregulation of MAPK pathway activation compared to control tumors. Dual knockdown also resulted in the highest PI3K-AKT pathway, although this effect was not statistically significant.

4.3 Discussion

RASSF1A plays an indispensable role in homeostasis in large part by restraining Ras-mediated transformation [129]. Canonical RASSF1A signaling is primarily centered on induction of apoptosis in response to Ras activation [9, 127]. Indeed, the growth-inhibitory response to mutant Ras activity constitutes a molecular hurdle that must be overcome in order for transformation to occur [271]. Hints of a more pleiotropic role for RASSF1A in modulating Ras activity have been published throughout the years [13, 14, 16, 17]. Here, we provide further evidence for a novel, regulatory role for RASSF1A in Ras signaling.

Figure 15. Effects of RASSF1A/DAB2IP suppression on Ras activation. Cells were seeded at low density in 10 cm dishes and growth for 72 hours, after which whole cell lysates were prepared. Ras-GTP was precipitated for 1 hour using the Ras binding domain (RBD) of Raf-1 conjugated to agarose. Precipitated Ras-GTP and total Ras levels were determined using a pan-Ras antibody (Cytoskeleton Inc.).

Figure 16: Effects if RASSF1A/DAB2IP suppression on *in vitro* Ras signaling.

Cells were seeded at low density and growth for 48 hours, after which whole cell lysates were prepared. AKT activation was determined via Western blot analysis of serine-473 phosphorylation.

Figure 17. *In vitro* growth curves of NCI-H1792 and NCI-H1437 matched sets of cells. Cells were plated at 10^4 cells per well in 9 wells of 6-well plates. Triplicate wells were counted 24 hours after plating as day 0, then again on days 3 and 7. Cell numbers are reported as mean $\pm$ s.e. **** $p < 0.0001$

Figure 18: NCI-H1437 xenograft growth curve. Cells were seeded at low density in several large flasks and incubated for 48 hours to achieve log-phase growth. 10^6 cells in 1X PBS were injected subcutaneously into the left flank of NRG mice. Resultant tumors were measured using calipers. Tumor volume was calculated by the formula $V = \frac{L \times W^2}{2}$. Volumes are reported as mean $\pm$ s.e. n = 5 or 6. $p \leq *0.05$, **0.01, ****0.0001.

Figure 19: Effects of RASSF1A/DAB2IP suppression on *in vivo* Ras signaling. NCI-H1437 xenograft tumors were harvested from sacrificed animals and lysates were prepared. Equal protein masses of each tumor lysate were analyzed for MAPK and PI3K-AKT activity via ERK 1/2 T-202/Y-205 and AKT S-473 phosphorylation, respectively. Bands were quantified using ImageJ and activation fold change was calculated by normalization to control cells. n= 5 or 6. $p \leq *0.05$, $**0.01$.

Having demonstrated the ability of RASSF1A to upregulate DAB2IP, an important RasGAP, we speculated that this modulation of DAB2IP protein levels would be transduced through Ras mitogenic activity and affect the growth of our matched sets of NSCLC cells. We anticipated that the effects on Ras signaling and cell growth would be apparent in both cell lines, despite the NCI-H1792 cells harboring an oncogenic K-Ras mutant, for several reasons. First, the specific mutation found in NCI-H1792 cells, G12C, actively—and rapidly—cycles between GTP- and GDP-bound states, suggesting that it retains some sensitivity to GAP action [115, 272]. Second, we have reported that loss of RASSF1A potentiates mitogenic signaling mediated by mutant K-Ras *in vivo* [14]. Finally, the remaining wild-type Ras alleles are certainly still susceptible to GAP activity, and their activation may support the oncogenic action of the mutant allele [137].

In both wild-type and mutant Ras cell lines, we observed striking upregulation of Ras-GTP and downstream activation of AKT upon co-suppression RASSF1A and DAB2IP. Analysis of growth in these cells, however, gave disparate results. In the mutant Ras cells, suppression of RASSF1A alone significantly enhanced 2D growth compared control cells in agreement with the Ras activity detected in these cells. Dual inactivation of RASSF1A/DAB2IP had an insignificant effect over that of RASSF1A suppression alone, despite these cells exhibiting the strongest Ras activation in the pulldown and signaling assays. One possible explanation for this is that concomitant suppression of RASSF1A and mutation of Ras maximally stimulates growth, such that further stimulation of Ras from suppression of DAB2IP confers no additional benefit. The interplay between

RASSF1A and DAB2IP has not been studied previously in any capacity. However, this explanation is in agreement with clinical observations in lung cancer patients, in that those tumors with dual mutation in *KRAS* and hypermethylation of the *RASSF1A* promoter are highly aggressive and confer a poor prognosis [273].

In wild-type Ras cells, we observed a synergistic effect between RASSF1A and DAB2IP suppression on 2D growth. Excited by this result, we chose to further examine the biology of these cells *in vivo*. Here, we found that suppression of RASSF1A alone significantly increased Ras activation and growth in NCI-H1437 xenografts, and this effect was compounded by addition of the DAB2IP shRNA. The discrepancy between the *in vitro* and *in vivo* results remains unresolved; however, the effect of RASSF1A on DAB2IP protein levels may be responsible. DAB2IP is known to inhibit angiogenesis through inhibition of VEGFR-2 [164]. Angiogenesis is critical to tumor growth, as oxygen and nutrient availability at the center of the tumor become limited by diffusion in avascular tissue once the tumor is just a few cubic millimeters in size [274, 275]. Hypoxia at the core of a tumor induces the expression of hypoxia-inducible factors (HIFs), transcription factors that regulate genes that aid cells in handling hypoxic stress [276]. Loss of DAB2IP has been shown to upregulate HIF-1 α and HIF-2 α [193, 277, 278]. Thus, suppression of DAB2IP confers a growth and survival advantage *in vivo* that is not otherwise apparent experimentally. Since knockdown of RASSF1A caused a greater reduction in DAB2IP protein levels than the DAB2IP shRNA, disinhibition of VEGF/HIF signaling may contribute to *in vivo* growth in these cells.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

