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Coordinate regulation of the WNT and Hippo pathways by the novel Ras effector NORE1A.

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COORDINATE REGULATION OF THE WNT AND HIPPO PATHWAYS BY THE NOVEL
RAS EFFECTOR NORE1A

By

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B.S., University of Louisville, 2004

M.S., University of Louisville, 2013

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in Partial Fulfillment of the Requirements
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Doctor of Philosophy

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Department of Biochemistry and Molecular Genetics
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A Dissertation Approved on

August 3, 2015

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Dr. Geoffrey J. Clark, Dissertation Director

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Dr. Thomas Geoghegan, Committee Member

DEDICATION

This dissertation is dedicated to my family for their love, encouragement, and guidance and to the memory of the lives taken far too early by cancer.

My Beloved Grandfather

Marvin LeRoy Schmidt

My Beloved Aunt

Ann Schmidt Walker

While they may no longer walk this earth with me, the lessons they taught of life and love remain with me always. I love you and miss you.

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I would like to take this opportunity to thank my lovely wife, Leslie Ann Schmidt, R.N., for her love, support, and encouragement. You walked every inch of this journey with me and I love you not only for the wonderful person you are, but also for what your support and love make of me as a husband and a man. I want to also take a moment to recognize and thank my incredible family. A grateful thanks goes to my mother, Vicky Lynn Schmidt, and to my father, Marvin Lee Schmidt, Sr., for giving me all the opportunities to grow and experience life to the fullest. They taught me to never settle and strive to be the best I can be in all aspects of life: academic, professional, and personal. Next, thanks to my brother and best friend, Jason McKinley Schmidt, for being a staple of strength, support, and comfort in my life. If trouble were to be found, we would more often than not find it together. To my grandparents, Jacqueline Nancy Schmidt, Anna Margaret Burgher, the late Marvin LeRoy Schmidt, and the late Marcus McKinley Burgher, Jr., my warmest thanks for always emphasizing to me the value of education and for your support, love, and encouragement throughout. My paternal grandmother once said: "Son, some people are not very intelligent, and that is ok. You, however, are very intelligent, and that, my son, is a gift...use it." I know there is a considerable amount of bias in this statement as I am her grandson. Nonetheless, I try hard to use the intellectual gifts God bestowed and my family molded to me to make a positive difference in the world. I also want to send my warmest thanks to my aunts and uncles for love and encouragement: Shirley and Marcus M. Burgher, III, Stephanie Burgher and Jay Garrett, Wendy I. and F. Ronalds Walker, and the late Ann Schmidt

Walker. A warm thank you goes to my cousins, Russell P. Walker and Marcus M. Burgher, IV, as well as my extended family including my mother and father-in-law, Michael and Pamela McClaren, and sister-in-law, Rebecca McClaren, for all of your motivation and love. A warm thank-you and sincere gratitude goes out to my lab mates, especially to Thibaut Barnoud and Jessica Mezzanotte. Tim, you were always there with a supportive attitude, a well-timed joke, and a smile whenever I needed a boost. Jessica, you were my sounding board, my extra set of hands, my calming and encouraging voice, my fan club, and above all else, a very special friend. Though distance may separate us later in our careers and in life, you both will always have a special place in my heart. Thanks you, Dr. Howard Donninger, for all the advice and support on my research and for helping me keep my as sane as possible during the tougher days in the lab. If I ever became stuck on mundane detail, Howard was always able to get me to see the forest for the trees. I would also like to thank my committee members, Dr. Bob Mitchell, Dr. Barbara Clark, Dr. Thomas Geoghegan, and Dr. Jaydev Dholakia for your assistance during my academic program. And finally, a very special thank you goes to Dr. Geoffrey J. Clark. Without you, Geoff, none of this would have been possible. You took a chance on me allowing me to pursue higher education and supported me throughout with advice, levity, and motivation. I will never forget what you have done for me, and my family.

ABSTRACT

COORDINATE REGULATION OF THE WNT AND HIPPO PATHWAYS BY THE NOVEL RAS EFFECTOR NORE1A

Marvin "Lee" Schmidt, Jr.

August 3, 2015

Ras is the most frequently activated oncogene found in human cancer, but its mechanisms of action remain only partially understood. Ras activates multiple signaling pathways in order to promote transformation, but can also exhibit a potent ability to induce growth arrest and death. NORE1A (RASSF5) is a direct Ras effector that acts as a tumor suppressor promoting senescence and apoptosis. Its expression is frequently lost in human tumors and its mechanism of action remains unclear. Here I show that NORE1A forms a direct, Ras regulated, complex with β -TrCP, the substrate recognition component of the SCF- β -TrCP ubiquitin ligase complex. NORE1A also binds GSK-3 β resulting in enhanced kinase activity, which is required to phosphorylate SCF- β -TrCP substrates before their recognition by the ubiquitin ligase complex. Thus, by acting as a scaffold between β -TrCP and GSK-3 β , NORE1A allows for Ras to both qualitatively and quantitatively enhance the proteosomal degradation of SCF- β -TrCP targets, such as β -catenin and TAZ. However, this control is substrate specific, as I κ B, another substrate of SCF- β -TrCP, is not sensitive to NORE1A promoted degradation. Thus, I identify a

completely novel signaling mechanism for Ras that allows the coordinate regulation of the Wnt/ β -catenin and Hippo pathways, and potentially multiple other SCF- β -TrCP targets. The mechanism is frequently impaired in tumors by loss of NORE1A expression and provides an explanation for the observation that β -TrCP can act as a tumor suppressor or an oncogene in different cell systems.

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CHAPTER I

BACKGROUND AND INTRODUCTION

1.1 - Overview

Members of the Ras family of small GTP-ase proteins control multiple signaling pathways responsible for growth, migration, adhesion, cytoskeletal integrity, survival, and differentiation and come in three closely related isoforms, H-Ras, K-Ras, and N-Ras [1, 2]. Mutations at codons 12, 13, and 61 render all three Ras isoforms into a constitutively active state [1]. These *Ras* mutations have been shown to induce tumors in experimental systems and can be found in approximately 30% of human cancers [3, 4]. Constitutively activated Ras can stimulate multiple mitogenic signaling pathways, including the Raf/MAPK, Ral, and PI3K pathways, resulting in oncogenic transformation [5]. While Ras does indeed activate transformative pathways, it can also activate growth suppressive pathways promoting apoptosis and Oncogene Induced Senescence (OIS) [6-8]. However, the mechanisms by which Ras can promote apoptosis or OIS and how these mechanisms are subverted to facilitate tumor development remain poorly understood. Current research has shown that Ras induced cell death appears to be mediated by members of the RASSF family of tumor suppressors, which bind activated Ras and serve as death effectors [9]. RASSF5, more commonly referred to as NORE1A, was the first member of the RASSF family to be characterized and has been implicated in tumor suppressor signaling through the Hippo and p53 pathways [10, 11].

NORE1A is expressed in most normal tissues, but its expression is lost in many tumors due to epigenetic inactivation or calpain-mediated proteolysis [10, 12]. In tumors where NORE1A expression was lost, restoration of NORE1A expression diminished the tumor phenotype [13]. Moreover, transient expression of NORE1A results in enhanced apoptosis and senescence [13, 14]. NORE1A is considered to be a *bona fide* tumor suppressor, as a hereditary genetic defect in NORE1A predisposes the human carriers to kidney cancer [15]; however, the biological functions of the Ras/NORE1A interaction remain mostly uncharacterized.

Research and data detailed in this thesis describes the discovery of a novel signaling mechanism in which Ras/NORE1A suppresses excessive growth signaling and promotes cellular senescence by regulating two growth and survival signaling cascade endpoints via poly-ubiquitination and degradation in the 26S proteasome. Here I will show that NORE1A can bind the E3 ubiquitin ligase, β -TrCP. This interaction is regulated by Ras and powerfully enhances the degradation of the terminal executor of Wnt signaling, β -catenin. Furthermore, I show that NORE1A can also bind GSK-3 β and enhance its phosphorylation of β -catenin. Considering that substrates, especially β -catenin, must be phosphorylated by GSK-3 β before recognition and ubiquitination by β -TrCP, this observation provides a potential explanation on why NORE1A can efficiently regulate β -catenin protein stability. After characterizing NORE1A's role in Wnt regulation, I investigated other signaling pathways regulated by both GSK-3 β and β -TrCP that are implicated in cancer. Regulation of the Hippo and Wnt pathways are known to be linked [16, 17], so I decided to also investigate the Hippo signaling pathway, specifically focusing on the functional elements of the signaling cascade, YAP and TAZ. Initial experiments found that NORE1A suppressed TAZ mediated gene transcription, while having little effect on YAP transcriptional activity. TAZ has a GSK-3 β phosphodegron, while its counterpart, YAP, does not [18]. This observation suggested a

possible mechanism allowing for the differential regulation TAZ over YAP exists. My experimental work goes on to show that NORE1A indeed specifically regulates TAZ protein levels independently of canonical Hippo signaling by enhancing GSK-3 β phosphorylation and β -TrCP mediated ubiquitination of TAZ. I also present evidence that NORE1A is a scaffolding element that is integral in stabilizing GSK-3 β and β -TrCP in a protein complex, providing a mechanistic explanation on how NORE1A enhances both phosphorylation and ubiquitination of substrates. Finally, I show that the loss of NORE1A removes a key protection element against excessive growth stimulation and resulting in a marked increase in steady state levels of β -catenin and TAZ. Thus, I identify a novel function for NORE1A operating as a Ras regulated scaffold for kinases and the β -TrCP ubiquitin ligase complex, resulting in enhanced activity towards key oncogenic targets and suppressing excessive growth and survival stimuli.

1.2 - The Discovery of the Ras Oncogene

Ras genes were initially discovered and characterized as viral genes transduced from the genome of rodents [19]. Interestingly, these genes are shown to drive the highly oncogenic characteristics of RNA tumor viruses [19]. The study of these viral genomes, their conserved eukaryotic cellular complements, along with the 21 kDa (p21) proteins produced by these genes helped define the fundamental properties of cellular Ras proteins [19]. The first report characterizing mutationally activated and highly transforming human *Ras* genes was made in 1982 [20]. When it was then determined that *Ras* mutations are found in very high frequency in human cancers, including three of the “Big Four” most deadly cancers in the United States: Lung, Colon, and Pancreatic Cancers; research turn its focus onto Ras and its role in human cancer [19].

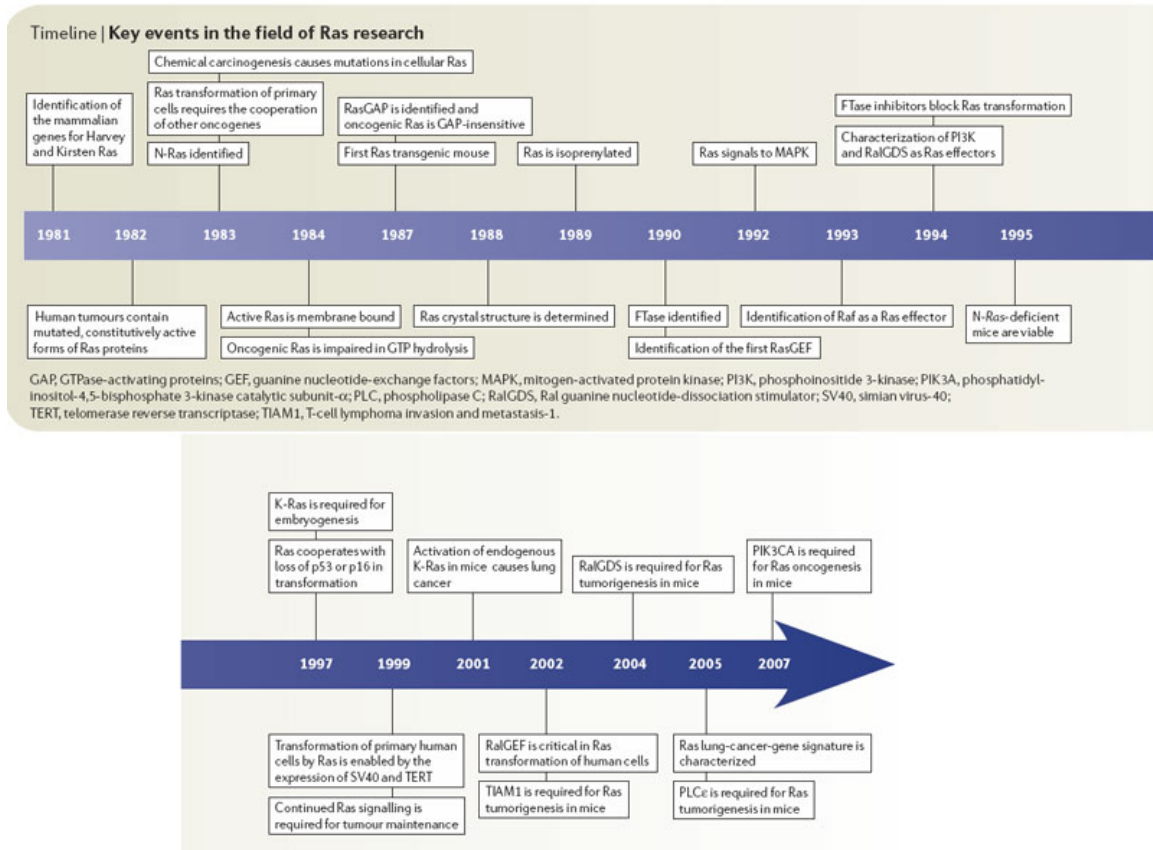
K-, H-, and N-Ras are the founding members of a superfamily of Ras-related small GTPases that now number more than 150 proteins [19]. Characterization of these proteins helped establish that small GTPases are operate as multi-functional regulators of numerous essential cellular processes [19]. When considering how complex the modes of regulation of the Ras superfamily are, it is easy to see how these proteins are involved in a spectacularly diverse range of biological processes [21]. Although early Ras research was established from a long history of research focused on retroviruses, it was the discovery of mutationally activated *Ras* genes in human cancer that would ignite an extensive effort in research focused on understanding Ras protein structure, biochemistry, and biology [19].

Identification of Ras from acutely transforming retroviruses:

The origins of *Ras* research begins in 1964 when Jennifer Harvey found that a sample of purified murine leukemia virus, isolated from a rat suffering from leukemia, induced the formation of sarcomas in newborn rodents [22, 23]. The tumorigenic properties of these sequences began to be defined in the early to mid 1970's when Ed Scolnick and colleagues determined that the Harvey (Ha-MSV) and Kirsten (Ki-MSV) viral strains were actually recombinant viruses with sequences collected from the rat genome [23, 24]. These genes were subsequently identified in the human genome and determined to be key players in human tumor pathogenesis [20]. In 1973, Ed Scolnick and colleagues predicted that the tumorigenic properties of these sarcoma viruses likely resulted from the transduction of normal cellular rat sequences into their own genomics [25]. Unfortunately, at that time of his prediction, the tools necessary to prove this theory were not available [19].

In the beginning, the genes we now refer to as *Ras* were named as variants of the *src* oncogene [19]. They were named *Ras* (**RA**t **SAR**coma) in part because their ability to induce rat sarcomas. The discovering scientist's names then became the basis for distinguishing each of them from the other: Harvey and Kirsten viral *Ras* genes, or *H-ras* and *K-ras*, gives rise to the proteins Ha-Ras, or H-Ras, and Ki-Ras or K-Ras [19]. What would then set the field of *Ras* research on its end was a report by Varmus, Bishop, Vogt, and colleagues that characterized the viral *src* oncogene as a normal chicken gene transduced by the virus into its own genome, thus transforming a normal gene into a strong oncogenic element [19, 26].

Scolnick and colleagues extensively studied *Ras* throughout the late 1970's and early 1980's. This work was then published explaining the eukaryotic origin of the viral *H-ras* and *K-ras* genes, demonstrating that these genes encode 21 kDa proteins that bind GDP and GTP, and described these proteins as being integrated into the plasma membrane [27-29]. However, these initial discoveries failed to explain key questions about *Ras* signaling including the framework in which *Ras* proteins function, what proteins affect signals toward *Ras*, and how *Ras* proteins pass signals to downstream targets in the cell [20]. Early experimental work was slow and complicated because *Ras* is controlled through several different positive and negative regulators and can act upon numerous downstream effectors, each with a defined pattern of tissue-specific expression and distinct set of intracellular functions. In the 1980's, several different assays were used to detect genetic mutations in *Ras* and were key in discovering the roles *Ras* performs in the pathogenesis of human cancers (**Figure 1**). These studies determined that mutations in *Ras* occur in approximately one-third of all human tumors, with the highest incidences found in cancers of the pancreas (90%), colon (50%), thyroid (50%), lung (30%) and leukemias (30%) [30]. However, for most tumor types, the role of mutated *Ras* is still unclear and will require a more thorough understanding of the



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Figure 1. Key Events in the Field of Ras Research - A timeline detailing key moments in the discovery and characterization of Ras. Discoveries in the 1980's explained how Ras is activated and operates as a small GTP-ase protein. In the 1990's, Ras effectors were beginning to be identified and their effects on the cell characterized. From the 2000's and ongoing today, research continues to work to explain how Ras plays a role in tumorigenesis.

mechanisms that regulate Ras along with a better understanding of how Ras acts upon secondary messenger systems [30].

Identification of Ras as an Oncogenic Human Gene

Although the high oncogenic capability of acutely transforming retroviruses was well known, the realization that human cancers are not induced by such infections quelled the fervor for studying them as a basis of human cancer development [19]. The researchers took a new course, establishing a new field of study based on the discovery that biologically active eukaryotic DNA could be inserted into mammalian cells by a process termed “transfection” [31, 32]. The cornerstone study showing the success of this type of laboratory procedure and demonstrating that transfected DNA could induce transforming properties was the use of NIH-3T3 mouse fibroblasts as the transfected cells [33]. While NIH-3T3 cells are immortalized, they retained some of the normal growth characteristics of normal cells including density dependent growth inhibition, a high dependence of serum growth factors, failure to grow in soft-agar experiments, or form tumors when inoculated into immunocompromised mice [19, 33]. When Weinberg and colleagues published a study in 1979 showing that when the NIH-3T3 cells were transfected with DNA isolated from chemically transformed rodent fibroblasts, they underwent morphologic transformation, the field was once again reinvigorated to determine the role of Ras in human cancer [34]. Soon after, other labs published similar reports using the same experimental approach described by Weinberg. In 1981, Krontiris and Cooper demonstrated that DNA isolated from the human EJ bladder carcinoma line also had transforming capabilities [35], and Weinberg’s group also detected transforming activity in carcinoma and leukemia cell lines [19, 36, 37]. In

response to these findings, researchers were rejuvenated and focused on the goal of identifying and isolating transforming human oncogenes.

In 1982, the two concurrent fields of Ras study, which were centered on acute transforming viral genes and transforming human genes, unexpectedly collided. Three groups concurrently determined that the transforming genes identified in the NIH-3T3 transfection experiments were actually the same *Ras* genes previously discovered in the Kirsten and Harvey sarcoma viruses [38-40]. Further study focused specifically on these sequences of *Ras* and found that the oncogenic variants contained a very specific set of conserved point mutations. These activating *Ras* mutations commonly result in amino-acid replacements at residues 12, 13, and 61 [41]. In 1983, a third member of the mammalian family of *Ras*-related genes, *N-ras*, was successfully captured and cloned from neuroblastoma and leukemia cell lines [42]. Remarkably similar to the *H-ras* and *K-ras* variants, *N-ras* was also found to contain activating point mutations in human tumors [43].

1.3 - Discovering Point Mutations of Ras in Human Cancers

Studies using the NIH-3T3 transfection assay, and later analyses using DNA sequencing assays, detected mutationally activated *Ras* genes in a broad range of human tumor cell lines as well as in primary patient tumors [19]. The most conspicuous of these discoveries was the remarkably high frequency of *Ras* mutations were found in colon, lung, and pancreatic cases [44-47]. Moreover, in addition to mutations at codon 12, *Ras* mutations were later identified at codons 13 and 61 [19]. While there are other mutations found in *Ras*, these occurrences are very rare. In fact, mutations at codons 12, 13, and 61 comprise 97-99% of all *Ras* mutations identified in human tumors and comprise the three “hot spots” of *Ras* activation [19].

The Multi-Hit Model of Oncogenic Transformation

It was not long before the hypothesized role of *Ras* in the initiation of cellular transformation was questioned. The ability of mutant *Ras* genes alone to transform NIH-3T3 cells into invasive and tumorigenic cells gave an initially simple and very misleading picture of the genetic basis of cancer [19]. Complicating the issue, another study showed that freshly isolated rodent embryo cells would not transform when they expressed a mutant *Ras* oncogene on its own [48]. Soon after, three different manuscripts were published noting the ability of H-Ras-12Val to transform primary cells that immortalized by the expression of another oncogene, such as *myc*, SV40 large T antigen, or the adenovirus *E1A* gene [20, 48, 49]. These discoveries fell in line with the Knudson hypothesis of a multi-hit model of carcinogenesis and further suggested that mutated, constitutively active Ras proteins can only transform cells that have previously undergone an oncogenic insult [50, 51].

The requirement for a cooperative second oncogenic hit was later supported by cell culture studies of primary human fibroblasts and epithelial cells where hTERT-mediated immortalization, inactivation of the p53 and Rb tumor suppressors, and protein phosphatase 2A inactivation were all found to help drive Ras-dependent transformation [52, 53]. This requirement for complementary oncogenic genetic alterations is consistent with the accumulation of genetic mutations in human colon and pancreatic cancers combined with the observation that cancer incidences increase with age [19].