The work presented in this dissertation identifies a novel role for RASSF1A in the regulation of the RasGAP DAB2IP. It tracks the transduction of this regulatory event from its start in regulating Ras activation, through the modulation of Ras mitogenic signaling, down to its terminal phenotypic output of suppressing tumor growth. In so doing, it furthers our mechanistic understand of RASSF-mediated tumor suppression and begins to answer the lingering question of why loss of RASSF1A enhances the mitogenic activity of Ras.

We found that RASSF1A forms an endogenous complex with the RasGAP DAB2IP. Unexpectedly, this interaction was not regulated by Ras. Preliminarily, our data suggest that RASSF1A stabilizes DAB2IP protein; however, this was not mediated through inhibition of proteasomal or lysosomal degradation of DAB2IP.

RASSF1A has previously been shown by our group and others modulate the activation of Ras mitogenic pathways [13, 14, 17]. However, the mechanisms underlying these phenomena are not completely clear. Here, we demonstrate a clear upregulation of Ras-GTP in response to RASSF1A suppression. Thus, the

effects of RASSF1A on Ras signaling are not caused by the complex interactions between RASSF1A and mitogenic Ras effectors, or at least not fully, but involves direct control of Ras activation. To our knowledge, this is the first example of a downstream effector of Ras modulating an upstream regulator of Ras.

Of note, the effect of RASSF1A on Ras activation is not limited to only wild-type Ras tumors, but can be observed in mutant Ras cancer cells as well. The implications of this novel regulatory event may not be immediately apparent. Gene therapy is not yet the standard of care in cancer treatment, and therefore knowing that one of the most frequently inactivated tumor suppressors can regulate the most frequently activated oncoprotein does not immediately reveal a useful therapeutic target. However, the renewed quest to effectively drug Ras as cancer treatment suggests that this regulatory event may be of therapeutic import after all.

Novel inhibitors of K-Ras^{G12C} function by trapping mutant Ras in the inactive state, demonstrating that the mutant is not completely devoid of GTPase activity and may still be stimulated by RasGAPs. In fact, the notion that Ras mutants are locked in an active state has recently been brought into question, as other common Ras mutants (G13D and Q61L specifically) have also been shown to retain intrinsic GTPase activity that is, though diminished, still comparable to wild-type Ras [279]. Therefore, RASSF1A-mediated loss of GAP expression may dampen the hydrolytic potential of Ras mutants, thereby reducing the pool of Ras-GDP available for inhibitors to bind. These novel inhibitors may rely on the intact regulatory machinery of Ras. In the same vein, though in diametrical opposition to the former in approach, a 2019 report showed that growth of lung cancer cells

could be inhibited by treatment with a small molecule agonist of K-RAS [280]. Pharmacological activation of K-Ras restricted cell growth by inducing apoptosis and autophagy. Loss of RASSF1A/DAB2IP dually uncouples Ras from its apoptotic effectors and substantially elevates the basal activation state of Ras. Hypothetically, then, RASSF1A/DAB2IP loss may elevate Ras activation and suppress Ras-mediated apoptosis to such a degree that further activation by the small molecule is incapable of eliciting an apoptotic response. As drug discovery in the Ras field continues, the importance of these little-known (and perhaps still unknown) regulatory mechanisms controlling Ras may become more apparent.

5.2. *Future Directions*

The original goal of this work was to confirm and characterize a novel binding interaction between RASSF1A and DAB2IP. While generating the reagents necessary to investigate the biological significance of the RASSF1A-DAB2IP interaction, we made another novel—and unexpected—discovery: RASSF1A upregulates DAB2IP. Thus, we turned our attention to investigating the effects of this novel regulatory event on growth and signaling in cancer.

In the course of our studies, we did elucidate a novel mechanism of RASSF1A-mediated regulation of Ras activation. However, several questions remain as to the function of the RASSF1A-DAB2IP complex. First, mapping the site of interaction on both DAB2IP and RASSF1A would allow for the generation of binding-defective mutants. These mutants would be critical to elucidating what

biological effects are directly attributable to the RASSF1A-DAB2IP interaction itself. RASSF1A and DAB2IP have several overlapping activities, including inhibition of AKT, induction of apoptosis, and regulation of inflammation. Therefore, it is important to distinguish which effects are due to the binding event versus the additive output of two independent mechanisms.

Additionally, the ability of other RASSF proteins to bind and/or regulate GAPs may be a worthwhile investigation. RASSF proteins are capable of dimerizing with each other, and therefore may cooperatively mediate tumor suppression [281]. Moreover, RASSF proteins exhibit some redundancy in effector binding and signaling activity [9, 37]. Indeed, we have seen that other RASSF family members can bind DAB2IP in overexpression systems (unpublished observation). Thus, regulation of small GTPases may be a common attribute of classical RASSF proteins rather than a unique property of RASSF1A.

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Publications

Harrell Stewart D, Hobbing K, Schmidt ML, Donninger H, Clark GJ. The role of RASSF proteins in modulating RAS driven lung tumors in vivo. J Thorac Dis 2019 May; 11(Suppl 9): S1436-1437. doi: 10.21037/jtd.2019.03.60. PMID: 31245154

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Activating Protein DAB2IP and modulates RAS activation
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