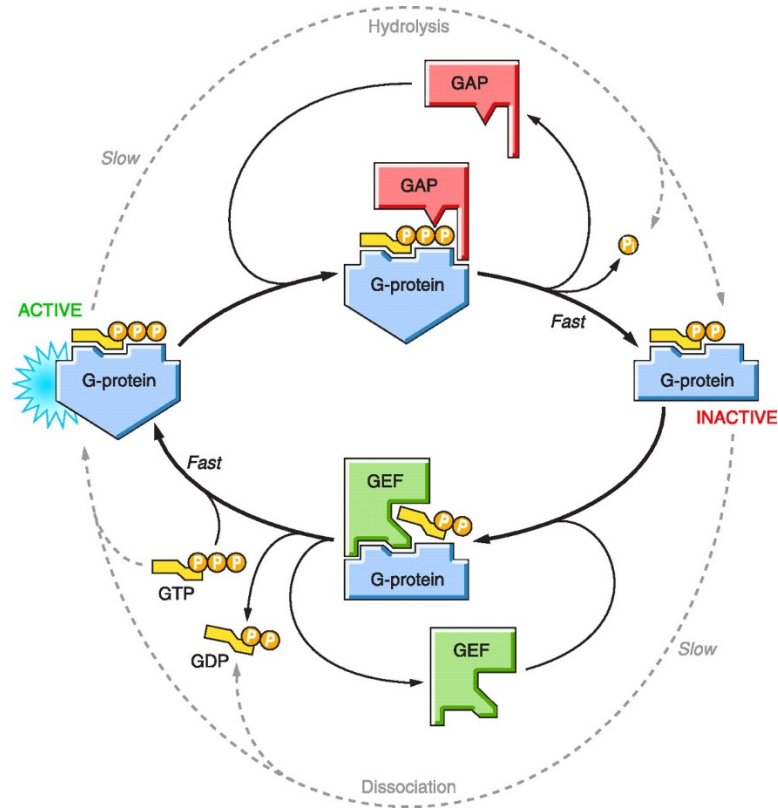
A particularly perplexing issue centers on the preferential mutational activation of specific *Ras* isoforms in different cancers. There are reports showing that certain types of human cancers are specifically associated with mutations in either K-, H-, or N-Ras. More specifically, K-*Ras* mutations were frequently observed in pancreatic and colon

cancers [54], H-*Ras* mutations were mostly associated with bladder carcinomas [55], and finally N-*Ras* mutations were described as being primarily localized to lymphoid malignancies [56] and melanomas [57]. However, the reasons for the observed tissue specificity remain unclear.

1.4 - Mutants of the Ras Oncoprotein and the GTPase Cycle

Once the molecular basis of *Ras* activation was shown to be caused by DNA mutation resulting a protein structure alteration, research focused on the origins of cancer at a molecular level [19]. In 1980, Edward Scolnick and colleagues investigated the biochemical features of the H-*Ras* protein. They initially described the binding of *Ras* proteins to guanine nucleotides, and determine that *Ras* may function analogously to the heterotrimeric G proteins [20, 58]. Heterotrimeric G-proteins were known to possess an intrinsic GTP hydrolysis activity, simply termed “GTP-ase” activity [59]. In G-proteins, it is the bound guanine moiety that governs its activity. When a G-protein is bound to a GTP molecule, the protein is typically in the active state. To shuttle to an off, or inactive, position, the G-protein will hydrolyze the GTP to a GDP. Reactivation of the protein occurs when the GDP is exchanged for the more abundant cellular GTP molecules [59].

A few years later, additional biochemical evidence showed that *Ras* proteins were indeed GTP-ases (**Figure 2**). Several groups demonstrated that mutated *Ras* oncoproteins differ functionally from their normal counterparts because their GTP-ase activity was impaired [60-62]. Soon after these discoveries, another group identified an oncogenic link between the Gly → Val substitution found at the 12th residue of H-*Ras* and GTP hydrolysis [63]. Moreover, mutations of the 61st residue were also found to be oncogenic due to disruption of the intrinsic GTP-ase activity of the H-*Ras* protein [64].



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Figure 2. The Ras GTP/GDP Activation Cycle - Diagram of the Ras GTP/GDP activation cycle. When Ras is bound to a GDP moiety, the enzyme is inactive. GEFs (Guanine Exchange Factor) interact with Ras and facilitate the exchange of the bound di-phosphate to a tri-phosphate co-factor. The Ras protein is active in this state. While Ras is able to hydrolyze the GTP to a GDP on its own, the process is quite slow. However, GAPs (GTPase Activating Protein) greatly enhance this speed of this reaction, returning Ras to the inactive state.

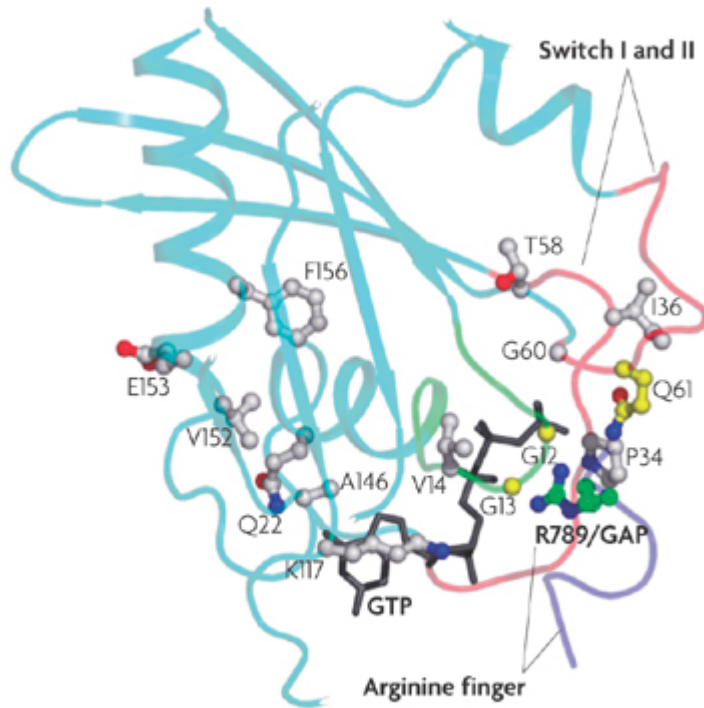
Thus, the dysfunction in GTP hydrolysis leads to an excessive activation of the Ras protein. However, increasing degrees of GTP-ase inhibition did not always correlate with increasing levels of oncogenic transformation, suggesting that compromised GTP hydrolysis is necessary, but not sufficient, for aberrant Ras activation [20, 65].

1.5 - A Closer Look into Ras Activation

In latter half of 1984, published work concentrating on the functional aspects of the regulation of Ras activation started to appear in the scientific literature. The breakthrough study investigating Ras activation observed that an increase in GTP-binding of cellular H-Ras occurred in response to treatment with epidermal growth factor (EGF) [66]. Multiple groups subsequently showed that activation or inhibition of Ras proteins by growth factors and cytokines suggested that Ras proteins, in a fashion similar to G-proteins, function as signal transducers from membrane-associated receptors [67-70].

Indeed, studies soon showed that Ras cycles similarly to G-proteins. Luckily, G-proteins were much better characterized than Ras, and share important structural and sequential homologies, which simplified much of the biochemical and structural research on Ras [71, 72]. A more comprehensive picture of Ras protein structure emerged when it was crystallized; showing the conformation of the GDP and GTP bound states as well as structures of the oncogenic mutants of Ras [73-75]. These studies helped explain the molecular features involved in the intrinsic GTP-ase activity of Ras and how mutations result in defects in the Ras activation switch.

The three-dimensional structure of Ras consists of six β -sheets and five α -helices that are connected by a series of ten loops (**Figure 3**) [76]. Five of these loops



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Figure 3. Three Dimensional Structure of the Ras Protein - Model of the 3-dimensional structure of the Ras protein. This model highlights the importance of the 12th, 13th, and 61st residues typically found mutated in constitutively activated Ras. The structure consists of six β -sheets and five α -helices that are linked by a series of ten loops. Mutations in residues 12 and/or 13 prevent GAPs from de-stabilizing and hydrolyzing the GTP to a GDP. The GTP-gamma-phosphate is stabilized by residues 16, 21, 35, 60, and 61. Of these, residue 61 coordinates the nucleophilic attack necessary for the hydrolysis of GTP to GDP.

are found on one lobe of the protein and have functional roles in binding GTP and regulating GTP hydrolysis. The GTP γ -phosphate is stabilized by interactions with residues Lys16, Tyr21, Thr35, Gly60, and Gln61 on loops 1,2, and 4 [73]. Of these residues, Gln61 appears to coordinate the nucleophilic attack that is necessary for the hydrolysis of GTP to GDP by stabilizing the transition state of this reaction [73]. Accordingly, mutations in Gln61 significantly reduce the intrinsic GTP hydrolysis rate, locking Ras in a constitutively active state and making it oncogenic.

Structural analysis of Ras in the GDP and GTP states revealed two highly dynamic regions, termed Switch I and Switch II [73, 74, 76, 77]. Switch I contains residues 30-40 and Switch II contains residues 60-76. Both regions are required for Ras to interact with upstream and downstream signaling elements. Binding of GTP shifts the conformation of Switch I by shifting Thr35 inward [76]. This allows Thr35 to interact with the GTP- γ -phosphate as well as the required Mg^{2+} ion cofactor. In much the same way, the bound GTP also changes the orientation of Switch II through interactions with Gly60 [76]. Functionality of both switches is required for the Ras protein to successfully cycle between active and inactive states.

While Ras contains intrinsic GTP-ase activity, kinetic assays show that it is slow and inefficient. In fact, differences in GTP-ase activity of Ras compared with its oncogenic mutants are modest and do not correlate with the degree of transformation found in tumors [78]. Thus, there must be other cellular elements facilitating Ras's GTP hydrolysis action. The discovery of GAPs (GTP-ase Activating Proteins) solved this dilemma. These proteins alter the conformation of Ras to greatly enhance its GTP-ase activity, shifting Ras back to an "off" conformation (**Figure 2**). The explanation began with an investigation of H-Ras in complex with the p120GAP GRD (GAP-Related Domain) [79]. Binding of the variable loop of GAP α 7 to Switch I of Ras establishes specificity between GAP and Ras [79]. This interaction is followed by a high affinity

interaction with the GAP's FLR motif (Phe-Leu-Arg), which stabilizes the two switch domains provoking a rapid acceleration of GTP hydrolysis [79].

Conversely, there are proteins that activate Ras by facilitating the exchange of GDP for GTP molecules. These proteins are called GEFs (Guanine Nucleotide Exchange Factors) and they facilitate the exchange of the GDP molecule in favor of a GTP molecule (**Figure 2**). Because of this interaction, an α -helical hairpin of the GEF separates the Switch I and Switch II domains of Ras, resulting in side-chain rearrangements centered on Ala59, which inserts itself into the Mg^{2+} binding cleft [80]. Additional structural shifts found in the phosphate-binding loop cause a massive enhancement of GDP ejection rates and preferential replacement with a GTP molecule in the nucleotide binding site [80].

1.6 - Post-translational Modifications of Ras

A pioneering study centered on viral form of H-Ras showed that the protein localized strongly to the cell membrane [81]. Soon afterwards, another report demonstrated that the viral H-Ras protein localized to the cell membrane in response to lipid modifications on the protein's C-terminus [82]. In 1984, a study described a correlation between lipid modifications and Ras function and showed that viral H-Ras proteins had to undergo a lipid modification and integrate into cellular membrane in order to drive cellular transformation [83]. This hypothesis also held true for the eukaryotic H-Ras protein, and the investigation concluded that C-terminal processing and membrane recruitment of Ras is a prerequisite to its subsequent biological activation [84].

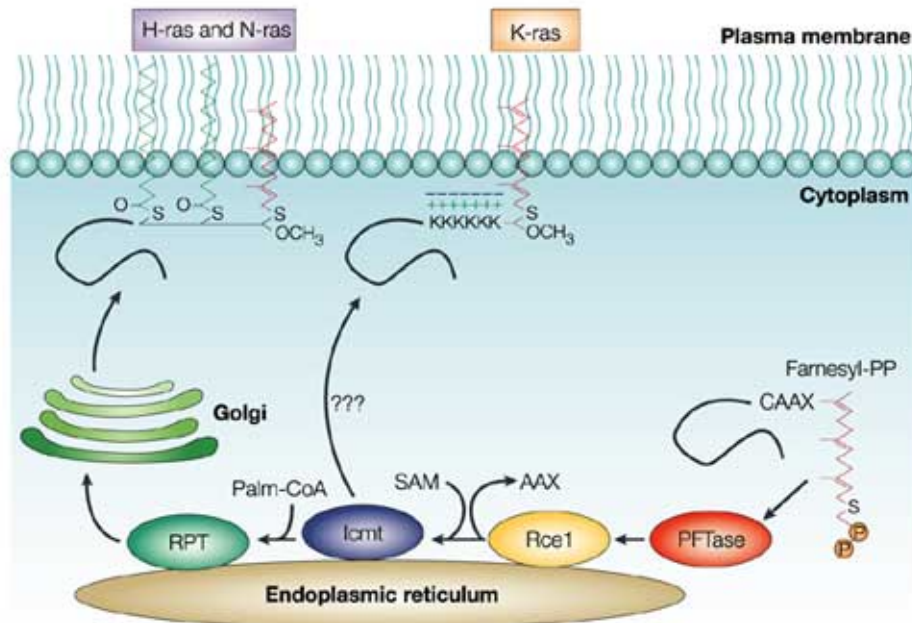
The biochemical reactions that lipidate Ras took several years to fully identify and required a wide array of biological and biochemical experimental systems. Even though there is a divergence in the C-terminus among the Ras family members, they

each contain a conserved CAAX sequence (**Figure 4**). A farnesyl pyrophosphate moiety is covalently attached to the cysteine residue of a cytosolic Ras protein by a farnesyltransferase (FTase) [85-87]. The farnesylation is followed by proteolytic cleavage of the last three amino acids by the Ras-converting enzyme-1 (RCE1), and finally isoprenylcysteine carboxyl methyltransferase 1 (ICMT1) methylates the last Cys residue [88]. At this point, a palmitoyltransferase (PTase) transfers a palmitoyl moiety to the Cys residues slightly upstream of the C-terminus, and the insertion of Ras into the membrane is stabilized [88].

While C-terminal processing of the CAAX sequence appeared to be essential for the association of Ras with the cellular membrane, studies of alternate splice variants of Ras identified a second signaling domain that allows for full membrane recruitment and full Ras function [88]. K-Ras-4B contains a string of positively charged Lys residues upstream of the C-terminus. This structural feature can be sufficient to attach the protein to the membrane. However, the prenylated H-Ras, N-Ras, and K-Ras-4A each require a further palmitoylation step where a palmitoyl moiety is attached to the C-terminal Cys residue before anchoring into the membrane is fully stabilized. Enzymes that catalyze these modifications later become attractive targets for the development of “Anti-Ras” drug therapies [20].

1.7 - Ras Activates a Wide Range of Effectors

Even though Ras was heavily studied during the 1980's, at that time Ras's role in cellular signaling remained largely unknown. It changed with a key observation from a study using a microinjection of Ras proteins into rat-embryonic fibroblasts that resulted in a remarkably fast activation of phospholipase A, an enzyme that hydrolyzes



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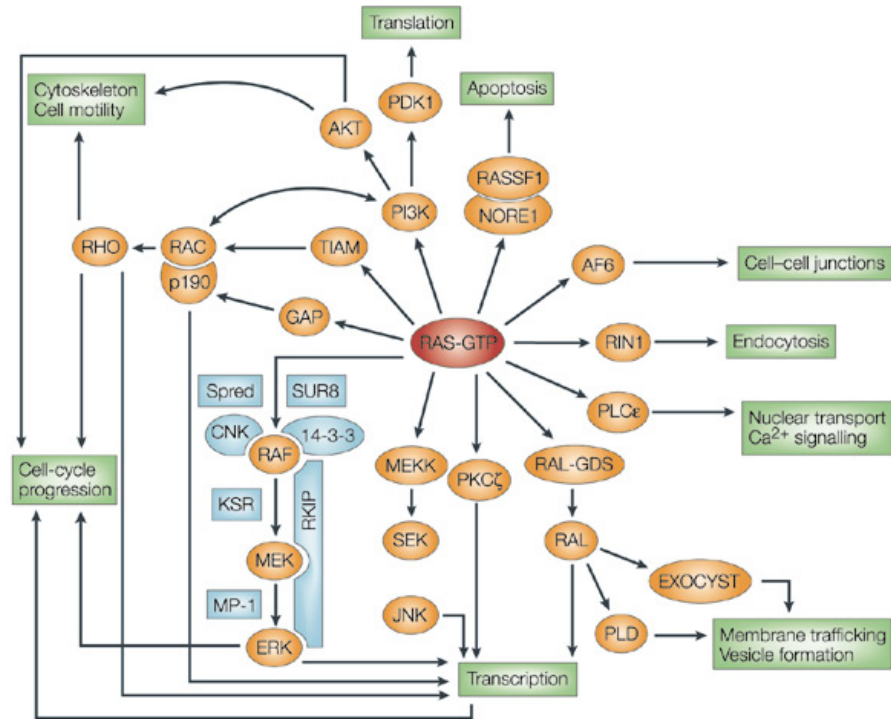
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Figure 4. Ras Farnesylation - All Ras family members contain a CAAX sequence on the C-terminus. A farnesyl pyrophosphate moiety is covalently attached to a cytosolic Ras protein by a farnesyltransferase (FTase). This is followed by the proteolytic cleavage of the last three amino acids of Ras. Finally, the last Cys residue is methylated and a palmitoyltransferase (PTase) transfers a palmitoyl moiety onto the Cys residues slightly upstream of the C-terminus, which facilitates and stabilizes Ras's insertion into the membrane.

phospholipids into fatty acids and is implicated in the production the secondary messenger, arachidonic acid [89]. Subsequently, additional evidence supported the hypothesis that excessive Ras activation leads to oncogenic transformation [90-92]. At the time, it was determined whether these cellular responses were direct downstream reactions to Ras signaling, or pleiotropic effects from over-expression of Ras [90-92]. In 1988, a landmark report was published demonstrating that specific inhibition of a downstream target of Ras disrupts Ras-induced signaling, suggesting that downstream cellular effectors are critical for Ras to induce its biological effects [93]. Thus, the term “Ras Effector” was coined for proteins that Ras acts on to propagate cellular signaling **(Figure 5)**.

The first bona fide mammalian Ras effector identified was the RAF1 Ser/Thr kinase [94-97]. In response to mitogenic stimulation, RAF1 was shown to activate the mitogen-activated protein kinase (MAP Kinase) signal cascade [98]. All three Raf isoforms (cRaf, B-Raf, and A-Raf) are capable of direct activation of the MAPK/extracellular signal-regulated kinases (MEK1/2 kinases) [98]. These, in turn, activate the extracellular signal-regulated kinases (ERK1/2), which subsequently activates the E26-transcription factor proteins (ETS) among other proteins. ETS targets genes involved in cell proliferation and differentiation. However, over-stimulation of ETS targets is tumorigenic and results in an oncogenic transformation. This pathway is a basic template for many signaling cascades that arise at the plasma membrane and terminate in the nucleus. However, exactly how Raf was recruited to the membrane and activated by mitogenic receptors remained a mystery until a yeast two-hybrid study identified Ras and Raf as direct binding partners [96]. Further experiments showed that Ras-GTP binds preferentially with Raf when compared to Ras-GDP, suggesting that Ras is the link that transmits signals from mitogenic receptors into the MAPK pathway, and establishing Raf as a bona fide Ras Effector [96].



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Figure 5. Ras Activated Effectors - Ras activates a wide range of effectors in the cell. While most of these drive growth and proliferation, there are some that activate apoptosis and senescence. The challenge in understanding Ras signaling lies in determining what is the net result of the Ras signal is and how different cellular contexts can change the result of Ras stimulation.

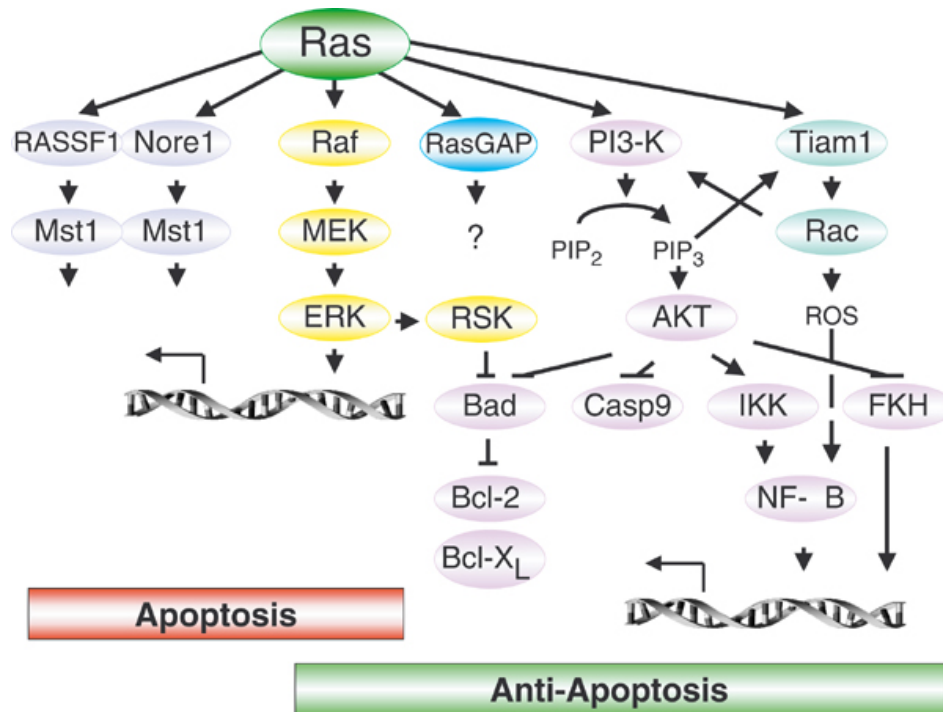
Another Ras effector discovered by a yeast two-hybrid screen is phosphoinositide 3-kinase (PI3K) [99]. PI3K is involved in the activation of AKT as well as regulation of the actin cytoskeleton by growth factors such as PDGF and insulin [100-102]. Just as Ras requires activation of the MAPK pathway to induce transformation, it also requires activation of PI3K to induce transformation of NIH 3T3 cells [99]. These studies laid the groundwork for future experiments concluding that the anti-apoptotic effects of Ras stimulation were driven by the pro-survival activities of PI3K signaling that propagates through a cascade involving the Ser/Thr kinase AKT and the transcription factor NF- κ B (nuclear factor- κ B) [103-105].

Another direct Ras effector, RalGDS (*ral* guanine nucleotide dissociation stimulator), was also discovered using a yeast two-hybrid system in 1993 [106]. RalA and RalB share 46-51% sequence identity with Ras and are members of the Ras superfamily of small GTP-ases [107]. RalGDS was found to specifically catalyze nucleotide exchange on both RalA and Ral B, but not on other small GTP-ases [108]. Activation of RalGDS by Ras results in a cooperative activation of Ral. It is believed that active Ras drives transformation primarily through the binding and activation of RAF, PI3K, and RalGDS. However, Ras can bind to numerous other downstream effectors and modulate many cellular activities including endocytosis, actin organization, cytokinesis, autophagy, gene transcription, and second messenger production [108].

In 2001, phospholipase C- ϵ (PLC ϵ) was identified as a direct effector of Ras [109]. PLC ϵ is a key mediator of both Ca²⁺ signaling and activation of Protein Kinase C (PKC). Activation of PLC ϵ enables it to cleave phosphatidylinositol 4,5-bisphosphate into inositol-1,4,5-triphosphate and diacylglycerol (DAG). This cleavage releases Ca²⁺ and activates PKC. PLC ϵ contains a CDC25 domain, also known as a RasGEF domain. This suggests that molecule upstream of Ras acting as an exchange factor. In fact, PLC ϵ has been shown to activate the Ras-MAPK pathway [110].

About a year passed following the identification of PLC ϵ as a Ras effector before Channing Der and colleagues published a study that TIAM1 (T-cell lymphoma invasion and metastasis-1) is also a member of the Ras effector family [111]. TIAM1, a Rac-specific GEF, preferentially binds GTP-bound Ras through a conserved Ras-binding domain leading to activation of Rac in a PI3K-independent manner [111]. Thus, TIAM1 can operate as an effector that directly mediates the Ras activation of Rac. Rac, much like Ras, is a small GTP-ase signaling protein and has a role in regulating of cell motility, growth, and invasion.

Ras interaction/interference protein-1 (RIN1), acute lymphoblastic leukemia-1 (ALL-1) fused gene on chromosome 6 (AF-6), and the Ras association domain-containing family (RASSF) proteins are some of the lesser-known and more poorly defined Ras effectors. While AF-6 was shown to be involved in mediating transformative cytoskeletal effects of Ras [112], the other two protein families (RIN1 and RASSF) have an unexpected ability to block Ras transformation (**Figure 6**). RIN1 was originally described as a Ras effector in 1991 and was the first Ras binding partner with the ability to block Ras transformation [113, 114]. The affinity of the interaction between Ras and RIN1 is remarkably high and suggests that RIN1 competes with Raf for Ras binding [115]. Other models propose that RIN1 triggers endocytosis of Ras-stimulating growth factor receptors, such as EGFR [116]. Loss of this negative Ras effector has recently been discovered in breast-tumor cell lines, and restoration of RIN1 expression blocked anchorage-independent growth *in vitro* and tumor formation *in vivo* [117]. Similarly to RIN1, loss of expression of another family of negative Ras effectors, the RASSF family, has also been implicated in tumorigenesis.



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Figure 6. Balancing Ras Signaling - Small subset of Ras effectors that show the importance of negative Ras effectors. Perhaps one of the roles of negative Ras effectors is to protect the cell from over-stimulation of Ras by activating pro-apoptotic genes. Accordingly, loss of these negative Ras effectors removes key protection elements against Ras mediated transformation. However, the mechanisms of negative Ras action have not been fully characterized.

1.8 - RASSF Family Proteins

Raf and PI3K interact with Ras through their Ras-binding domains, termed RBD and PI3K-RBD, respectively [118]. However, there is another group of Ras effectors which share a conserved motif identified as the RaIGDS/AF-6 Ras association (RA) domain [119]. This domain is found in RaIGDS, AF6, and more recently in the Ras-association domain family (RASSF). The RASSF family of tumor suppressors is a group of non-catalytic proteins encoded by ten genes, each expressing multiple splice variants [120]. The RASSF family currently consists of RASSF1, RASSF2, RASSF3, RASSF4, NORE1A (RASSF5), RASSF6, RASSF7, and RASSF8. The founding member was the Novel Ras Effector 1A (NORE1A), designated RASSF5, which binds directly to Ras in a GTP-dependent manner [120]. Characterization of RASSF1 proteins followed closely behind that of NORE1A and they received much attention after both of them were identified as important physiological tumor suppressors [121].

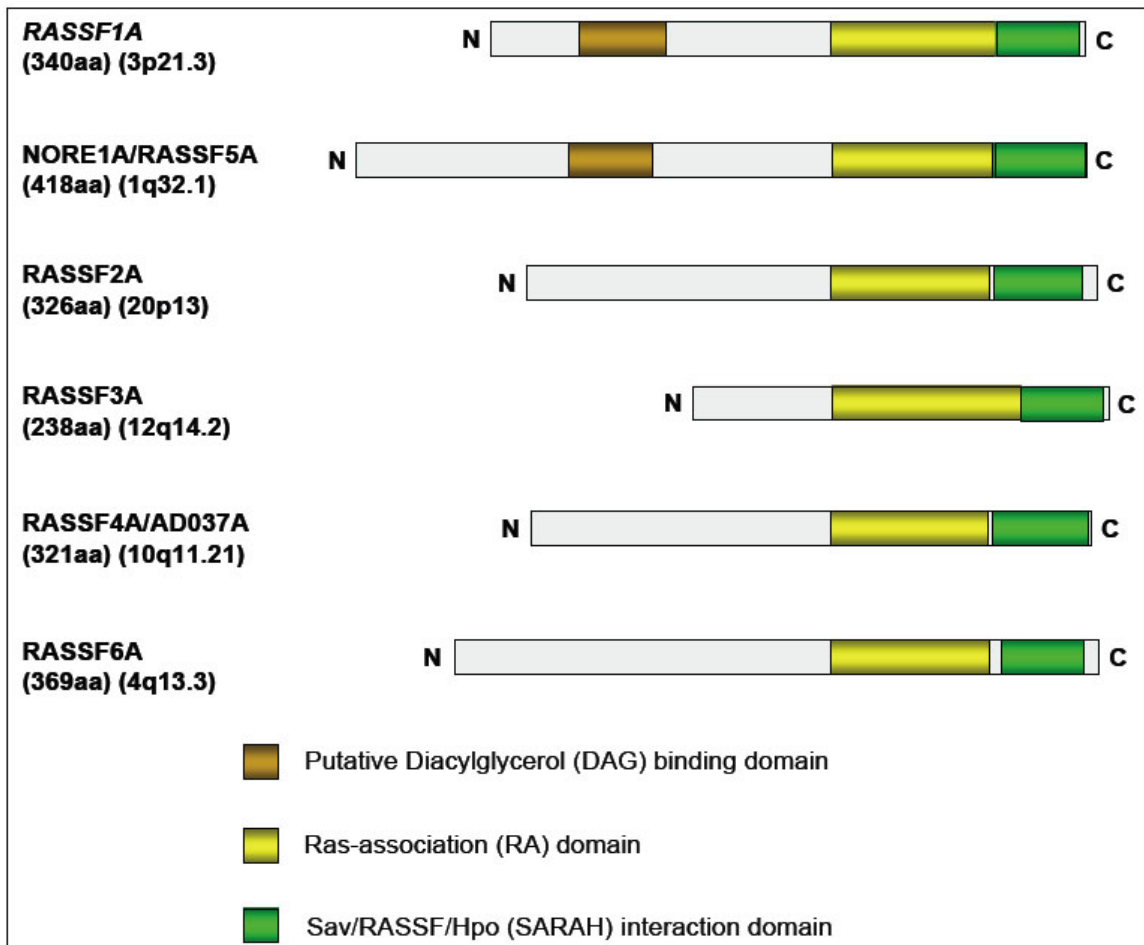
The role of RASSF1A in tumor suppression was first identified when Loss of Heterozygosity (LOH) studies centered on lung, breast, and kidney tumors identified several loci on chromosome 3p that likely contained one or more tumor suppressor genes [118]. Homozygous deletions in lung and breast tumor cell lines narrowed the region of 3p21.3 down to a 120kb region in which 8 genes were coded [122, 123]. The genes CACNA2D2, PL6, 101F6, NPRL2/G21, ZMYND10/BLU, RASSF1/123F2, FUS1, and HYAL2 were each subjected to in-depth genetic analysis; however, these tests failed to identify any mutations [118]. While no candidate point mutations were found, the promoters of the RASSF family were often found to be excessively methylated in tumors, resulting in their suppressed transcription.

Members of the RASSF family of proteins share a set of highly conserved domains that are integral in defining how they are activated and how they function in the

cell. The first 6 members of the RASSF family, RASSF1-RASSF6, all contain a variable N-terminus but share conserved C-terminal motifs containing a Ras-association (RA) domain of the RaIGDS/AF-6 class and a specialized coiled/coil domain named SARA as it mediates interactions with Salvador, RASSF, and Hippo proteins (**Figure 7**) [118]. The RA domain is responsible for interactions with Ras and other small GTP-ases, while the SARA domain mediates protein-protein interactions that are integral in interactions with the Hippo pathway inducing cell cycle arrest and apoptosis [10]. Even though the RASSF family all share considerable structural homology, studies show that individual members of this family perform unique functions in tumor suppression [124]. Although RASSF1A has been the best studied, another member of the RASSF family, RASSF5 or NORE1A, appears to be a powerful tumor suppressor, although its full understanding of its tumor suppressive functions has not been achieved.

1.9 - The Tumor Suppressor NORE1A (RASSF5)

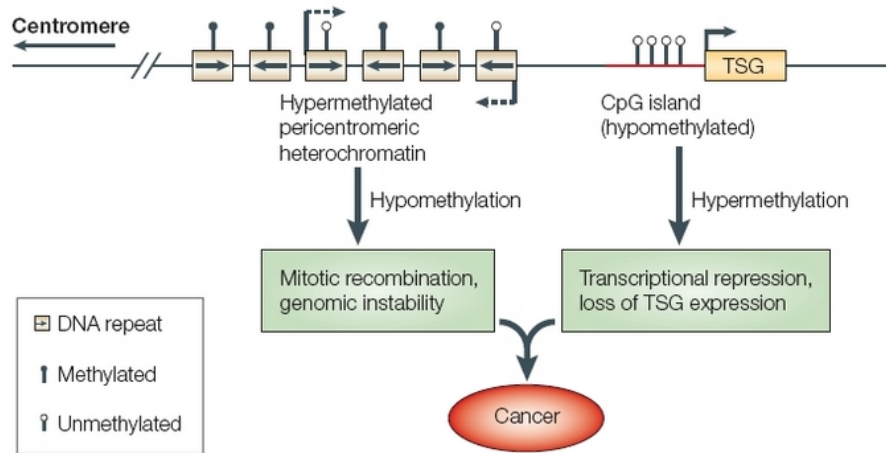
NORE1A (Novel Ras Effector 1 or RASSF5) was the first member of the RASSF family discovered and is roughly 50% identical to the relatively well-characterized RASSF1A tumor suppressor [125, 126]. In human tumors, NORE1A expression is frequently downregulated by promoter hypermethylation or calpain mediated proteolysis (**Figure 8**) [11, 12, 126, 127]. Additionally, the NORE1A gene locus has been reported to undergo a Loss of Heterozygosity (LOH) in some primary tumors [128]. Moreover, loss of NORE1A via a genetic translocation results in a familial human cancer syndrome [15]. Studies on NORE1A *in vitro* show that exogenous expression of NORE1A can promote apoptosis and cell cycle arrest [10, 11, 129]. In human tumor cell lines deficient for NORE1A expression, restoration of endogenous levels of NORE1A



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Figure 7. The RASSF Family of Proteins - The RASSF Family of tumor suppressors is comprised of a group of non-catalytic members encoded by ten genes, each expressing multiple splice variants. The first 6 members of the RASSF family share a set of highly conserved domains which are integral in defining how these proteins are activated and how they function. The first is a Ras-association (RA) domain where Ras associates with these proteins. The second is a specialized coiled-coil structure called a SARAH domain (Salvador-RASSF-Hippo).



Phillips, T. (2008) The role of methylation in gene expression. Nature Education 1(1):116

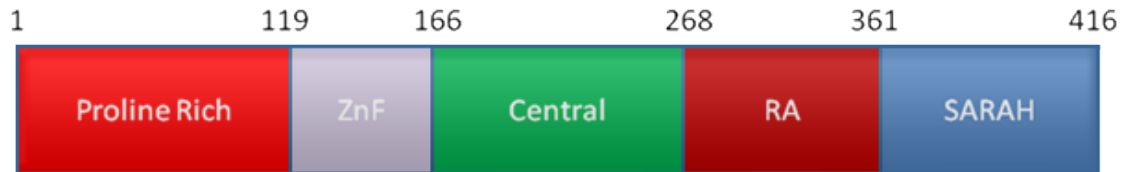
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Figure 8. Methylation of the CpG Islands Silences Gene Expression - This figure shows a representative region of genomic DNA in a normal cell and how methylation can affect transcription. The region shown contains a repeat-rich hypermethylated pericentromeric heterochromatin (Left) and an actively transcribed promoter of a tumor suppressor gene associated with a hypomethylated CpG island (Right). In tumor cells, repeat-rich heterochromatin becomes hypomethylated and this results in genomic instability. Conversely, hypermethylation of CpG islands results in a tumorigenic phenotype by effectively silencing the gene under the control of this promoter sequence.

effectively blocks the tumorigenic phenotype [11]. Based on these results, NORE1A seems to function as a legitimate human tumor suppressor [9, 130, 131].

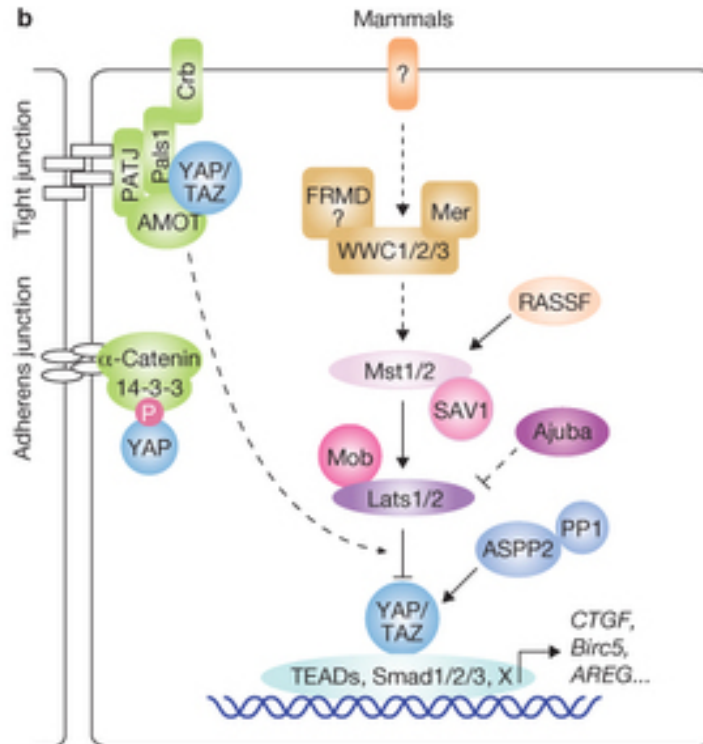
Like other members of the RASSF family, NORE1A contains a Ras Association (RA) domain and was identified as a direct Ras binding protein in a yeast two-hybrid screen (**Figure 9**) [120]. NORE1A and Ras form an endogenous complex in cells, and NORE1A binds to the Ras oncoprotein in a GTP-dependent manner with affinities similar to other known Ras effectors [10, 132]. Therefore, NORE1A is said to be a *bona fide* Ras effector. Mutations in Ras leading to constitutive activation are known to be transforming; however, activated Ras can also actuate pro-apoptotic and senescent pathways [7, 14]. NORE1A has been confirmed as a major Ras senescence/apoptosis effector; however, the mechanisms underlying this activity are poorly understood [10, 14].

Much of the early research on NORE1A was focused on its loss of expression and established a correlation of this event in cancer. In 2004, the first report detailing a mechanism of NORE1A mediated tumor suppression was released [129]. Using yeast two-hybrid screens, NORE1A was shown to bind the MST1/2 kinases [133]. The MST kinases are the core components of the Hippo pathway which is responsible for the regulation of cellular apoptosis and senescence (**Figure 10**) [134]. In the Hippo pathway, MST kinases drive the signaling cascade by activating the LATS kinases which in turn phosphorylate the terminal transcriptional co-activators, YAP and TAZ [134]. The phosphorylation of YAP and TAZ results in their cytoplasmic sequestration and eventual degradation [134]. Loss of YAP or TAZ transcriptional activity results in the induction of apoptosis (YAP) and an irreversible cessation of the cell cycle termed senescence (TAZ) [134].



Schmidt L, C.G., RASSF5 (Ras association (RalGDS/AF-6) domain family member 5). Atlas Genet Cytogenet Oncol Haematol, 2011.

Figure 9. Domains of the NORE1A Protein - The different protein domains of NORE1A are shown above. The N-Terminus is composed of a Proline Rich domain followed by a Cysteine Rich Domain (CRD a.k.a. ZnF) and Central region to the protein. The RA domain is found between residues 268 and 361. This domain binds Ras in a GTP dependent manner. Finally, the C-Terminus contains the SARAH domain where NORE1A interacts with other RASSF proteins and elements of the Hippo signaling pathway.



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Figure 10. The Hippo signaling pathway - The Hippo pathway activates apoptosis/senescence when the terminal transcription co-activators YAP and TAZ are degraded. On stimulation, the MST1/2 kinases phosphorylate the LATS1/2 kinases. These mark YAP and TAZ for recognition and ubiquitination by the ubiquitin ligase, β -TrCP. However, the potential exists for non-canonical regulation of this pathway by other kinases and ubiquitin ligases.

The Avruch group suggested that Ras/NORE1A drives apoptosis by modulating MST kinase activity. Unfortunately, this study unfortunately failed to conclusively show that NORE1A was actually enhancing YAP/TAZ phosphorylation [133]. Ironically, Avruch would later publish work that contradicts this report and shows that NORE1A and RASSF1A do not activate the MST1/2 kinases; instead he hypothesizes that NORE1A and RASSF1A control Hippo signaling by altering substrate availability and localization for MST1/2 activation [135]. Therefore, the role of NORE1A in Hippo signaling remains controversial at this time.

Complicating the issue further, activation of the MST kinases is generally considered an apoptotic event; however, NORE1A appears to focus more on mediating senescence rather than apoptosis [14, 130]. Cellular senescence can be activated by several mechanisms, including activation of p53, Rb, and TAZ [136-138]. My lab recently published that NORE1A induces cellular senescence through control of p53 transcriptional activity via HIPK2 [14]. A close analysis of the data suggests that p53 activation is not the only factor that NORE1A modulates to activate senescence. Perhaps the reason NORE1A is such a potent activator of cellular senescence is because it can activate multiple senescent pathways.

1.10 - NORE1A and Ras in (Oncogene) Induced Senescence

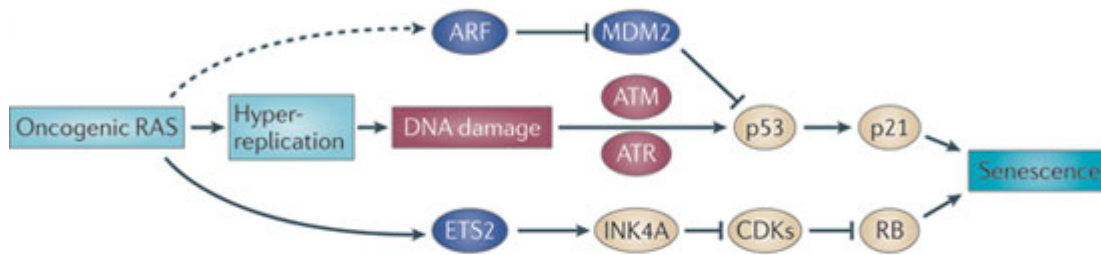
Normal, untransformed cells are not capable of dividing indefinitely because they will eventually undergo a process referred to as cellular senescence [139]. This permanent cell cycle arrest restraining uncontrolled proliferation and tumorigenesis of cells and is thought to be an evolutionarily derived protection in higher eukaryotes, particularly mammals, preventing them from developing cancer [139, 140]. Cells that

have undergone senescence remain metabolically active; however, they exhibit characteristic changes ranging from altered morphology to increased senescence-associated β -galactosidase activity [140]. Senescence was first described in cultured cells that had exhausted their growth potential after a number of passages *in vitro*. In 1997, a study was published showing how ectopic expression of oncogenic H-Ras in normal fibroblasts permanently arrested the cell cycle, suggesting that the senescence response is a fail-safe mechanism protecting the cells from tumorigenic transformation [8, 139].

Oncogene Induced Senescence, abbreviated "OIS", occurs in the G1 phase of the cell cycle and is associated with a significant increase in the protein levels of p53, p21, and p16 [8]. Oncogenic activation of Ras activates p53 inducing the senescent activity of p16 which activates cell-cycle arrest [8]. Previous reports demonstrated that cells lacking functional Rb overexpress p16 [141]. However, these cells were insensitive to p16 mediated cell cycle arrest and continued to proliferate [8]. Therefore, Ras-induced senescence can be bypassed by inactivating the Rb and p53 pathways (**Figure 11**) [142].

Studies indicate that Ras-induced senescence is not an immediate product of Ras signaling, but rather a cellular response to aberrant or excessive Ras activity [8]. When primary human and rodent fibroblasts were microinjected with isolated Ras protein, they proliferated and did not immediately enter senescence until after a lag period lasting about two days post injection [143-145]. This delayed response is consistent with a homeostatic mechanism that suppresses proliferation after prolonged hyper-stimulation by Ras mediated, at least in part, by p53 and p16 [8].

Activating Ras-induced senescence requires a complex web of multiple, independent signaling mechanisms including ATM, p38, Arf, p16, and FOXO [142].



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Figure 11. Ras (Oncogene) Induced Senescence Mechanisms - Ras stimulation in normal cells results in an initial wave of proliferation followed by irreversible growth arrest (senescence). Concurrent with growth arrest, there is accumulation of p53 (via bypass of MDM2 regulation) and p16 (a CKDI that prohibits G1 to S phase transition and enhances Rb function) proteins.

These mechanisms converge on activation of two proteins, p53 and Rb [142]. Yet, activation of p53 and Rb alone is not sufficient to activate cellular senescence [142]. Rb and p53 are activated in quiescent cells, but these cells are very different from senescent cells because their cessation of the cell cycle is reversible. Perhaps this indicates that senescence requires a threshold level of p53 and Rb activity be reached to activate the senescent response [142]. Furthermore, in order for p53 and Rb to achieve this threshold, these proteins potentially require a collaboration of multiple signaling mechanisms [142]. Thus, signal intensity, tissue type, and tissue microenvironment might all be factors determining whether the cell activates or suppresses the senescent response [142].

In normal cells, p53 transcriptional activity increases in response to Ras induced senescence [8]. The mechanism remained unknown until 2009, when Diego Calvisi and colleagues linked the senescent Ras effector, NORE1A, to p53 activation [130]. The study used genomic microarray analysis of HEK-293ecr kidney cells with NORE1A expression artificially manipulated to endogenous levels and found alterations in gene expression that closely mimicked those found with other known tumor suppressors [130]. One target, in particular, was the cyclin-dependent kinase (cdk) inhibitor p21^{CIP1} [130]. Like NORE1A, over-expression of p21^{CIP1} induces G₁ arrest, which made it a potential target for NORE1A mediated signaling [146]. In fact, the study determined that NORE1A actually enhances expression of p21^{CIP1} via the p53 tumor suppressor [130].

While the study by Calvisi and colleagues identified a plausible physiological role in the regulation of cell cycle arrest by p53, a new study identified NORE1A as a key component of Ras driven senescence signaling to p53 [14]. The HIPK2 kinase is a tumor suppressor that performs a wide range of functions within the cell. It has been shown to regulate apoptosis by directly phosphorylating p53 at the S46 residue, enhancing p53's affinity for pro-apoptotic gene promoters [147, 148]. Yet, HIPK2 can

also recruit acetyltransferases CBP/p300 and PCAF which can acetylate p53 and modulate its transcriptional activity [148, 149]. Interplay between acetylation and phosphorylation of p53 likely determines whether p53 activates a more apoptotic or senescent cellular program. CBP/p300 acetylates p53 at residue K382 and PCAF targets K320 [14]. Acetylation at K382 in combination with phosphorylation at S46 shifts p53 into an apoptotic program; however, without S46 phosphorylation, this K382 acetylation shifts p53 towards a cellular senescence program [150, 151]. Furthermore, acetylation of K320 has been reported to enhance p53 association with the p21^{CIP1} promoter, enhancing its expression which results in the activation of cellular senescence [152]. In the presence of Ras/NORE1A signaling, HIPK2 promotes the pro-senescent transcriptional program by acetylating p53 at K382 and suppressing the phosphorylation of p53 at S46 [14]. Moreover, NORE1A induces the pro-senescent acetylation of p53 at K320 [14]. Thus, cellular levels of NORE1A indeed play an important role in defining how the cells quantitatively and qualitatively modulate p53 in response to different stimuli, specifically hyper-active oncogenes [14].

1.11 - Discovering a Novel Role of NORE1A in Tumor Suppression

To gain a better understanding of the mechanism of action of NORE1A, a yeast two-hybrid screen using full length NORE1A as bait identified several potential binding partners. One protein in particular, which scored quite high, was the E3 ubiquitin ligase, β -TrCP. This potential target is intriguing as it is involved in regulating several powerful oncoproteins including I κ B, β -catenin, and MDM2, and its loss of function has been implicated in several cancers [153-156]. In human tumors, β -TrCP is often found over-expressed; however, it fails to properly regulate key oncogenic pathways including NF-

κ B and Wnt/ β -catenin [157]. Adding to this, mutations are rarely, if ever, found in β -TrCP [158]. Thus, other factors that affect β -TrCP ubiquitin ligase function including, but not limited to, the absence of adapter molecules, loss of cooperating kinases, and/or loss of protein scaffolding molecules are likely absent in cancers. Therefore, we hypothesize that NORE1A is a central element involved in β -TrCP mediated protein ubiquitination and serves as a scaffolding molecule stabilizing collaborating elements to β -TrCP, including the kinase, GSK-3 β .

This dissertation will present data showing that NORE1A does in fact form a direct, endogenous complex with β -TrCP. This complex results in the efficient ubiquitination and degradation of the Wnt signaling endpoint, β -catenin. NORE1A also forms an endogenous complex with the GSK-3 β kinase, which is responsible for the phosphorylation of substrates, marking them for recognition by the β -TrCP ubiquitin ligase complex. A mutant of NORE1A that is unable to bind GSK-3 β fails to efficiently regulate β -catenin, suggesting that NORE1A is serving as a scaffold molecule for GSK-3 β and β -TrCP. Additionally, I will show data demonstrating that NORE1A can “split the Hippo” by specifically regulating TAZ over its closely related partner, YAP. In Hippo signaling, activation of the MST kinase cascade results in the ubiquitination and degradation of the terminal transcriptional co-activators YAP and TAZ. However, newer research reported that GSK-3 β can specifically phosphorylate one of the Hippo pathway endpoints, TAZ. While NORE1A was initially implicated in Hippo signaling via interaction with the MST kinases, a mutant of NORE1A that fails to bind MST still effectively regulates TAZ protein levels in the cell. Thus, the NORE1A mediated regulation of TAZ can occur outside of canonical Hippo signaling. Furthermore, the mutant of NORE1A that fails to bind GSK-3 β fails to regulate TAZ, suggesting that NORE1A is also working with GSK-3 β and β -TrCP to effectively control TAZ. Suppression of TAZ protein levels is

known to be a senescent event, so this novel Ras/NORE1A signaling mechanism provides another way for NORE1A to drive oncogene induced senescence. Finally, data will show that NORE1A is a scaffold for GSK-3 β and β -TrCP, stabilizing them into a novel protein regulatory complex. Subsequent loss of NORE1A results in an impairment of this complex, and knockdown of NORE1A results in increased steady state levels of both β -catenin and TAZ protein levels, resulting in a more aggressive oncogenic phenotype.

CHAPTER II

MATERIALS AND METHODS

2.1 - Overview

Isolating and characterizing the growth suppressive functions of important human tumor suppressor proteins presents several challenges. These challenges are compounded by the fact that many signaling pathways have a significant amount of overlap and cross talk. However, I designed a set of experiments that isolate these signaling elements and provide key insight into these signaling networks. Work detailed in this dissertation employs a wide variety of molecular biology and cell/tissue culture techniques. The goal of these experimental methods is to identify and further and define the function of the negative Ras effector NORE1A in human tumor suppression. This section will review these materials and methodology in detail. These discussions will be grouped in different classes of procedures including molecular biology, cell culture, and biological assays used in this study.

2.2 – Molecular Biology

Polymerase Chain Reaction – DNA amplification for sub-cloning purposes was performed using a standard thermocycler by Bio-Rad (Model T100). PCR reactions were performed using standard PCR master mixes (Invitrogen) with the Platinum Taq

Polymerase (Invitrogen). Reaction parameters consisted of a 5 minute segment at 95°C to perform the initial melting of the DNA. Then the reactions were cycled at 95°C for 1 minute, annealing temperatures between 55° and 62°, and 72°C for 1 min/1kb amplified for 35 cycles. In each case, the annealing temperatures varied slightly with respect to the specific primers individual predicted annealing temperatures. After the cycling, the reactions were allowed to undergo the elongation state at 72°C for 7 minutes and then the reaction was held at 4°C until further processed.

TA/TOPO Cloning – Amplified DNA fragments were subcloned into pCR2.1-TOPO (Invitrogen) or pGEM-T-EASY (Promega) as each specific protocol dictated. All PCR reactions were sequenced after TA/TOPO cloning to ensure no errors were introduced by the Taq polymerase during the PCR amplification step.

Restriction Enzyme Digests – DNA subcloning requires restriction enzyme digests to allow the alteration of recombinant DNA plasmids. All enzymes used are from New England Biolabs and were used in reactions consisting of DNA, proper prescribed buffers (as required), BSA (as required), and enzymes. Restriction digests were allowed to incubate for 1 hour at the proper temperature which was most commonly 37°C, yet some enzymes require 25°C.

DNA Ligation – To recombine DNA fragments together, a DNA ligation reaction was used. In order for two DNA fragments to be joined together, they must be digested with the same restriction enzymes. After they are digested and purified, the two DNA fragments are added to the reaction along with a 10x ligation buffer (NEB) diluted to a final concentration of 1x. T4 DNA ligase (NEB) was then added to the reaction and the reaction was allowed to sit overnight at 4°C.

Agarose Gel Electrophoresis – DNA from PCR and restriction enzyme digests were run on 1% agarose gels to separate the linearized fragments by size. Agarose gels were made by adding powdered agarose (SeaKem LE agarose from Lonza Cat#50004) to a concentration of 1% by weight into a 1x TAE (Tris-Base, Acetic Acid, EDTA) solution (Diluted from a 10x TAE solution purchased from MediaTech Cat#46-010-CM). Ethidium Bromide solution (Invitrogen) was then added to the gel solution after melting and before casting to allow for the visualization of DNA. Gels were then exposed to DC electric current at roughly 80mA (110V) allowing proper separation of DNA fragments. In some instances, retrieval of DNA from the agarose gel was required. To achieve this, the DNA bands were excised and placed into a GenEleute Column (Sigma Cat# 56500) and processed as the protocol prescribed.

Bacterial Transformation – Chemically competent bacteria were procured from Invitrogen and I used several types of different competency levels including subcloning efficiency DH5 α , Max Efficiency DH5 α , and Chemically and Electrically Competent TOP10 *E. coli* (Invitrogen Cat# 18265-017, 18258-012, C5050-03, and K4580-01). For standard whole plasmid transformation, it is more cost effective to use the subcloning efficiency DH5 α . However, when performing plasmid ligations and TOPO/TA cloning, it is best to use a higher competency bacterium (Max Efficiency or TOP10 *E. coli*) as the mass of complete ligated plasmid is much lower than that of a whole plasmid transformation. To chemically transform a plasmid into chemically competent bacteria, the bacteria was mixed with 200ng up to 1 μ g of plasmid DNA on ice for 30 minutes. The mixture was then transferred into a 42°C water bath for 30 seconds. After the heat shock, the bacteria/plasmid mixture was returned to the ice for 2 minutes. 250 μ L of

SOC media (Corning Cellgro Cat# 46-003-CR) was then added to the mixture and placed at 37°C for 1 hour. After the outgrowth stage, the mixture was plated on LB agar plates containing antibiotics (typically Ampicillin at a final concentration of 100µg/ML or Kanamycin at a final concentration of 50µg/mL) matching the resistance markers on the transformed plasmid. The plates were incubated at 37°C overnight. The next day colonies were selected and grown in LB broth media, again supplemented with the proper antibiotic at the correct final concentrations listed above, and once again incubated overnight at 37°C in a shaker set at 300 RPM. After the colonies were expanded and grown in the LB media, the cultures were processed and the plasmids were extracted and purified from the bacteria as described below.

Plasmid Purification (Preparation) – DNA plasmids were extracted and purified from *E. coli* cultures using kits from Qiagen (Mini-Scale Kit Cat# 27106 and Midi-Scale Kit Cat# 69506 and Midi-Scale Plasmid Kit by Sigma Aldrich Cat# NA0200-1KT). Mini scale plasmid preparations started with a 5mL culture inoculated from a single bacterial colony. This inoculation was grown overnight in LB media at 37°C in proper antibiotics per the plasmids resistance marker. The entire culture was then pelleted using a bucket centrifuge spun at 5000 RPM for 15 minutes. Following centrifugation, the media was decanted and the entire pellet resuspended in buffer provided by the kit. From there, the plasmid extraction and purification were carried out according to manufacturer's protocol. For Midi-Scale plasmid extractions and purifications, a single colony was isolated from an LB agar plate and grown for 8 hours in 5mL of LB media containing proper antibiotics at 37°C. The entire culture was then added to 50mL of fresh LB media containing necessary antibiotics and incubated overnight at 37°C. The next day, the culture was centrifuged at 5000 RPM for 20 minutes. The supernatant was decanted

and the remaining pellet was then processed according to the specific Midi-Scale kit's protocol.

2.3 – Plasmids

pCR2.1-TOPO – This TA cloning system purchased from Invitrogen has a TOPO-Isomerase attached to each end of the pCR2.1 DNA plasmid. Isolated PCR fragments can be inserted into this vector due to the fact that the Taq polymerase leaves an “A” overhang on polymerized DNA fragments. The vector is designed with “T” overhangs allowing for base pair recognition and subsequent isomerization with TOPO to seal PCR fragments into the plasmid. Colonies can be selected with Blue/White x-gal screenings to aid in the selection of positive transformants.

pGEM-T-Easy – Working similarly to pCR2.1-TOPO, this system employs a T-4 DNA ligase instead of a bound isomerase. In some instances, this TA vector system was more helpful as pCR2.1-TOPO has an internal BglII site, which drops a non-specific 1.5kb band when digested with BglII that sometimes interferes with the retrieval of inserts with BglII cloning sites. pGEM-T-Easy contains no such BglII site, and allows for easier cloning of elements requiring BglII restriction digests.

pEGFP-C1 – This mammalian expression construct is driven by a CMV promoter and allows for an N-Terminal GFP (Green Fluorescent Protein) tag to be fused to cDNAs inserted into the multiple cloning site. We purchased this plasmid from Clontech.

pmKate2-C – This plasmid backbone is practically a base for base copy of pEGFP-C1 except this expression construct expresses an RFP (Red Fluorescent Protein) tag fused with cDNAs inserted into its multiple cloning site. This construct was purchased from Evrogen.

pCDNA3 – One of the more ubiquitous mammalian expression plasmids used in research today, this construct was purchased from Invitrogen. It pairs a CMV promoter upstream on a multiple cloning site. One of its downfalls is that it does not have a segment that expresses a fused epitope tag. Thus, the need to create a pCDNA3 construct with fused epitope tags would become useful in future experiments.

pCDNA3-HA – Using annealed polylinkers inserted into the multiple cloning site of pCDNA3, the construct now was altered to have an in-frame BamHI fused to a 5'-HA epitope tag which allows for insertion of genes by a BamHI/EcoRI digest. Expression of inserted genes results in a fused HA tag on the N-terminus of proteins.

pCDNA3-Flag – Similarly to the pCDNA-HA plasmid, the pCDNA-Flag expression construct was created in much the same way, except the inserted polylinker expresses the Flag epitope tag. The construct allows for inserts to receive an N-terminal Flag tag fused to any expressed protein that is in frame with the 5' BamHI restriction site. Again, this construct is designed for inserts to be ligated by restriction digests of BamHI/EcoRI.

NORE1A – Full length human *NORE1A* cDNA was obtained from Origene (Rockville, MD). The *NORE1A* cDNA clone was then PCR amplified with primers “hNore1a5” with the sequence 5’ – GCAGATCTATGGCCATGGCGTCCCCGGCCATC – 3’ and “hNore1a3” with the sequence 5’ – GCGAATTCTTACCCAGGTTTGCCCTGGGATTC – 3’ to yield a 1273 base pair DNA fragment with 5’-BglII and 3’-EcoRI restriction sites. The fragment was TOPO cloned into pCR2.1-TOPO for sequencing and subcloning applications. Post-analytical confirmation, the pCR2.1-TOPO-*NORE1A* plasmid was digested with BglII and EcoRI and ligated into a BamHI/EcoRI digest of a pCDNA3 (Invitrogen) mammalian expression construct containing an in-frame 5’-HA tag as well as a pCDNA3 construct containing an in-frame 5’-Flag epitope tag. The BglII/EcoRI digested *NORE1A* fragment was also ligated into both GFP (pEGFP-C1) and RFP (pmKate2-C) digested with BglII and EcoRI.

NORE1A 92-94A Mutant – I developed a triple point mutant of *NORE1A* mutating the 92nd to 94th amino acids from Arg to Ala. To achieve this, two separate *NORE1A* fragments were created and ligated together disrupting the 92-94th amino acids. The first fragment, termed “Upper Fragment” was PCR amplified from the full length *NORE1A* cDNA with primers “hNore1a5” and “Nore3’RMut” with sequence 5’ – CTGAGCTCCAGGCGCTGCGGCCAGTCTCTGCTGCAGACCAGG – 3’. This yielded a 297 base pair DNA fragment was TOPO cloned into pCR2.1-TOPO for sequencing and subcloning applications. The “Lower Fragment” was amplified using primers “Nore5’RMut” with sequence 5’ – CTGGAGCTCCCCGACCCCGCGACGTG – 3’ and “hNore1a3” yielding a 982 base pair band. This fragment was also TOPO cloned into pCR2.1-TOPO for sequencing and subcloning applications. The mutation was made using primer over-write from the Nore3’RMut primer which made the mutation and

installed a SacI restriction site. To complete the mutated cDNA, the Upper Fragment was ligated into pEGFP-C1 using BglII and SacI. The Lower Fragment was then inserted into pEGFP-C1-NORE1A-RMut-UpperFragment by restriction digests with SacI and EcoRI and subsequent T4 DNA ligations. Combining the Upper and Lower Fragments allowed for the mutation of the 92-94th amino acids and completion of the cDNA. The completed NORE1A92-94A mutant was then subcloned from pEGFP-C1-hNORE1A92-94A Mutant (BglII/EcoRI) into pCDNA3-HA (BamHI/EcoRI), pCDNA-Flag (BamHI/EcoRI), and pmKate-2C (BglII/EcoRI).

NORE1AΔSARAH – The SARAH domain of NORE1A lies on the C-terminal end of the protein. To remove this domain, full length NORE1A was PCR amplified with primers “hNore1a5” and “hNore1aDeltaSARAH3” with sequence of 5’ – GCGAATTCTTATTAATTCTCCTTTAGCACAAAGC – 3’. The resulting DNA fragment was then TA cloned into pGEM-T-Easy for sequence analysis and subcloning applications. After sequence analysis showed that the polymerase produced an error free product, the pGEM-T-Easy-NORE1AΔSARAH was digested with BglII and EcoRI and ligated into pCDNA-HA (BamHI/EcoRI), pCDNA-Flag (BamHI/EcoRI), pEGFP-C1 (BglII/EcoRI), and pmKate2-C (BglII/EcoRI).

H-Ras12Val – Constitutively activated H-Ras was expressed with two different tags. pCGN-HA-H-Ras12V was created by cloning the full-length H-Ras12V cDNA as a BamHI/EcoRI fragment into the pCGN-HA vector [159]. This cDNA fragment was also sub-cloned into pmKate-2-C using that vector’s BglII/EcoRI sites yielding the pmKate-2-C-H-Ras12V expression construct [131].

shNORE1A – NORE1A shRNA constructs (Cat# RHS4531-EG83593) were obtained from Open Biosystems (Rockford, IL).

β-catenin – GFP-β-catenin was generated by a PCR amplification of the human β-catenin cDNA (Addgene #16828) with primers “B-Cat5’BamHI” with sequence 5’ – GCAGGATCCATGGCTACTCAAGCTGATTTG – 3’ and “B-Cat3’Sall” with sequence 5’ – GCGTCGACCTTACAGGTCAGTATCAAACCAG – 3’ making a 2.7kb PCR fragment. The PCR fragment was TA cloned into pGEM-T-Easy (Promega) for sequencing and subcloning applications. After sequence verification, pGEM-T-Easy-β-catenin was digested with BamHI and Sall and ligated into pEGFP-C1 using the same restriction sites.

β-TrCP - β-TrCP human cDNA was obtained from Addgene (#4489). Using primers “B-TrCP5’BamHI” with sequence 5’ – GCGGATCCATGGACCCGGCCGAGGCGGT – 3’ and “B-TrCP3’Sall” with sequence 5’ – GCGTCGACTTATCTGGAGATGTAGGTGTATGTTTCG – 3’, the cDNA clone from addgene was PCR amplified and the product was TA cloned into pGEM-T-Easy for sequence confirmation and subcloning applications. After sequence verification, the pGEM-T-Easy-β-TrCP plasmid was digested with BamHI and Sall and ligated to pEGFP-C1 using the same restriction sites.

β-TrCPΔFBOX – The dominant negative β-TrCP, GFP-β-TrCPΔFBOX, was a generous gift from Dr. Tianyan Gao, Associate Professor of Molecular & Cellular Biology at the University of Kentucky, who kindly shared his plasmid with us.

TAZ – We procured the full length, wild-type HA-TAZ mammalian expression plasmid from Addgene (#32839).

YAP – We purchased a YAP1 expression construct, pEGFP-C3-YAP1, from Addgene (#17843) and an RFP-YAP2 was a generous gift from Dr. Howard Donninger, Assistant Professor of Medicine at the University of Louisville.

GSK-3β - We obtained the HA-GSK-3β and an un-repressible HA-GSK-3β-S9 mammalian expression constructs from Addgene (#14753 and #49491).

pGL3-BAR-Luc – This luciferase reporter construct containing a β-catenin Activating Region (BAR) promoting element upstream of a luciferin response element allowed us to examine β-catenin transcriptional activity in cell systems. This plasmid was a kind gift from Randall Moon at the University of Washington, WA.

8xGTIIIC-Luc – This reporter for TEAD transcriptional activity was obtained from Addgene (#34615) and was used to examine YAP and TAZ transcriptional activation of the TEAD transcription factor.

TK-renilla – The renilla luciferase control plasmid was obtained from Promega (Madison, WI). This construct was co-transfected with all luciferase reporters to provide a readout for transfection efficiency in luciferase assays as the Renilla reporter is constitutively expressed in mammalian cells. Furthermore, while the main reporter plasmid responds to the luciferase reaction buffer, renilla does not. Instead, the TK-renilla construct reacts to the “stop and glo” reagent, allowing for the use of a dual reporter system and separate RLU readouts contained in the same reaction mix.

2.4 – Antibodies

Mouse-Anti-HA – The antibody against the HA epitope tag was purchased from Covance (Cat# MMS-101P). For detection of proteins in on a Western blot, the antibody was diluted at 1:5000 in 5%Milk/TBST (TBS-Tween - 50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween 20) and the blot was incubated in the antibody solution overnight at 4°C. The blots were then probed with a Sheep-Mouse-IgG-HRP (Amersham Cat# NA931-1ML) secondary antibody diluted at 1:20000 in TBST for one hour at room temperature and detected by West Pico Enhanced Chemi-Luminescence (Pierce Cat#34080).

Mouse-Anti-GFP – The antibody detecting the GFP-epitope tag was purchased from Santa Cruz Bio-Technology (Cat# SC-9996). For detection of proteins on a Western blot containing a GFP epitope tag, the GFP-antibody was diluted at 1:200 in a 5%Milk/TBST solution. The blot was incubated in the antibody solution overnight at 4°C. After incubation in the primary antibody, the Western blot was exposed to a Mouse-IgG-HRP

secondary (Amersham) at a dilution of 1:15000 in TBST for 1 hour at room temperature and detected by West Pico ECL.

Rabbit-Anti-RFP (Kate) – For detection of the Katushka (pmKate-2-C RFP Epitope Tag), I used the Anti-tRFP antibody from Evrogen (Cat#AB233,234). This rabbit polyclonal antibody was incubated overnight at 4°C at a dilution of 1:2000 in a 5% BSA/TBST solution. The blots were then probed with a Goat Anti-Rabbit secondary antibody (KPL) at a dilution of 1:10000 in TBST for 1 hour at room temperature and detected using West Pico ECL (Pierce).

Mouse-Anti-Flag – For detection of the Flag epitope tag, I used an Anti-Flag primary antibody was purchased from Sigma Aldrich (Cat# F1804). This antibody was diluted at 1:1000 in a 5%Milk/TBST solution and incubated on the Western blot overnight at 4°C. After incubation in the primary antibody, the Western blot was exposed to a Mouse-IgG-HRP secondary (Amersham) antibody at a dilution of 1:15000 in TBST for 1 hour at room temperature and detected by West Pico ECL.

Rabbit-Anti-Human- β -TrCP – Detection of endogenous β -TrCP was performed by an Anti- β -TrCP primary antibody purchased from Cell Signaling Technology (Cat# 4394). Western blots were incubated in the Anti- β -TrCP antibody at a dilution of 1:2500 in a 5% BSA (Sigma Aldrich Cat# A3059-100G)/TBST solution overnight at 4°C. After incubation in the primary antibody, the blot was then exposed to a Goat Anti-Rabbit-HRP secondary antibody (KPL Cat# 374-1506) at a dilution of 1:20000 in TBST for 1 hour at room temperature. The blot was developed using West Pico ECL.

Rabbit-Anti-Human- β -catenin – Endogenous β -catenin proteins were detected on Western blots using an Anti- β -catenin antibody from Cell Signaling Technology (Cat# 8814). The antibody was diluted into a 5%BSA/TBST solution at 1:2000 and incubated on the blot overnight at 4°C. The blot was then exposed to a Goat Anti-Rabbit-HRP secondary antibody (KPL) at a dilution of 1:20000 in TBST for 1 hour at room temperature and developed using West Pico ECL.

Rabbit-Anti-Human-phospho- β -catenin (Ser33/37 Thr41) – To detect the fraction of β -catenin that was phosphorylated by GSK-3 β , I used an antibody against Phospho- β -catenin on residues Ser33/37 and Thr41 purchased from Cell Signaling Technology (Cat# 9561). Western blots were exposed to this antibody diluted at 1:2500 in a 5%BSA/TBST solution overnight at 4°C. The Goat Anti-Rabbit-HRP was subsequently applied at a dilution of 1:20000 in TBST for 1 hour at room temperature and the blot was detected using West Pico ECL.

Mouse-Anti-NORE1A – Monoclonal NORE1A antibodies were a gift from A. Khokhlatchev (University of Virginia, Richmond, VA) and were raised against human NORE1A amino acid residues 119-416. This antibody was used for detection of NORE1A on Western blots and also for immunoprecipitations (IP). For Western blot detection, the antibody was added to a 5% Milk/TBST solution at a 1:500 dilution and incubated with the blot overnight at 4°C. The blot was then probed with a mouse-IgG-HRP (Amersham) secondary antibody for 1 hour and detected with a West Pico ECL. For immunoprecipitations, 1mg of whole cell lysate was incubated with 10 μ L of the antibody in 1mL final volume of modified RIPA buffer overnight and then

immunoprecipitated on Mouse conjugated agarose beads. For full IP protocol, see section “2.7 - Immunoprecipitations”.

Rabbit-Anti-Human-GSK-3 β - Endogenous GSK-3 β was detected on a Western blot by an antibody from Cell Signaling Technology (Cat#9315) diluted 1:2000 in a 5%BSA/TBST solution. The antibody solution was incubated with the Western blot overnight at 4°C followed by exposure to a Goat Anti-Rabbit-HRP secondary antibody diluted at 1:15000 in TBST for 1 hour at room temperature and detected using West Pico ECL.

Rabbit-Anti-Human-TAZ – When Western blotting for endogenous levels of TAZ protein, an Anti-TAZ antibody purchased from Cell Signaling Technology (Cat# 4883) was found to perform well. This antibody was diluted at 1:1500 in a 5%BSA/TBST solution and incubated with the blot overnight at 4°C. Following the overnight incubation, a Goat Anti-Rabbit-HRP secondary antibody was added at a dilution of 1:20000 in TBST for 1 hour at room temperature and the blot was detected using West Pico ECL.

Rat-Anti-Mouse-HRP TrueBlot Secondary Antibody – Rockland’s TrueBlot product line is an HRP conjugated immunoblotting detection reagent that does not detect heavy and light chain IgG bands. This technology is very useful when using agarose-conjugated beads that are bound with mouse or rabbit secondary antibodies and yields a much cleaner blot. In some instances, this secondary antibody was used instead of the standard HRP-mouse secondary antibody listed above. When the Mouse TrueBlot (Rockland Cat# 18-8817-33) antibody was used, it diluted at a 1:2000 dilution in a 5% Milk/TBST solution and incubated on the blot for 2 hours and detected with the West Pico ECL.

Mouse-Anti-Rabbit-HRP TrueBlot Secondary Antibody – Used in similar applications to the Mouse TrueBlot secondary antibody, this Rabbit-HRP (Rockland Cat#18-8816-33) secondary also does not detect the heavy and light chain IgG from Rabbit secondary antibody conjugated agarose beads. It is also used at a 1:2000 dilution in 5% Milk/TBST solution and exposed on the blot for 2 hours and developed with West Pico ECL.

2.5 – Cell Lines

HEK-293 – This cell line composed of human embryonic kidney cells was derived from an aborted human embryo and acquired from ATCC. These cells transfect very easily and allow for a nice functional platform for transgenic experimentation. This line is cultured in DMEM (explained in the next section) supplemented with 10% FBS (Fetal Bovine Serum).

HEK-293T – The HEK-293T cells are derived from HEK-293 cells, but they have been altered to stably express the SV40 Large T Antigen that can bind SV40 enhancers commonly found in expression vectors to increase protein production. We purchased these cells from ATCC. The Large T Antigen also suppresses p53 and Rb tumor suppressive activity allowing for a more tolerable phenotype to transgenic studies. These cells are cultured in the same way as the HEK-293 cells.

NCI-H1299 – Also known as H1299, this cell line is a human non-small cell lung carcinoma derived from a lymph node and we purchased this line from ATCC. Similarly to other immortalized cells, the H1299 line can divide indefinitely. These cells have a homozygous partial deletion of the p53 gene, and thus do not express the p53 tumor

suppressor. Furthermore, this cell line also contains a point mutant NRAS-Q61K resulting in a constitutively activated Ras signaling phenotype. These cells are cultured in RPMI cell growth medium supplemented with 10% FBS.

A549 – These cells are adenocarcinomic human alveolar basal epithelial cells derived from the removal and culture of cancerous lung tissue and acquired from ATCC. This line also contains a point mutation in KRAS (G12S) resulting in the constitutively activation of KRAS signaling. This line, however, was ideal for studies on cellular senescence as they are wild-type for p53 and Rb signaling, two elements known to be necessary for the induction of senescence. These cells are cultured in DMEM supplemented with 10% FBS.

HBEC-3KT – These normal Human Bronchial Epithelial Cells were a generous gift from Dr. Jerry Shay, The Southland Financial Corporation Distinguished Chair in Genetics, Department of Cell Biology, University of Texas Southwestern Medical Center, and were used to examine how knockdown of specific members of the RASSF family of tumor suppressors along with Ras stimulation results in oncogenic cellular transformation [14]. These cells require a special growth medium and I selected Keratinocyte-SFM medium purchased from Invitrogen.

MCF-10A – This cell line is a non-tumorigenic epithelial cell line derived from human mammary gland/breast tissue. These cells are immortalized without the use of viral oncogenes giving them primary cell phenotypes. I used this cell line extensively to perform endogenous co-immunoprecipitations of proteins and makes for an excellent system to examine how loss of tumor suppressors along with Ras stimulation can result in an oncogenic phenotype in several different biological assays. These cells are

responsive to insulin, glucocorticoids, cholera enterotoxin, and epidermal growth factors. MCF-10A require a specialized growth media and were cultured in a 50%/50% mixture of DMEM and Ham's Nutrient Mixture F-12 supplemented with horse serum, insulin, and EGF.

2.6 – Cell Culture and Transfections

All cell culture was processed inside a laminar flow biological safety cabinet per protocol from the Department of Environmental Health and Safety, the University of Louisville, and the Occupational Safety and Health Administration. All cells were cultured on T-75 and T-25 cell culture flasks (TPP), 100mm and 60mm cell culture dishes (Greiner Bio-One), and 6, 12, and 24 well cell culture plates (Greiner Bio-One). Different cells cultured sometimes require different culture media as previously listed. The descriptions of the media used is as follows:

DMEM – Dulbecco's Modified Eagle's Medium was purchased from Corning Cellgro (Cat# 10-013-CV) and is composed of 4.5g/L glucose and supplemented with L-glutamine and pyruvate. The DMEM was further supplemented with 10% FBS (Valley Biomedical) and 1% Pen-Strep Antibiotic (Corning Cellgro Cat# 30-002-CI).

RPMI – RPMI 1640 cell culture medium was purchased from Corning Cellgro (Cat# 10-040-CV) with L-glutamine. This medium was further supplemented with 10% FBS (Valley Biomedical) and 1% Pen-Strep Antibiotic (Corning Cellgro).

Keratinocyte-SFM – HBEC cells require a special growth media formulation and I purchased this medium designed for keratinocytes from Invitrogen (Cat# 17005-042).

This kit contains Keratinocyte-SFM medium (Cat# 10724-011) which was supplemented with provided supplements 25mg Bovine Pituitary Extract (BPE) (Cat# 13028-014), 2.5µg human recombinant EGF (Cat# 10450-013), and 1% Pen-Strep Antibiotic (Corning Cellgro).

DMEM/Ham's-F12 50/50 Mix – This media is a 50%/50% mixture of two different cell culture growth mediums, DMEM and Ham's Nutrient Mixture F-12 and was purchased from Corning Cellgro (Cat# 15-090-CV). This medium was further supplemented with 5% horse serum (Sigma Aldrich Cat# H1270), human insulin (Sigma Aldrich Cat# I9278-1ML) to a final concentration of 10ug/mL, and recombinant epidermal growth factor (Invitrogen Cat# PHG0311) to a final concentration of 10ng/mL, and hydrocortisone (Sigma Aldrich Cat# H6909) to a final concentration of 0.5ug/mL.

PBS – Phosphate Buffered Saline was purchased from Corning Cellgro (Cat# 21-040-CV and #46-013-CM) and used to wash cells and other routine cell culture applications as required.

Trypsin EDTA 0.25% - We purchased this trypsin solution (Corning Cellgro Cat# 25-053-CI) to remove adherent cells from culture dishes and flasks. Media was removed by aspiration from cell culture dishes and washed once with 1xPBS. The PBS was then aspirated and the trypsin-EDTA solution was then added, covering the bottom of the dish with a thin layer of solution. The dishes were then placed back into the incubator for 3-5 minutes allowing the trypsin to remove adherent cells. After the cells were removed from the flask, the trypsin-EDTA was neutralized with supplemented culture medium and the cells were pelleted by centrifugation (5 minutes at 1500 RPM). The cell pellets were resuspended in culture medium and plated as the application required.

RIPA Buffer – For applications where we were not investigating protein/protein complexes, I lysed cells in a RIPA Buffer (Sigma Aldrich Cat# R0278). This buffer contains 150mM NaCl, 1.0% IGEPAL® CA630, 0.5% sodium deoxycholate, 0.1% SDS, and 50mM Tris pH 8.0. This buffer allows for efficient cell lysis and exceptional protein stabilization for analysis on a Western blot.

G418 Sulfate – For selection of cells expressing a G418 resistance marker, G418 sulfate (Corning Cellgro Cat# 30-234-CR) was added to growth media at a final concentration of 500µg/mL.

Puromycin – For selection of cells expressing a puromycin resistance marker, Puromycin dihydrochloride (Sigma Aldrich Cat# P8833-10MG) was added to cell culture media at a final concentration of 1µg/mL

MG132 – To inhibit the 26S proteasome, cells can be treated with a chemical named MG132 (Sigma Aldrich Cat# C2211-5MG). To make a working stock of MG132, stock powder was dissolved in DMSO to a final concentration of 10mM and used in cell culture media at a final concentration of 10µM for 8-24 hours. The drug is reversible, so care must be taken to not let the drug effects fade and time experiments properly.

Cycloheximide – Cycloheximide (CHX) is an antibiotic produced by the *S. griseus* species that inhibits protein biosynthesis in eukaryotic cells. It inactivates the transferase II enzyme that is involved in peptide chain elongation. It is used in studies to analyze protein stability. Stock powder (Sigma Aldrich Cat# P8833-10MG) was dissolved in 100% EtOH to a final concentration of 20mg/mL. For use in cell culture to

inhibit transcription, CHX was added to the cell culture medium at a final concentration of 20µg/mL.

Farnesyl Transferase Inhibitor (FTI) – FTI-277 was obtained from CalBiochem (LaJolla, CA) and was used to chemically inhibit Ras signaling by impeding its ability to bind the cell membrane. The inhibitor was added to cell culture media at a final concentration 10µM.

Cell Transfections - Cells were transfected using several options available in the laboratory. Commonly the HEK-293, HEK-293T, H1299, and MCF-10A cells were transfected with JetPRIME (Polyplus) transfection reagent. A549 cells were transfected with Lipofectamine 3000 Transfection Reagent (Invitrogen) as the JetPRIME reagent did not have sufficient efficiency when transfecting this line. Transfection reagent specifics and methodology are listed below.

JetPRIME™ - The JetPRIME™ DNA transfection reagent (VWR Cat# 89137-972) is described by the manufacturer as a novel and versatile cationic polymer-based reagent designed to ensure high DNA transfection efficiency. JetPrime™ forms positively charged complexes with DNA that can penetrate the cell membrane through endocytosis and release DNA into the cytoplasm of the cell via the “proton sponge mechanism”. Plasmids mostly reach the nucleus when the nuclear envelope disappears during mitosis. 1-2µg of specific DNA plasmids were mixed in 100µL (35mm dish) to 200µL (60mm dish) of the provided JetPrime™ Transfection Buffer. To this mixture, 2µL of JetPRIME™ was added per 1µg of DNA to be transfected. The mixture was then vortexed for 15 seconds and the transfection mixture was allowed to incubate for 10 minutes at room temperature. The entire mixture was then added to the dish and

allowed to sit for at least 6 hours. After 6 hours, culture media could be changed if complications with cytotoxicity arise.

Lipofectamine 3000® - This transfection reagent is a proprietary lipid formulation purchased from Invitrogen (Cat# LC3000015). Lipofectamine 3000® is especially designed for hard to transfect cells, and I found this to be much more successful at transfecting A549 cells than when I used JetPRIME™. To begin the process, cells were seeded to be 70-90% confluent prior to addition of transfection reagent. Two tubes each containing 125µL of Opti-MEM (Invitrogen Cat# 31985062) were prepared. The Lipofectamine 3000® was then added to each tube, 3.75µL in the first tube and 7.5µL in the second. Next, a DNA master mix was prepared by diluting 1-2µg of each DNA plasmid into 250µL Opti-MEM and adding 2µL of P3000™ per 1µg of DNA. This solution was mixed well and then 125µL was added to each previously prepared tube containing the Lipofectamine 3000® reagent. These mixtures were then allowed to sit for 5 minutes at room temperature, making a high and low lipid dose of transfection for each dish. After the incubation, both high and low mixtures were added to the proper dish and allowed to sit on the cells for 6 hours. After this time, the culture media was changed and the cells incubated overnight to begin expressing recombinant plasmids.

2.7 – Immunoprecipitation

One of the best ways to detect and examine how proteins interact with each other is the co-Immunoprecipitation assay. This assay uses an antibody, typically conjugated to an agarose bead, to bind and precipitate a specific protein. The precipitation is then analyzed for the presence or absence of other proteins that were bound to the precipitated protein. This assay presents several challenges from

insufficient cell lysis, inadequate purification of proteins, non-specific precipitation, and high background detection. Caution must be taken at every step to minimize these issues.

Immunoprecipitation of Over-Expressed Proteins – Transfected cells were lysed 24 to 48 hours post-transfection in a modified RIPA buffer. Modified RIPA buffer is composed of 50mM Tris-HCl pH7.4, 200mM NaCl, and 1% NP-40. Cells were plated in 60mm dishes for over-expression studies and 350 μ L of modified RIPA buffer was added to each dish to begin the lysis process. Cells were then scraped and collected in microfuge tubes and placed on a rotator for 3 hours at 4°C. After rotation, each sample was then slowly passed through a 25 gauge syringe needle 4 times and then centrifuged at 10,000 RPM for 5 minutes at 4°C. The supernatant was then removed from the insoluble pellet and quantified using a Bio-Rad protein quantification assay (Bio-Rad Cat# 500-0006). To a new microfuge tube, 1mg of total protein was added and modified RIPA buffer was added to reach a final volume of 1mL. Agarose beads were then added matching the specific epitope tag of one of the over-expressed proteins. For HA precipitations, I used 5 μ L of HA-conjugated agarose beads prepared as the manufacturer's protocol prescribed (Sigma Aldrich Cat# A2095-5ML). To precipitate the Flag epitope tag, 5 μ L of Anti-Flag M2 Affinity Gel (Sigma Aldrich Cat# A2220-5ML) was prepared according to the manufacturer's protocol and added to the cleared cell lysate. And finally, for precipitation of the GFP epitope tag, GFP-Trap® (Allele Biotech Cat# ABP-NAB-GFPA100) was used according to manufacturer's protocol. In each case, the cleared cell lysate was rotated overnight at 4°C with the proper agarose conjugate. The next day, the bead/lysate mixture was centrifuged for 3 minutes at 3000 RPM at 4°C to pellet the agarose. The supernatant was then removed by aspiration and the beads were washed with 1mL of modified RIPA buffer, repeating this process three times. After the

final wash, the beads were resuspended in 10 μ L of standard RIPA buffer and 5 μ L of 4x LDS (Lithium Dodecyl Sulfate) sample running buffer purchased from Invitrogen (Cat# NP0007). The IP samples and lysate controls were then run on an SDS-PAGE gel and analyzed by Western blot.

Immunoprecipitation of Endogenous Proteins – Conversely to over-expressed proteins, endogenous proteins do not contain epitope tags and must be immunoprecipitated slightly differently than over-expressed proteins. Cells were lysed in a modified RIPA buffer and again 1mg of total cell lysate was set-aside in a final volume of 1mL. Then a primary antibody against the target protein was added and rotated overnight in the protein lysate at 4°C. The next day, an agarose conjugated secondary, typically mouse or rabbit (Rockland Cat# 00-8800-25 and 00-8811-25), antibody was added and rotated for 2 hours at 4°C to capture the primary antibody/target protein complex. Then mirroring the previously described method, the agarose beads were collected by centrifugation at 3000 RPM for 3 minutes at 4°C. The beads were washed 3 times with 1mL of modified RIPA buffer. Following the wash steps, the beads were resuspended in 10 μ L of standard RIPA buffer and 5 μ L 4xLDS sample running buffer. The IP along with controls were analyzed by Western blot.

2.8 – Western Blotting

Sample Preparation – Cell lysates and protein samples were combined with a 4x LDS Sample Buffer (Invitrogen Cat# NP0008) containing 10% β -Mercaptoethanol (Sigma Aldrich Cat# M6250-100ML) to a final concentration of 1x. The protein sample/LDS mixture was then incubated at 95°C for 10 minutes to allow for the denaturation of the

protein sample, allowing for a more consistent separation of size in the polyacrylamide gel.

SDS-PAGE – Prepared protein samples were then loaded on a pre-cast 4-12% Tris-Glycine Polyacrylamide Gel (Invitrogen Cat# EC60352BOX) which is immersed in 1x Tris-Glycine SDS Running Buffer (Invitrogen Cat# LC2675-5). The gel was run at 150V until the loading dye reached the bottom of the gel. The gel was then removed from the running buffer and prepared for transfer onto a nitrocellulose membrane.

Nitrocellulose Transfer – The protein gel was transferred onto a 0.2 μ m pore size nitrocellulose membrane by electrophoresis in 1x Tris-Glycine Transfer Buffer (Invitrogen Cat# LC3675) supplemented with 20% methanol for 3 hours at 20V DC power.

Western Blot Detection – After transfer, the nitrocellulose filter was blocked in a 5% Milk/TBST solution for 30 minutes and then the blot was incubated in primary and secondary antibodies as previously explained earlier in this chapter. After primary and secondary antibody probes were complete, the blots were detected using an ECL solution and exposed to chemiluminescent detection film.

2.9 – Biological Assays

Luciferase Assay – Dual luciferase assays were performed using reagents from Promega's Dual Luciferase Assay Kit (Cat# E1960) per the manufacturer's protocol. Cells were transfected for 24-72 hours depending on the reporter construct used before being lysed in 200 μ L of the provided passive lysis buffer. 20 μ L of sample was processed with 70 μ L of each buffer in sequence to give a primary RLU (Relative Light

Units) reading following by a secondary standardization RLU reading on a Lumat 9507 machine from Berthold Technologies. Data was analyzed with the RLU1 value divided by the RLU2 value resulting in a numerical figure representing relative promoter activation.

Senescence Assay – One of the hallmarks of cells that have undergone senescence is an increase in β -galactosidase activity. The Senescence Detection Kit from BioVision (Cat# K320-250) is designed to histochemically detect β -gal activity in cultured cells and tissue sections. A549 cells were plated onto 12-well culture dishes at 40,000 cells per well initial seeding. The next day, the cells were transfected with expression constructs to analyze their effects on the induction of cellular senescence. 72 hours post-transfection, the cells were processed with the Senescence Detection Kit per the manufacturer's protocol. Five random fields were counted in each well and the number of blue cells counted was divided by the total number of cell counted in the entire field. The result yields a value that shows percentage of cells in senescence.

CHAPTER III

RAS REGULATES SCF- β -TrCP ACTIVITY AND SPECIFICITY VIA NORE1A

3.1 - Introduction

Mutations in the *Ras* oncogene can be detected in roughly one third of human cancers [23]. When activated, the Ras oncoprotein drives growth and transformation by binding and activating multiple effectors that stimulate multiple signaling pathways. Three of the best characterized cascades are the Raf, PI-3Kinase, and RalGDS effector pathways [160]. However, Ras can also impact other signaling pathways important to proliferation and development, such as the Wnt/ β -catenin signaling pathway [161]. The interaction of the Ras and the Wnt/ β -catenin signaling pathways is quite complex and appears to occur at multiple levels [162-164]. An interesting dichotomy exists, as Ras has been reported to exhibit both positive and negative effects on the pathway [162-164]. Moreover, although synergistic activation of transformation by Ras and Wnt/ β -catenin has been reported, other reports have identified an antagonistic role in transformation [163, 164]. How Ras impacts the Wnt/ β -catenin pathway is still not fully understood. Nor is the balance of cellular factors that dictate whether a net positive or negative effect is observed for Ras on Wnt/ β -catenin pathway signaling.

Similarly to many other oncogenic signaling pathways, members of the Wnt signaling pathway can be subdivided in positively and negatively acting components [165]. More often than not, the negative acting components are found mutated to a loss

of function status in cancer, while the positively active components are found constitutively activated [165]. Improper regulation of the Wnt signaling pathway transcriptional co-activator, β -catenin, is an oncogenic force in human cancers. Beginning with the first observations that β -catenin overexpression could lead to malignant transformation and continuing today, the Wnt/ β -catenin pathway continues to evolve as a central mechanism in tumorigenesis [165].

In the canonical Wnt/ β -catenin pathway, β -catenin is the terminal executor, serving as both a nuclear transcriptional co-regulator and key component of adherens junctions [166]. In the absence of Wnt ligand signaling, a multi-protein complex consisting of APC, Axin, and GSK-3 β , phosphorylates β -catenin. This phosphorylation is necessary for the binding of β -TrCP, the substrate recognition component of the SCF- β -TrCP ubiquitin ligase complex. SCF- β -TrCP mediated ubiquitination of β -catenin results in its rapid degradation by the 26S proteasome [167]. In response Wnt ligand activation of the Frizzled receptor, the GSK-3 β phosphorylation complex is destabilized by Disheveled family proteins allowing for unphosphorylated β -catenin levels to quickly increase in the cytoplasm and translocate to the nucleus. The nuclear pool of β -catenin functions as a cofactor for transcription factors of the TCF/LEF family, modulating genes involved in growth and survival [168].

Beta-transducin repeats-containing proteins (β -TrCP) serve as the E3 substrate recognition subunits for the SCF- β -TrCP ubiquitin ligase complexes [157]. The ubiquitin proteasome system controls the degradation of the majority of regulatory eukaryotic signaling proteins, including proteins that perform central functions in tumorigenesis, like β -catenin, I κ B, YAP, and TAZ [157, 169]. E3 ubiquitin ligases determine the timing and specificity of ubiquitination for substrates and typically target proteins with a very specific set of post-translational modifications including phosphorylation or hydroxylation [157].

Beta-TrCP is expressed at relatively low levels in the cell and its function can be easily disrupted by the expression of a dominant negative construct, which lacks an F-Box domain making it unable to bind the other members of the SCF ubiquitin complex [170, 171]. Furthermore, the activity of β -TrCP can be controlled by several different cellular mechanisms that can alter its localization, relative abundance, and levels of phosphorylated substrates [157]. Interestingly, levels of β -TrCP are often elevated in tumor cells, yet in these cells β -TrCP fails to properly regulate its usual oncogenic substrates, including β -catenin [157, 172]. This suggests that presence or absence of other scaffolding elements can alter its ubiquitin ligase activity and/or specificity.

While cells constitutively synthesize β -catenin to remain ready to respond to incoming Wnt signaling, the high turnover rate of β -catenin via the SCF- β -TrCP ubiquitin ligase complex maintains the proper balance [173]. Dysfunction in the regulatory mechanism of β -catenin is found in many human cancers and mutated forms of β -catenin which cannot be ubiquitinated by the SCF- β -TrCP ubiquitin ligase are indeed oncogenic [168]. By enhancing down-regulation of β -catenin, β -TrCP can serve as a tumor suppressor [174]. However, the situation is more complex as β -TrCP has also been reported to have oncogenic potential in some situations [175, 176]. This may be due to its role in degrading non β -catenin targets such as Claspin [177].

Paradoxically, activated forms of Ras are not only powerfully transforming, but can also act as powerful stimulators of cell death pathways [7, 126]. Ras induced cell death appears to be mediated, at least in part, by members of the RASSF family of tumor suppressors, which bind Ras and function death effectors [126]. NORE1A (RASSF5) was the first member of the RASSF family to be identified [9]. It connects Ras to the pro-apoptotic Hippo pathway, but this does not seem to be essential for its tumor suppressor function [129]. It also modulates p53 [10, 11], which may explain its

ability to promote cell cycle arrest. NORE1A is expressed in most normal tissues, but its expression is lost in many tumors due to epigenetic inactivation, loss of heterozygosity, or deregulated proteolysis [10, 118]. NORE1A is considered a bona fide tumor suppressor, as a hereditary genetic defect in NORE1A predisposes the human carriers to kidney cancer [15]. However, many of the tumor suppressive functions of the Ras/NORE1A interaction remain largely uncharacterized.

We preliminarily identified a direct interaction between NORE1A and β -TrCP in a yeast two-hybrid screen. This suggests that either NORE1A is a substrate for SCF- β -TrCP or, more interestingly, might connect Ras to the control of the complex. As SCF- β -TrCP modulates the Wnt/ β -catenin pathway by targeting β -catenin for degradation by the proteasome [174], NORE1A might serve to link Ras to the control of β -catenin protein stability and transcriptional signaling.

Following the preliminary data, I sought to determine the role of NORE1A in the control of the SCF- β -TrCP ubiquitin ligase and Wnt/ β -catenin signaling. I now show that NORE1A forms a direct, endogenous, Ras regulated complex with β -TrCP allowing Ras to stimulate the activity of SCF- β -TrCP towards β -catenin. The activation of the SCF- β -TrCP ubiquitin ligase complex by Ras/NORE1A is substrate specific, as NORE1A had no effect on SCF- β -TrCP substrate I κ B. Moreover, NORE1A deficient lung tumor cells exhibit enhanced steady state levels of β -catenin and resist the growth inhibitory effects of β -TrCP. Therefore, the cellular levels of NORE1A may dictate how Ras modulates β -catenin and determine the substrate profile of the SCF- β -TrCP ubiquitin ligase. β -TrCP has been reported as an oncogene and as a tumor suppressor in different cell systems. The levels of NORE1A may dictate the ultimate activity of β -TrCP in a cell.

3.2 – Results

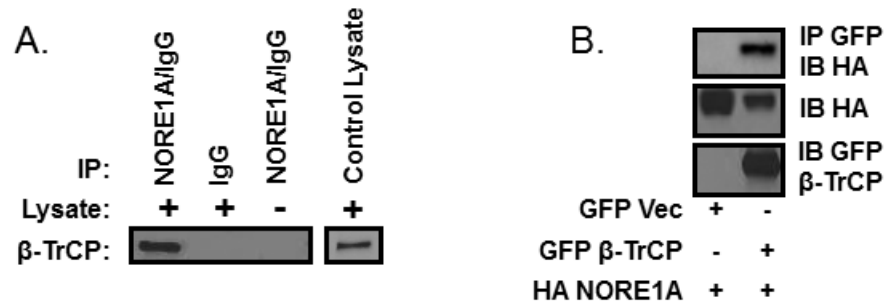
NORE1A forms a direct, endogenous, Ras regulated complex with β -TrCP

β -TrCP is the substrate recognition component of the SCF- β -TrCP ubiquitin ligase. It is expressed as two closely related isoforms, β -TrCP1 and β -TrCP2. A yeast two-hybrid screen (Myriad Genetics, Salt Lake City, UT), using full length NORE1A as bait, identified β -TrCP1 as a potential direct binding partner. To determine if this result was physiological, I immunoprecipitated lysates from MCF10A cells for NORE1A and immunoblotted for the presence of β -TrCP. Figure **(12a)** shows that NORE1A and β -TrCP could be detected in an endogenous complex. Further studies were then performed using exogenously expressed proteins. HEK-293 cells were co-transfected with expression constructs for NORE1A and β -TrCP1. The cells were then lysed and immunoprecipitated for β -TrCP1 and immunoblotted for NORE1A. The proteins could be co-immunoprecipitated **(Figure 12b)**.

NORE1A is a Ras effector protein, consequently I examined the role of Ras in controlling the interaction between NORE1A and β -TrCP1 in similar experiments. The presence of activated H-Ras in the co-transfection enhanced the association between NORE1A and β -TrCP1 proteins **(Figure 13)**. These blots were under-exposed in comparison to the blots of Figure 12b.

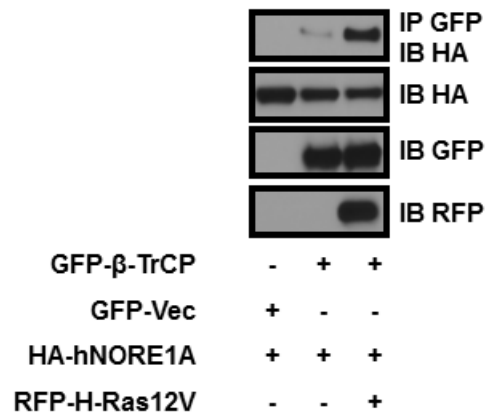
NORE1A promotes β -catenin degradation via β -TrCP

As β -TrCP is the substrate recognition component of the major ubiquitin ligase complex regulating protein levels of β -catenin [167], I examined the effects of NORE1A



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Figure 12. NORE1A Forms a Protein Complex with β -TrCP - (A) Whole cell lysates were prepared from MCF-10A cells and the protein extracts were immunoprecipitated (IP) with anti-NORE1A for 16 hours and then immunoblotting (IB) for β -TrCP. IgG incubated with MCF-10A lysate and Ig/NORE1A antibody incubated with lysis buffer serve as the negative controls. Blot of NORE1A levels is not shown due to interference from the IgG band. MCF10A lysate serves as the positive control. (B) HEK-293 cells were transfected with expression constructs for HA-NORE1A and GFP- β -TrCP1. Cell lysates were immunoprecipitated (IP) with GFP-Trap® Beads (Allele Biotech, San Diego, CA) and Immunoblotted (IB) for the presence of β -TrCP1.



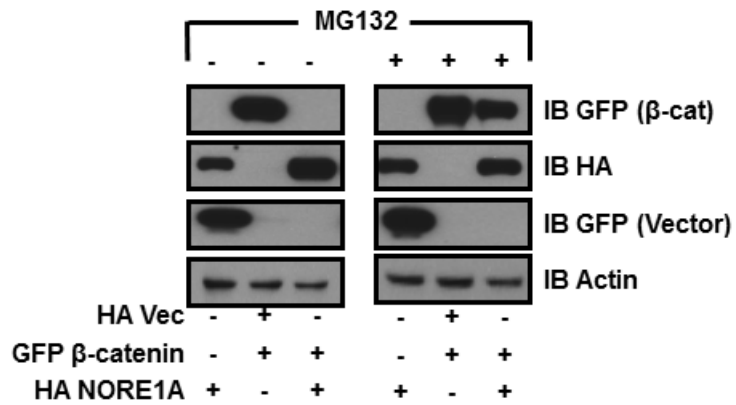
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Figure 13. Ras Regulates NORE1A Binding with β-TrCP - HEK-293 cells were transfected with expression constructs for HA-NORE1A and GFP-β-TrCP1 in the presence or absence of an activated form of RFP-H-Ras (12V). After 24 hours the cells were lysed and immunoprecipitated with GFP-Trap® beads before immunoblotting. A shorter exposure of the immunoprecipitation shows that the presence of activated H-Ras enhances NORE1A binding to β-TrCP.

expression on β -catenin protein stability using transient transfections performed in HEK-293 cells. Co-expression of NORE1A suppressed the expression of β -catenin to sub-detectable levels. This effect was largely blocked by the 26S proteasome inhibitor MG132 (**Figure 14**). To further support the MG132 experiment, the transient transfections of NORE1A and β -catenin were repeated once more in HEK-293 cells. 24 hours post-transfection, the cells were treated with cycloheximide. Dishes were lysed over a time course of 24 hours and samples examined by western blot. The results confirmed NORE1A was acting at a protein stability level (**Figure 15**). In order to determine if NORE1A and β -TrCP1 synergize to promote the degradation of β -catenin, I partially inhibited the proteasome by adding low levels of MG132 for 6 hours. Under these conditions, the ability of both NORE1A and β -TrCP1 to suppress β -catenin expression was impaired rather than abolished. This allowed us to detect a synergistic reduction in β -catenin levels when NORE1A and β -TrCP1 were transfected together (**Figure 16**). Furthermore, a dominant negative form of β -TrCP1, lacking the F-Box domain rendering it unable to confer ubiquitin onto substrates [178], blocked NORE1A mediated degradation of β -catenin (**Figure 16**).

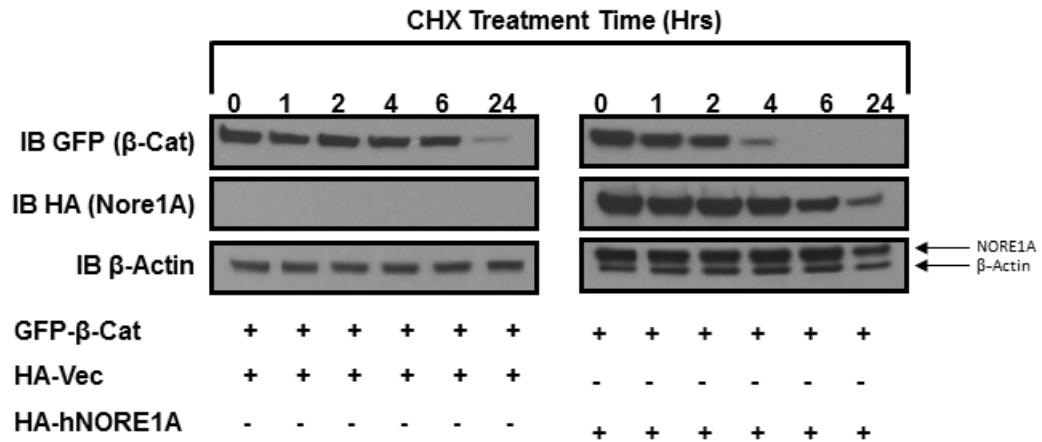
To examine the effects of Ras on the system, I included activated H-Ras in the transfections, again with attenuation of the proteasome with low levels of MG132. Activated Ras enhances NORE1A activity against β -catenin (**Figure 17**). As further support for the role of Ras in directly modifying the action of NORE1A, I included a NORE1A point mutant that is defective for Ras binding in the assay (NORE1ARAMUT) [179]. This experiment showed that the effects of Ras were due to its interaction with NORE1A (**Figure 17**).

NORE1A appears to be regulating β -catenin protein levels through the proteasome by interacting with the ubiquitin ligase, β -TrCP. If this were indeed the



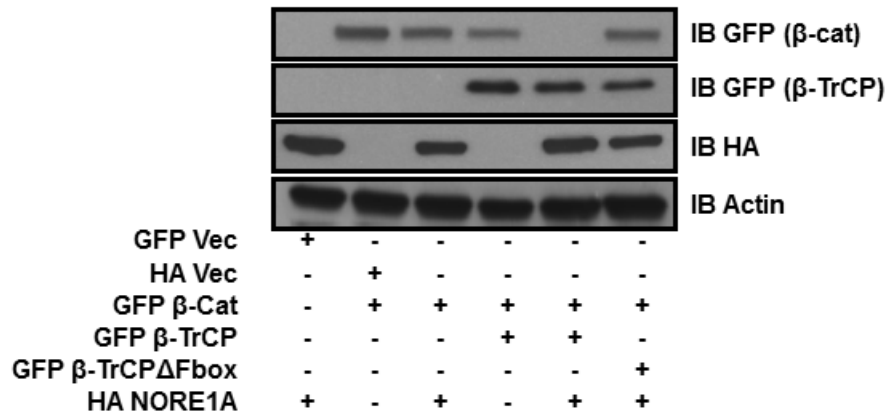
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Figure 14. NORE1A Degrades β -catenin Via the 26S Proteasome - HEK-293 cells were transfected with expression constructs for β -catenin and NORE1A. 24 hours post transfection, the cells were trypsinized and split into two groups and allowed to rest for an additional 24 hours. One group was then treated with the proteasome inhibitor MG132 for 5 hours and the other group was treated with DMSO also for 5 hours. The cells were then lysed and immunoblotted (IB) for levels of β -catenin.



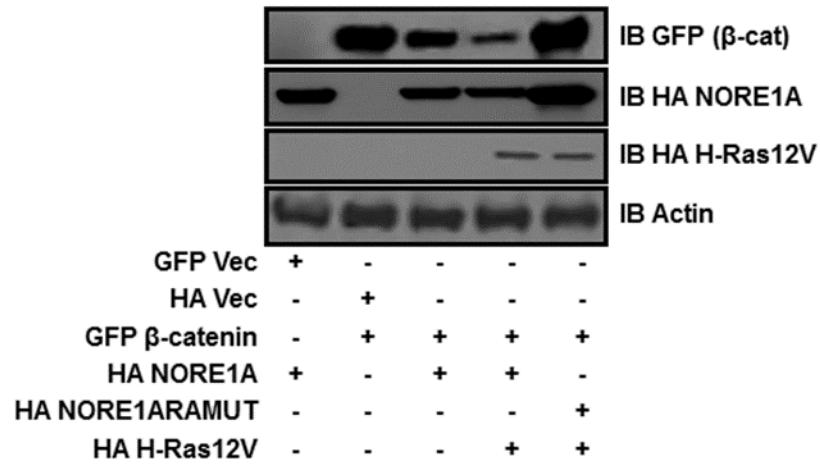
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Figure 15. NORE1A Decreases β-catenin Protein Stability Independent of Transcription - HEK-293 cells were transfected with vector or NORE1A and after 24 hours split into 6 dishes each. Cycloheximide was added after a further 24 hours and dishes lysed over a time course. Levels of β-catenin protein were measured by Western blot. Figure shown is a representative blot of two experiments.



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Figure 16. NORE1A Degrades β -catenin in Cooperation with β -TrCP - HEK-293 cells were transfected with expression constructs for NORE1A, β -catenin, β -TrCP1, and a dominant negative β -TrCP (Δ FBOX) which is unable to ubiquitinate target substrates. Low levels of MG132 were added to partially inhibit the 26S proteasome to attenuate the system and reveal differences between weak and strong activity.



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Figure 17. Ras Regulates NORE1A Mediated Degradation of β-catenin - HEK-293 cells were transfected with expression constructs for NORE1A, a NORE1A mutant unable to bind to Ras (RAMUT), β-catenin, and activated H-Ras. Relative levels of GFP-β-catenin were assayed by immuno-blot. Actin serves as a loading control.

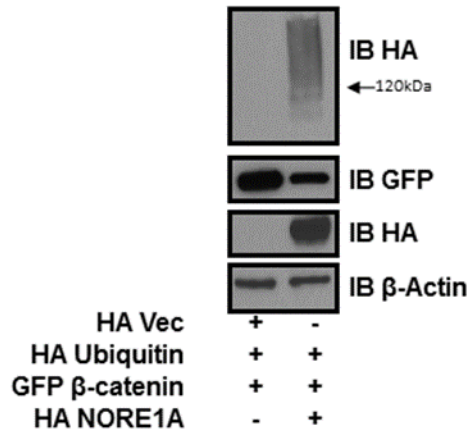
mechanism of action, it would require β -catenin to be polyubiquitinated by β -TrCP, which would mark it for proteasomal degradation. To confirm this, HEK-293 cells were transfected with expression constructs for NORE1A and GFP- β -catenin along with a HA-tagged ubiquitin and treated with MG132. Incorporation of ubiquitin into GFP- β -catenin could be detected (**Figure 18**).

NORE1A suppresses β -catenin signaling

Active β -catenin translocates to the nucleus where it binds the transcriptional repressors TCF/LEF, thereby activating specific gene transcription. To investigate if NORE1A has any effect on β -catenin transcriptional activity, NCI-H1299 cells, null for NORE1A expression, were transfected and stabilized with a pZIP-HA expression vector expressing NORE1A or an empty vector control. The cells were selected in 400 μ g/mL G481 solution until the selection process was complete. Then, using an artificial promoter sequence that is linked to the luciferase gene was used to analyze transcriptional activity for β -catenin activity [180]. This reporter, containing a β -catenin activation region (BAR-Luc), was co-transfected with an expression construct for β -catenin and a Renilla luciferase internal control into the matched pair of NCI-H1299 cells stably transfected with vector or NORE1A expression vector. The NORE1A expressing cells showed reduced β -catenin activity (**Figure 19a**).

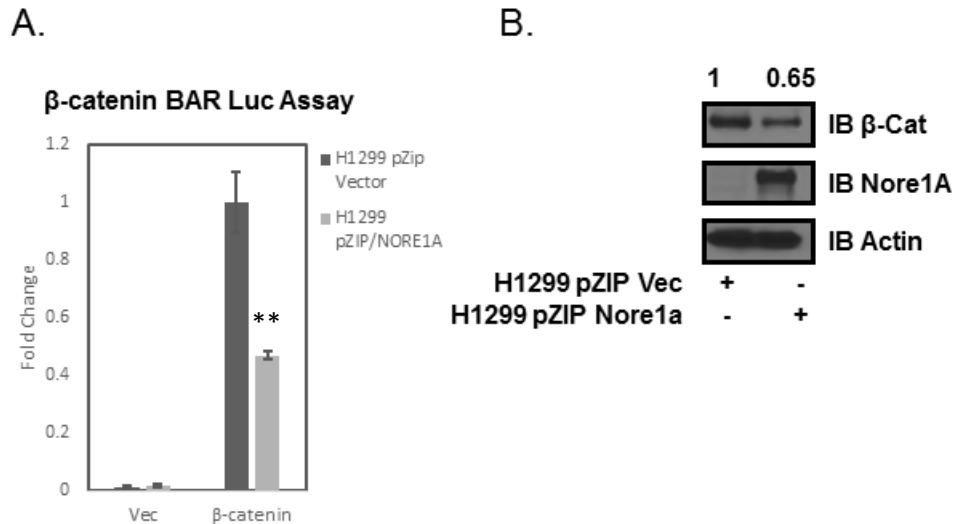
Loss of NORE1A expression enhances steady state levels of β -catenin

The above results suggest that the loss of NORE1A that is so frequently found in human tumors should uncouple Ras from the negative regulation of β -catenin. Thus, the levels of β -catenin in a Ras driven tumor cell should inversely correlate with NORE1A



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Figure 18. NORE1A Enhances β -catenin Ubiquitination - HEK-293 cells were co-transfected with expression constructs for NORE1A, β -catenin, and a HA-tagged Ubiquitin. 24 hours post-transfection, the cells were lysed and analyzed by Western blot for HA incorporation into GFP- β -catenin (~120 kD).



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Figure 19. NORE1A Suppresses Wnt Signaling - (A) NCI-H1299 cells stably transfected with vector or NORE1A were transfected with an expression construct for β-catenin along with a β-catenin responsive luciferase reporter (BAR-Luc) and a TK-Renilla internal control. Dual luciferase assays were performed and results standardized with the Renilla readout (M1/M2). n = 2 independent experiments, error bars show standard error, p = 0.02. (B) Immunoblot (IB) analysis confirming expression of HA-NORE1A. NCI-H1299 +/- for NORE1A expression shows differences in steady state expression of β-catenin. Whole cell lysates were prepared and endogenous levels of β-catenin were analyzed by immunoblot. Quantification was performed by densitometry using QuantityOne software by BioRad is shown above the blot as a ratio.

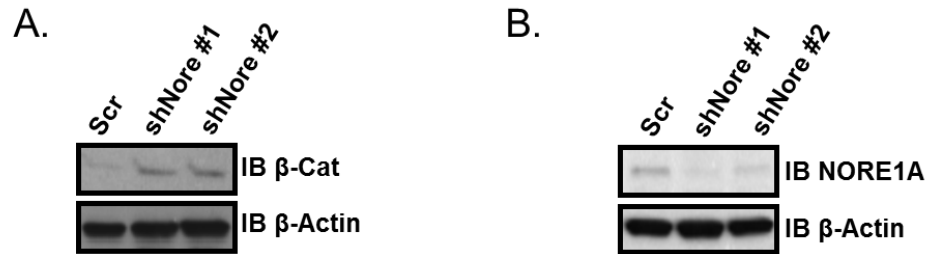
expression. To test this hypothesis, I examined the levels of endogenous β -catenin in the NCI-H1299 NORE1A +/- matched pair. These cells carry an activated Ras mutation and undetectable levels of endogenous NORE1A [181]. Figure (19b) shows that the NORE1A negative cells exhibited higher levels of endogenous β -catenin than the cells stably transfected with NORE1A. As further confirmation, I transfected HEK-293 cells with shRNA constructs against NORE1A and assayed for the levels of endogenous β -catenin protein. Protein levels of β -catenin were elevated in the shRNA transfected cells but not in the scrambled shRNA transfected cells (Figure 20a).

NORE1A differentially regulates β -TrCP targets

To determine if the effect of NORE1A on SCF- β -TrCP is a general activation or a specific activation towards a particular sub-set of targets, a second degradation target of β -TrCP1, I κ B[182], was chosen for analysis. HEK-293 cells were transfected with expression constructs for I κ B, β -catenin, and NORE1A. Western analysis of the protein levels showed that while I κ B levels remained the same in the presence of NORE1A, NORE1A suppressed β -catenin (Figure 21a). I κ B is a negative regulator of NF κ B [183]. If NORE1A promotes its degradation, I should expect NF κ B signaling to increase. Luciferase assays using HEK-293 cells stably transfected with an NF κ B luciferase reporter showed that NORE1A does not promote the activation of NF κ B (Figure 21b).

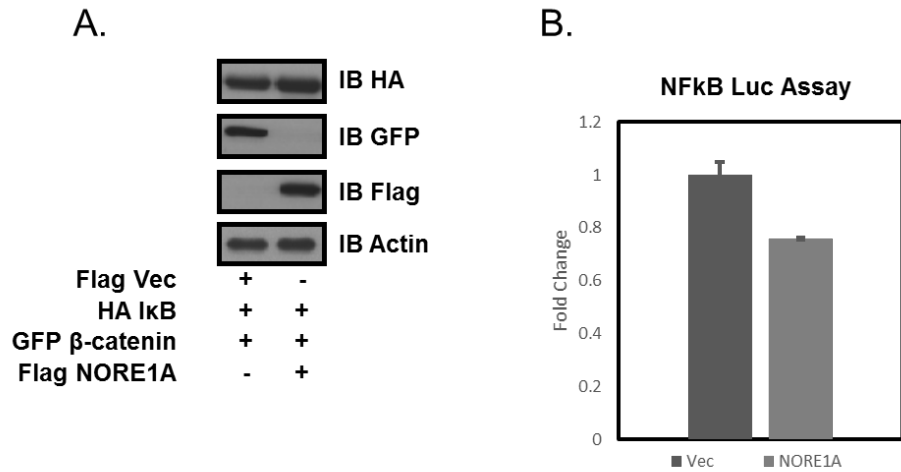
NORE1A levels dictate the biological activity of β -TrCP

β -TrCP has a range of targets and can act as an oncogene or a tumor suppressor depending upon the particular cellular milieu [174-177]. The co-factors determining if the net effect on growth of β -TrCP is positive or negative are not known.



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Figure 20. Loss of NORE1A Enhances Steady State β -catenin Protein Levels - (A) HEK-293 cells were transfected with shRNA constructs against NORE1A. Endogenous levels of β -catenin were analyzed by Western blot. (B) Stably NORE1A shRNA constructs were validated against endogenous NORE1A in HBEC-3KT cells.



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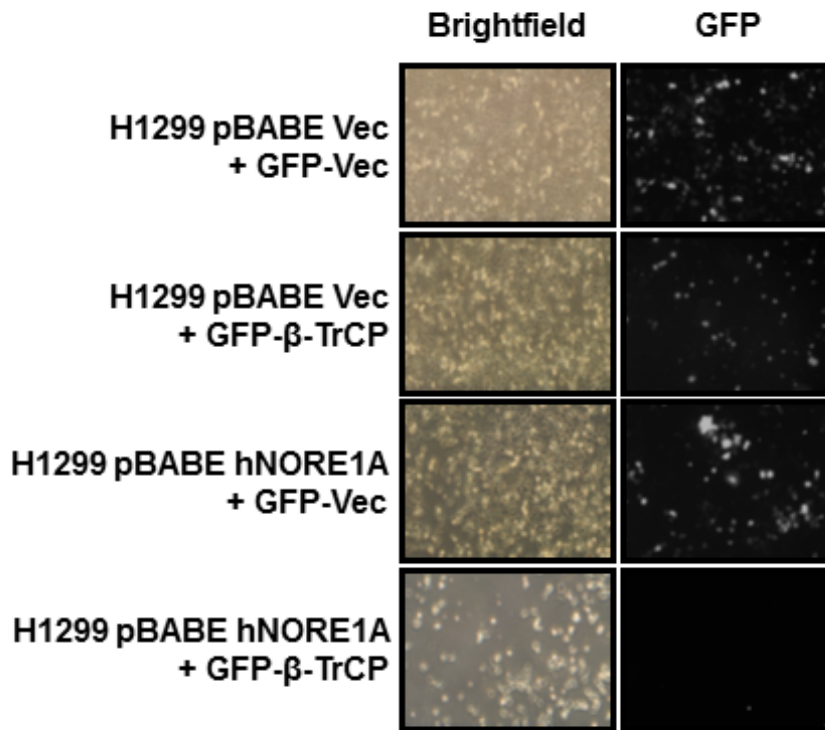
Figure 21. NORE1A/β-TrCP Mediated Ubiquitination is Substrate Specific - (A)

HEK-293 cells were transfected with expression constructs for NORE1A, β-catenin, and IκB. After 24 hours the cells were lysed and assayed for differential protein expression by Western blot. Figure is a representative blot of at least two experiments (B). HEK-293 cells stably expressing an NFκB luciferase reporter were transfected with HA-empty vector and HA-NORE1A. A slight suppression of activity was observed due to NORE1A but quantification showed this was not significant ($p = 0.12$). $n = 2$ independent experiments, error bars show standard error.

To determine if NORE1A may be one of these factors, I transfected the NORE1A +/- NCI-H1299 cell system with β -TrCP1 and selected for the marker carried by the β -TrCP1 construct. Figure (22) shows that β -TrCP1 was highly growth inhibitory in the NORE1A positive cells but only weakly inhibitory in the NORE1A negative cells. Quantification is shown in (Figure 23).

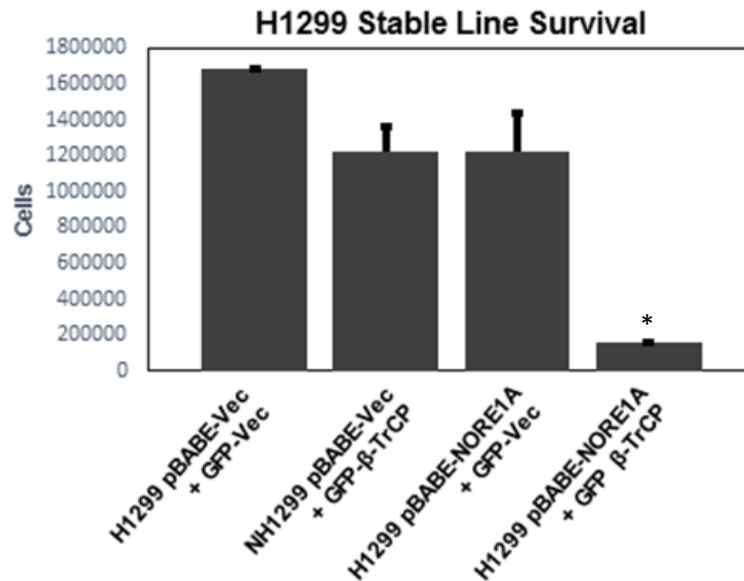
NORE1A is a key node in the regulation of β -catenin via Ras

Ras has been reported to exhibit both negative and positive effects on β -catenin protein levels [184, 185]. The factors that determine the net effect of Ras on β -catenin are not clear. To determine if NORE1A dictates the effects of Ras on β -catenin, I treated the NCI-NCI-H1299 NORE1A +/- cell system with a farnesyl transferase inhibitor (FTI) (Figure 24a) that inactivates Ras [186]. NCI-H1299 cells contain an activated form of Ras, which allowed us to examine the effects of endogenous Ras activation on endogenous β -catenin expression in a NORE1A positive or negative background. A “normal” cell is positive for NORE1A expression and does not have an activated Ras signaling pathway. The levels of β -catenin under these circumstances are shown in column four of Figure 24b. In the presence of a competent activated Ras (no FTI) in these NORE1A positive cells, the levels of β -catenin go down (column 2). However, when I remove the FTI from NORE1A negative cells, I observed that the levels of β -catenin increased (column 1) relative to the levels in the “normal” cells (column 4). Thus, the presence or absence of NORE1A may dictate the effects of Ras, positive or negative, on β -catenin protein levels.



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Figure 22. NORE1A/β-TrCP Cooperate to Control Cell Survival - NCI-H1299 cells stably expressing NORE1A or an empty vector were transfected with an expression construct for β-TrCP1 and selected with G418. Cell survival was slightly reduced in cells expressing NORE1A or β-TrCP alone. However, when both are co-expressed, cell survival is greatly decreased suggesting a synergistic relationship in tumor suppression functions.



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Figure 23. Quantification of NORE1A/β-TrCP Effects on Cell Survival - Figure 22 shows a representative experiment, Figure 23 shows quantification of live cells after 2 weeks. The data is the average to two separate assays performed in duplicate. The error bars on the graph represent standard error with $p = 0.03$.

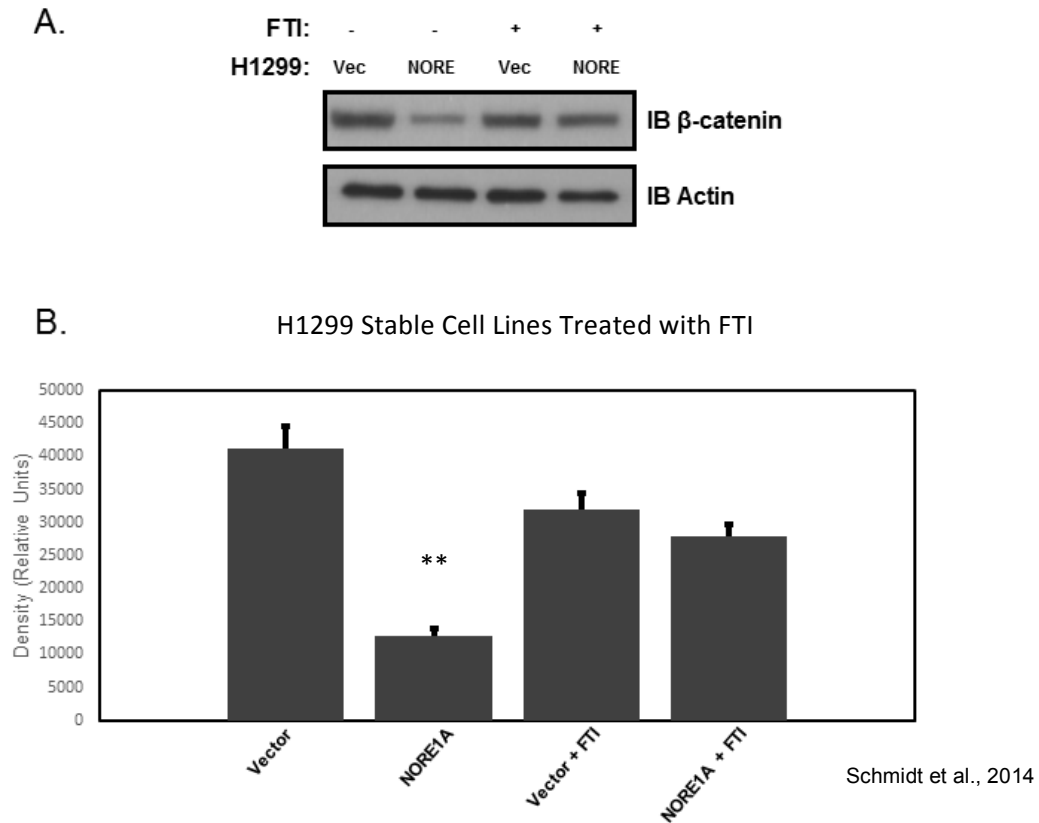


Figure 24. Inhibition or Ras Farnesylation Impedes NORE1A Mediated β -catenin

Control - (A) NCI-H1299 cells stably transfected with NORE1A or empty vector were treated +/- with a farnesyl transferase inhibitor for 24 hours to inhibit Ras signaling. Whole cell lysates were harvested and probed for total levels of endogenous β -catenin. A representative blot is shown in (A), quantification of two experiments is shown below in (B). Vector = Ras+/NORE1A-, NORE1A = Ras+, NORE1A+, Vector + FTI = Ras-/NORE1A-, NORE1A + FTI = Ras-/NORE1A+. A normal cell would be expected to be negative for activated Ras and positive for NORE1A. Thus, bar 4 is the reference bar. Quantification using densitometry was calculated on QuantityOne software from BioRad. Values from two separate assays were combined for quantification. The graph shows this data with standard error and $p = 0.02$.

3.3 - Discussion

NORE1A directly binds activated Ras and serves as a pro-apoptotic Ras effector [10, 11]. What's more, NORE1A suppresses tumor cell growth, is frequently inactivated in human tumors, and its inactivation is implicated in a hereditary cancer syndrome [15, 126, 187]. Therefore, it is likely to serve as an important human tumor suppressor. At one point, NORE1A was implicated in the regulation of Hippo signaling as it binds to the MST kinases; however, further study revealed that NORE1A fails to activate their kinase activity [10, 135]. Furthermore, deletion mutants of NORE1A that cannot interact with MST kinases retain the ability to inhibit cell growth and suppress the tumorigenic phenotype [129]. Thus, unknown tumor suppressor pathways independent of the classic Hippo pathway must also be modulated by NORE1A.

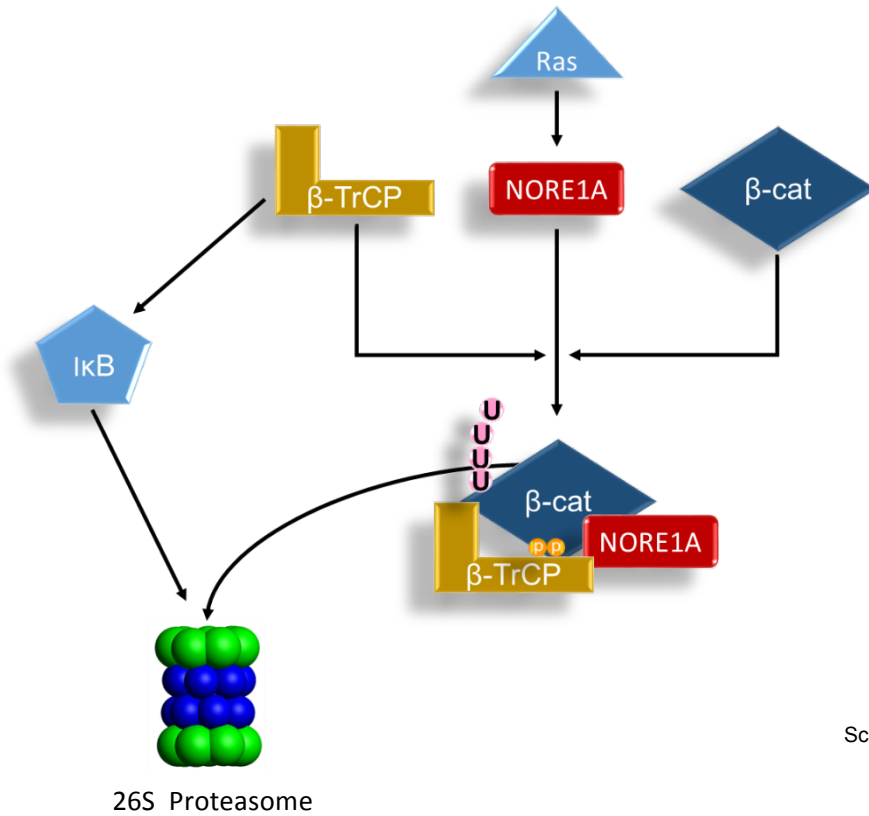
β -TrCP is the substrate recognition component for the SCF- β -TrCP ubiquitin ligase complex and exists as two closely related isoforms, β -TrCP1 and β -TrCP2. Under the influence of Wnt signaling, it binds and regulates the degradation of the proto-oncogene β -catenin [174]. β -catenin has a dual role in the cell serving both as a component of adherens junctions in the cytoplasm and as a transcriptional co-regulator in the nucleus modulating the function of the TCF/LEF transcription factors [188]. The Wnt/ β -catenin pathway plays key roles in development and tumorigenesis, and mutations in the system leading to the excessive accumulation of β -catenin have been identified at high frequency in human tumors [168, 188, 189].

Aberrant activation of the Ras pathway is one of the most common defects observed in human cancers [30]. Over a decade of study has revealed multiple, subtle links between the Ras and Wnt/ β -catenin pathways. The links are complex and remain

poorly understood as they invoke both synergistic and antagonistic relationships [164]. Indeed, Ras has been reported to suppress β -catenin levels but also to promote β -catenin transcription [184, 185]. The net effect appears to be context dependent. However, *in vivo* studies have confirmed that defects in the two pathways can synergize to promote cancer [161].

Here I show that Ras promotes the binding of NORE1A to β -TrCP1, the substrate recognition component of the SCF- β -TrCP ubiquitin ligase and this interaction promotes the degradation of β -catenin (**Figure 25**). As I identified NORE1A and β -TrCP1 in a yeast two-hybrid interaction screen, this interaction is likely to be direct. The interaction of NORE1A with β -TrCP1 explains how Ras can negatively regulate β -catenin and how such regulation may be defective in tumor cells that have suffered inactivation of NORE1A. By using FTIs to inhibit the endogenous activated Ras in my NCI-H1299 cell system, I were able to show that Ras down-regulates endogenous β -catenin protein levels in the presence of NORE1A but enhances them in its absence. Thus, the levels of NORE1A in a cell may play a major role in determining the net effect of Ras on β -catenin. However, it is not impossible that these experiments also impacted non-Ras farnesylated targets. So further experiments to examine the biological effects of the Ras/NORE1A/ β -TrCP1 interaction may be warranted.

At this point, the mechanism by which NORE1A specifically stimulates SCF- β -TrCP ubiquitin ligase activity via β -TrCP1 remains unclear. In order for substrates to be recognized by β -TrCP, they first must be phosphorylated. Therefore, I hypothesize NORE1A may potentially be acting as a scaffold for other substrate regulatory components, including GSK-3 β , Axin, and APC. Furthermore, in order for ubiquitin ligases to function properly, the E3 component requires a complex of several proteins that serve as substrate adapters. Thus, other potential proteins NORE1A may scaffold



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Figure 25. Diagram of the Proposed Novel NORE1A/β-TrCP Signaling Module –

Here I show my proposed Novel Ras/NORE1A/Wnt signaling pathway. I interpret my data to suggest that this Ras/NORE1A signaling pathway activates β-TrCP towards a subset of its substrates such as β-catenin but not IκB. This results in differential degradation of SCF-β-TrCP substrates by the proteasome in the presence of Ras/NORE1A signaling.

these E1 and E2 elements, specifically SCF (Skip/Cullin/F-Box) components, resulting in an enhanced stabilization of a functional ubiquitin ligase complex. Lastly, perhaps NORE1A functions in recruiting, scaffolding, and stabilizing specific β -TrCP substrates for ubiquitination including β -catenin, Claspin, REST, or DEPTOR, explaining the observed altered substrate specificity of β -TrCP often found in cancer.

β -TrCP1 can act as a tumor suppressor [174], as is expected since it can serve as a negative regulator of β -catenin, but has also been reported to exhibit oncogenic functions in some cellular environments [175, 176]. This may be due to its effects on substrates in addition to β -catenin. These include a range of proteins involved in cell cycle regulation and transcriptional control such as I κ B [190, 191], NF κ B [192], GLI2 [193, 194], REST [195, 196], ATF4 [197], CDC25A [198, 199], CDC25B [199], and Claspin [200, 201]. It seems likely that mechanisms exist to target β -TrCP to particular substrates under particular conditions. NORE1A may be part of such a mechanism as it activates β -TrCP1 towards β -catenin but not towards I κ B. As I found that the ability of β -TrCP1 to suppress growth of tumor cells is heavily dependent upon the presence of NORE1A, it may be the NORE1A status of a cell that dictates whether or not β -TrCP1 is oncogenic or tumor suppressive. It will be interesting to determine which other substrates NORE1A activates β -TrCP1 against.

NORE1A is not the first member of the RASSF family to be identified as binding to β -TrCP1. A splice variant of the RASSF1A protein, RASSF1C, can also bind [202]. However, unlike NORE1A, RASSF1C acts to somehow stabilize β -catenin protein levels and enhance β -catenin signaling. Perhaps it acts by competing with NORE1A for binding.

Another pathway that RASSF family members have been associated with is the Hippo pathway. This pathway is a major signaling mechanism involved in controlling organ size and apoptosis [203]. The canonical Hippo signaling pathway involves a kinase cascade where MST kinases phosphorylate LATS kinases which then act on the transcriptional co-regulators YAP and TAZ [204]. RASSF proteins, such as NORE1A, can bind the MST/Hippo kinases [10]; nevertheless, NORE1A does not enhance their activity toward the Hippo pathway executors YAP and TAZ by MST/LATS phosphorylation [135]. However, in addition to β -catenin, β -TrCP1 also binds and regulates the stability of YAP and TAZ [205]. Interestingly, the Hippo pathway intersects with the Wnt pathway at many levels. The first intersection discovered identified a point of β -catenin regulation where TAZ could interact with Dishevelled disrupting GSK-3 β phosphorylation of β -catenin, effectively stabilizing Wnt signaling [206]. Additionally, the Hippo pathway has been shown to enhance transcription of Wnt target genes by upregulating the ability of β -catenin to transcribe target genes [207]. Finally, a recent study demonstrated that YAP and TAZ are required elements for recruitment of β -TrCP to the β -catenin destruction complex and are degraded together with β -catenin [208]. Therefore, the interaction of NORE1A with β -TrCP1 may allow NORE1A to invoke powerful non-canonical Hippo pathway regulation outside of the MST1/2 kinases. This may prove to be a potent additional mechanism where the RASSF family proteins contribute to growth and survival control.

Thus, I identify a function for NORE1A acting as a Ras activated control element for β -TrCP that stimulates the ubiquitin ligase complex towards specific targets. These results may explain, mechanistically, many of the apparent contradictions in the relationship between the Ras and the Wnt/ β -catenin pathways.

CHAPTER IV

NORE1A INTERACTS WITH GSK-3 β ENHANCING β -CATENIN PHOSPHORYLATION

4.1 - Introduction

Signaling by the Wnt family of proteins is one of the core cellular mechanisms that coordinate cell proliferation, cell polarity, and tissue homeostasis [14, 209]. Mutations in the Wnt pathway are often associated with human birth defects and cancer [203]. The most studied aspect of the Wnt signaling mechanism is centered on the regulation of the transcriptional co-activator, β -catenin, which controls key developmental genetic expression programs [209]. In normal tissue growth and development, the cell requires coordination between signal transduction pathways such as the Hippo and Wnt/ β -catenin pathways. In normal cells receiving Wnt signal, Wnt promotes growth and proliferation [210]. However, in cancer, Wnt signaling is typically corrupted into an Wnt signal independent “on” state as a result of one of several genetic mutations, most of which center around proteasomal regulation of β -catenin [210].

In the canonical Wnt/ β -catenin signaling pathway, β -catenin serves as both a nuclear transcriptional co-regulator and core component of adherens junctions [166]. The cellular mechanism involved in the control of β -catenin is centered on the presence or absence of Wnt ligand. In the absence of Wnt ligand signaling, a multi-protein

complex consisting of APC, Axin, and GSK-3 β , phosphorylates β -catenin. The phosphorylation is a required step for recognition and binding by β -TrCP, the E3 component of the SCF- β -TrCP ubiquitin ligase complex. SCF- β -TrCP mediated ubiquitination of β -catenin results in its rapid degradation by the 26S proteasome [211]. When Wnt ligand activates this pathway, the GSK-3 β phosphorylation complex is disrupted by Disheveled family proteins allowing for unphosphorylated β -catenin levels to quickly increase in the cytoplasm and translocate to the nucleus [168]. Nuclear β -catenin functions as a coactivator for transcription factors of the TCF/LEF family, modulating genes involved in growth and survival [168]. While cells constitutively synthesize β -catenin to maintain a ready response to incoming Wnt signaling, the very quick turnover rate of β -catenin maintains a proper signaling balance [173]. Dysfunction in the regulatory mechanism of β -catenin has been described in many human cancers and mutated forms of β -catenin which cannot be phosphorylated by GSK-3 β are potentially oncogenic [168].

In the previous chapter, I characterized a novel, direct interaction between NORE1A and β -TrCP, the E3 component of a SCF ubiquitin ligase complex [212]. This Ras mediated complex effectively regulated β -catenin protein stability and established a novel role for NORE1A in the regulation of oncoprotein stability [212]. Accordingly, loss of NORE1A expression resulted in an increase in steady state levels of β -catenin and resistance to β -TrCP mediated growth inhibitory effects [212]. Nonetheless, before β -catenin can be recognized and ubiquitinated by β -TrCP, it first must be phosphorylated by GSK-3 β . NORE1A has no known enzymatic activity and appears to function in cells as a scaffolding protein [13, 130, 212, 213]. Perhaps a potential function of NORE1A in the regulation of β -catenin (Wnt pathway) works by scaffolding GSK-3 β to β -TrCP resulting in a more efficient protein regulatory mechanism. Correspondingly, loss of

NORE1A would remove a key negative Ras mediated regulatory element and may explain why Ras and Wnt are often found co-activated in many human cancers [214].

To determine if NORE1A is determining factor governing the effects of Ras on Wnt signaling, a series of studies were undertaken. Here I now show that NORE1A forms an endogenous complex with GSK-3 β . This novel protein/protein interaction between NORE1A and GSK-3 β results in a coordinated and enhanced GSK-3 β phosphorylation of β -catenin. I also show that when cells were treated with LiCl₂, a specific inhibitor of GSK-3 β activity, NORE1A lost the ability to enhance β -catenin degradation. Moreover, a triple point mutant of NORE1A that fails to bind GSK-3 β is deficient in suppressing β -catenin. The NORE1A/GSK-3 β mechanism appears to operate independently of Ras signaling and more than likely has much broader implications with respect to cellular tumor suppression. Furthermore, this novel interaction provides key insight into RASSF tumor suppression control via NORE1A.

4.2 - Results

NORE1A forms and endogenous complex with GSK-3 β

I previously published that NORE1A can negatively regulate β -catenin protein levels by interacting with the E3 ubiquitin ligase, β -TrCP [212]. However, NORE1A has been recently shown to interact with kinases involved in Hippo and p53 signaling [14, 133]. Thus the potential exists for NORE1A to interact with kinases involved in β -catenin regulation. While β -catenin is regulated by a dual-kinases system consisting of CK1 and GSK-3 β , the latter actually marks β -catenin for recognition and ubiquitination by β -TrCP [215]. Accordingly, I sought to determine if NORE1A and GSK-3 β interact. First,

HEK293T cells were transfected with expression constructs expressing HA-GSK-3 β and a GFP-NORE1A along with an activated H-Ras(12Val). Cells were lysed 24 hours post-transfection and immunoprecipitated against the GFP-epitope tag. NORE1A was found in complex with GSK-3 β and this interaction did not seem to be affected by the presence of Ras (**Figure 26**). To determine if this result was physiological, MCF-10A cells were lysed and an antibody against GSK-3 β was used to immunoprecipitate GSK-3 β . NORE1A was again found in an endogenous complex (**Figure 27**).

NORE1A enhances phosphorylation of β -catenin.

Since NORE1A and GSK-3 β were found in an endogenous complex, I next sought to determine if NORE1A enhances phosphorylation of β -catenin. H1299 cells, null for NORE1A expression, were transfected with either an empty vector or pZIP-HA-NORE1A. Transformants were selected using G418 yielding a stable population that was +/- for NORE1A expression. Using an antibody against specific GSK-3 β phosphorylated residues of β -catenin (Ser33/37/Thr41), cell lysates from H1299 cells that were +/- for NORE1A were analyzed by Western blot to see if NORE1A enhances GSK-3 β mediated phosphorylation of β -catenin. In the lysates from cells expressing NORE1A, there was a substantial increase in phosphorylated β -catenin (Ser33/37/Thr41) when compared to lysates expressing empty pZIP-HA-vector (**Figure 28**).

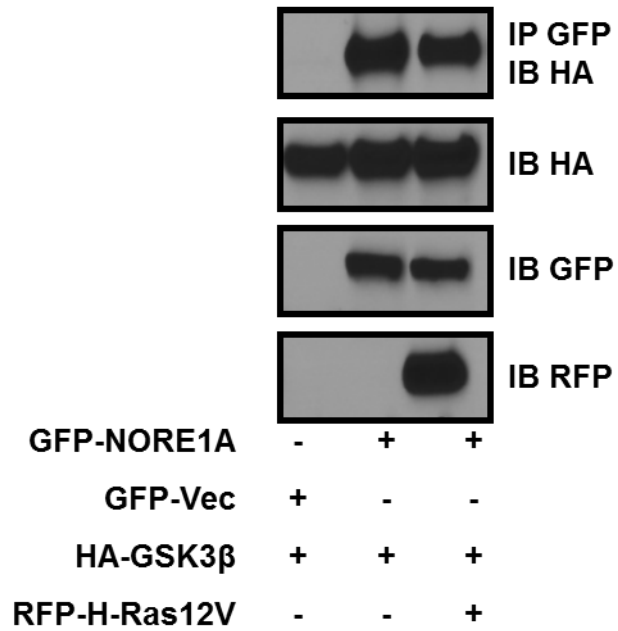


Figure 26. NORE1A Complexes with GSK-3β - HEK-293T cells were transfected with expression constructs for NORE1A, GSK-3β, and a constitutively activated H-Ras (12Val). NORE1A was immunoprecipitated using anti-GFP agarose beads and levels of bound GSK-3β was analyzed by Western blot. The results of this experiment show that NORE1A complexes with GSK-3β and this interaction does not appear to be affected by Ras.

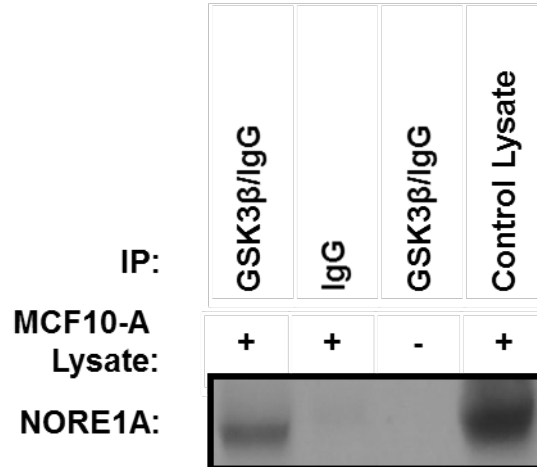


Figure 27. Endogenous Immunoprecipitation of NORE1A and GSK-3 β - Whole cell lysates were prepared from MCF-10A cells and the purified extracts were immunoprecipitated (IP) with anti-GSK-3 β for 16 hours and then immunoblotted (IB) for NORE1A. IgG incubated with MCF-10A lysate and Ig/GSK-3 β antibody incubated with lysis buffer serve as the negative controls. MCF-10A lysate serves as the positive control. NORE1A is detected in an endogenous complex with GSK-3 β .

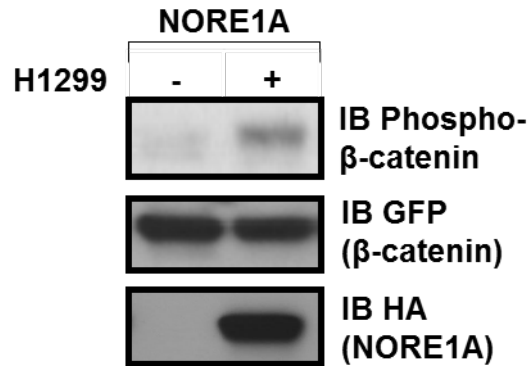


Figure 28. NORE1A Enhances β -catenin Phosphorylation on GSK-3 β Target Residues - H1299 cells, null for NORE1A expression, were transfected with either pZIP vector or pZIP-HA-NORE1A. Transformants were selected using G418 yielding a stable population that is either +/- for NORE1A expression. Cells were lysed and analyzed on a Western blot for phosphorylated β -catenin. In the cells expressing NORE1A, there is a marked increase in levels of phosphorylated β -catenin on GSK-3 β target residues.

Lithium Chloride prevents NORE1A mediated β -catenin degradation.

To determine if NORE1A is using GSK-3 β or another kinase to phosphorylate and drive β -catenin degradation, I chose Lithium Chloride to specifically inhibit GSK-3 β phosphorylation activity [216]. HEK-293 cells were transfected with expression constructs expressing β -catenin paired with either an empty vector or HA-NORE1A. As expected, in the presence of NORE1A, levels of β -catenin are difficult to detect (**Figure 29**). However, when LiCl₂ is added to the cell media for 24 hours, NORE1A loses the ability to suppress β -catenin protein levels when analyzed by Western blot (**Figure 29**). NORE1A can bind several cellular kinases, including HIPK2 [14]. HIPK2 is known to be able to regulate β -catenin as well [217]. However, LiCl₂ specifically inhibits GSK-3 β and does not disrupt HIPK2 kinase activity [218]. Thus, this result helps confirm that NORE1A is in fact working through GSK-3 β and not mediating β -catenin phosphorylation via other kinases like HIPK2.

Disruption of the NORE1A/GSK-3 β interaction suppresses the ability of NORE1A to downregulate β -catenin.

To further support the Lithium Chloride data, I sought to develop a point mutant of NORE1A that disrupted its binding to GSK-3 β . Using fragment mapping and site directed mutagenesis, a triple point mutant of NORE1A was developed mutating the 92-94 residues from Arg to Ala. HEK-293T cells were transfected with expression constructs for GSK-3 β paired with empty vector, NORE1A, and the triple point mutant (NORE1A 92-94A mutant). 24 hours post-transfection, the cells were lysed and the cell lysate was immunoprecipitated using GFP-conjugated agarose beads. The assay was then analyzed on a Western blot where GSK-3 β was found to complex strongly with NORE1A; however, GSK-3 β failed to complex with the mutant NORE1A (**Figure 30**).

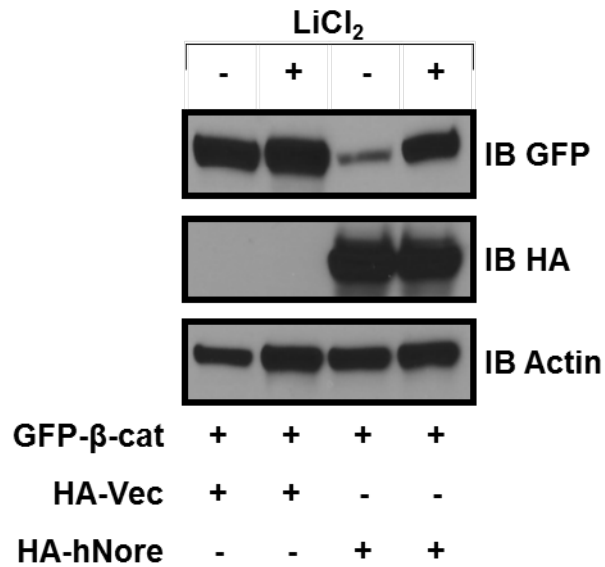


Figure 29. Inhibition of GSK-3β Kinase Activity Blocks NORE1A's Effects on β-catenin - HEK-293 cells were transfected with expression constructs expressing NORE1A and β-catenin. 24 hours post-transfection, the cells were treated with LiCl₂ for another 24 hours. LiCl₂ is known to be an effective inhibitor of GSK-3β kinase activity. In the cells treated with LiCl₂ at a final concentration of 10mM, NORE1A mediated downregulation of β-catenin is impaired. This result suggests that NORE1A is regulating β-catenin protein levels in by enhancing GSK-3β kinase activity.

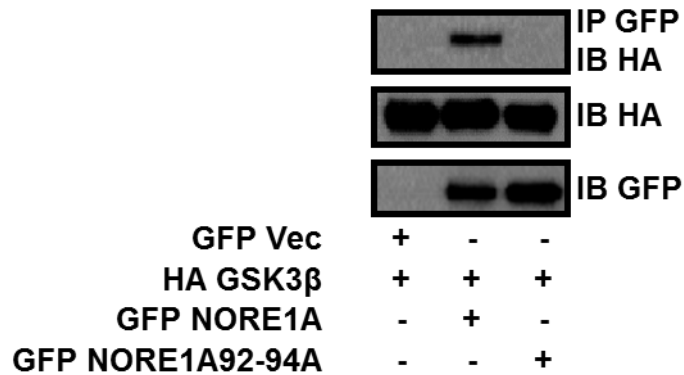


Figure 30. Development of NORE1A Point Mutant that Fails to Bind GSK-3 β - In order to further understand the cellular implications of the NORE1A/GSK-3 β interaction, mutations were made in NORE1A focused on disrupting its interaction with GSK-3 β . Deletion mutagenesis suggested that potential GSK-3 β binding region rested in the 92-94 amino acid residues of NORE1A. These residues were mutated to Alanine residues via PCR mutagenesis. Then HEK-293T cells were transfected with expression constructs for GSK-3 β and either wild-type NORE1A or the mutant NORE1A (92-94A). Cells were lysed 24-hours post-transfection and immunoprecipitated. Wild-type NORE1A was found in complex with GSK-3 β . However, the mutant NORE1A (92-94A) failed to bind GSK-3 β .

The mutant NORE1A was then employed to see if disruption of the interaction between NORE1A and GSK-3 β affects the observed downregulation of β -catenin. HEK-293 cells were transfected with expression constructs expressing either β -catenin and subsequently paired with an empty vector, wild type NORE1A, or the mutant NORE1A 92-94A. Results show the wild type NORE1A suppressed β -catenin protein levels, while the mutant NORE1A (92- 94A) was unable to suppress them (**Figure 31**). This is in agreement with the LiCl₂ experiment and again supports the idea that NORE1A is indeed coordinating GSK-3 β to regulate β -catenin.

4.3 - Discussion

The evolutionarily conserved Wnt signaling pathway is a vital signaling mechanism controlling a wide range of cellular processes including growth, development, tissue repair, and organogenesis [219]. In canonical Wnt signaling, Wnt ligands activate the pathway by binding to the Frizzled receptor [14]. Activation of this receptor stabilizes cellular levels of β -catenin and results in the formation of a transcriptional complex containing a DNA-binding factor known as lymphocyte enhancer factor (Lef), T-cell factor (Tcf) driving transcription of a set of growth and development genes [13]. However, a very intricate set of events is required before this complex can be stabilized and begin transcription. Wnt signaling involves the inhibition of a constitutively active degradation mechanism that keeps cytosolic “signaling” pool of β -catenin at low levels [13]. Disruption of this degradation complex allows β -catenin levels to quickly increase where it can then enter the nucleus to drive transcription of the Tcf/Lef family genes [13]. Conversely, in the absence of Wnt signaling, cytosolic β -

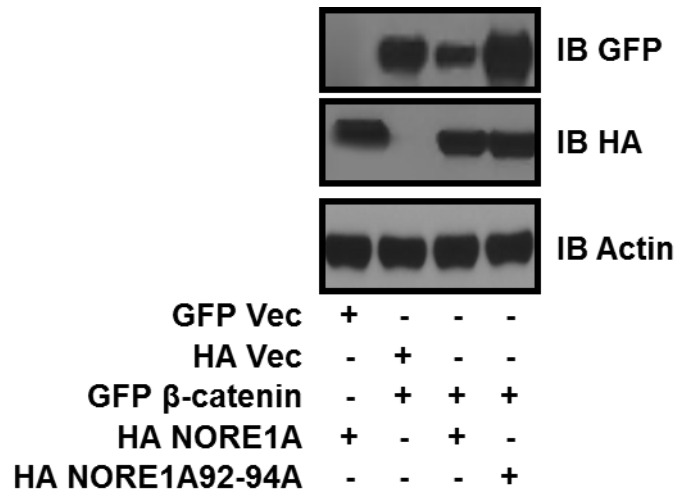


Figure 31. Disruption of NORE1A/GSK-3 β Impairs NORE1A Degradation of β -catenin - HEK-293 cells were transfected with expression constructs expressing β -catenin paired with empty vector, wild-type NORE1A, and the mutant of NORE1A that fails to bind GSK-3 β (NORE1A92-94A). In cells expressing β -catenin with empty vector, protein levels of β -catenin are high. As expected, in the presence of wild-type NORE1A, levels of β -catenin are suppressed. Intriguingly, when β -catenin was co-expressed with the 92-94A mutant, β -catenin was not degraded and appears to be stabilized to an extent suggesting that the 92-94A mutant may be acting as a dominant negative with regard to β -catenin proteasomal regulation.

catenin is rapidly degraded through ubiquitin mediated proteasomal degradation. The kinases CKI and GSK-3 β play central roles in controlling β -catenin levels [13]. Yet, studies show that cells require very strict coordination in regulating β -catenin protein levels to maintain homeostasis, and disruption of these mechanisms is often oncogenic [168].

Glycogen synthase kinase 3 (GSK-3 β) is a serine/threonine kinase that is an important component of numerous signaling pathways involved in the regulation of cell survival, protein synthesis, glycogen metabolism, cell mobility, and proliferation [220]. In addition to these functions, GSK-3 β also negatively regulates many proto-oncogenic proteins and cell cycle regulators via the ubiquitin proteasome system [221]. When considering the broad range of oncogenic targets, it is easy to assume that GSK-3 β functions to suppress tumorigenesis and indeed several studies support the view that GSK-3 β functions as a tumor suppressor. Oddly enough, unlike most other protein kinases, GSK-3 β is constitutively active. Thus, other factors that affect its proper function, including the presence or absence of scaffolding molecules, must exist to regulate target acquisition and are likely key in determining the oncogenic potential of a cell.

Since GSK-3 β negatively regulates many proto-oncogenic proteins and cell cycle regulators, a tumor suppressive role makes perfect sense [221]. However, some studies suggest that GSK-3 β may actually promote tumorigenesis and cancer development as GSK-3 β over expression has been observed in human ovarian, colon, and pancreatic cancers [222]. Hence, it remains controversial whether GSK-3 β is a tumor suppressor or tumor promoter. Therefore, the role GSK-3 β plays in cancer is likely complex and may be dependent on specific cellular circumstances or perhaps the presence or absence of cooperating tumor suppressor proteins like NORE1A.

GSK-3 β regulates many diverse substrates and signaling pathways, and the mechanisms that determine its tumor suppressive function are indeed complicated. One of the better-characterized roles of GSK-3 β on tumor suppression is centered on its control of β -catenin, the terminal executor of the Wnt signaling pathway. Phosphorylation of β -catenin by GSK-3 β targets it for ubiquitination by the substrate recognition protein β -TrCP and subsequent proteasomal degradation. Activation of Wnt signaling disrupts GSK-3 β mediated phosphorylation of β -catenin allowing cellular levels of β -catenin to increase. As β -catenin levels climb, the protein moves to the nucleus where it co-activates transcription of genes of the TCF/LEF gene group.

Over 15 years of study has revealed multiple, subtle links between the Ras and Wnt/ β -catenin pathways. The links are complex, occur at multiple levels, and remain poorly understood as they invoke both synergistic and antagonistic relationships [164]. Ras has been reported to suppress β -catenin levels but it has also been reported to promote β -catenin mediated gene transcription [184, 185]. The net effect appears to be situationally dependent. While the antagonistic effects are likely not tumorigenic, the synergistic effects likely are tumorigenic and *in vivo* studies confirmed that defects in the two pathways can promote cancer [161]. Data shown thus far supports my original hypothesis names NORE1A has the potential to be the link that determines whether Ras can activate or suppress Wnt.

In the previous chapter, I showed how Ras signaling enhances the binding of NORE1A to β -TrCP, enhancing ubiquitination of specific substrates regulated by the β -TrCP ubiquitin ligase [212]. β -TrCP is the E3 substrate recognition component for the SCF- β -TrCP ubiquitin ligase complex and is known to be the major ubiquitin ligase involved in the regulation of β -catenin protein levels. I now present evidence that NORE1A has deeper involvement in this protein regulatory mechanism. Here I show a novel

interaction between NORE1A and GSK-3 β and demonstrate that NORE1A can activate the kinase activity of GSK-3 β towards a specific substrate of the Wnt pathway, β -catenin. NORE1A appears to be working exclusively through the GSK-3 β kinase as a specific GSK-3 β kinase inhibitor, LiCl₂, blocks the ability of NORE1A to suppress β -catenin protein levels. Furthermore, a mutant of NORE1A that fails to bind GSK-3 β is deficient in properly regulating β -catenin protein levels. Taken together, these results suggest that NORE1A is working to control Wnt signaling by coordinating both phosphorylation and ubiquitination of β -catenin (**Figure 32**). NORE1A and GSK-3 β play an essential role in the Ras mediated regulation of Wnt/ β -catenin signaling and loss of NORE1A shifts the relationship between Ras and Wnt from antagonistic to protagonistic resulting in enhanced tumorigenesis.

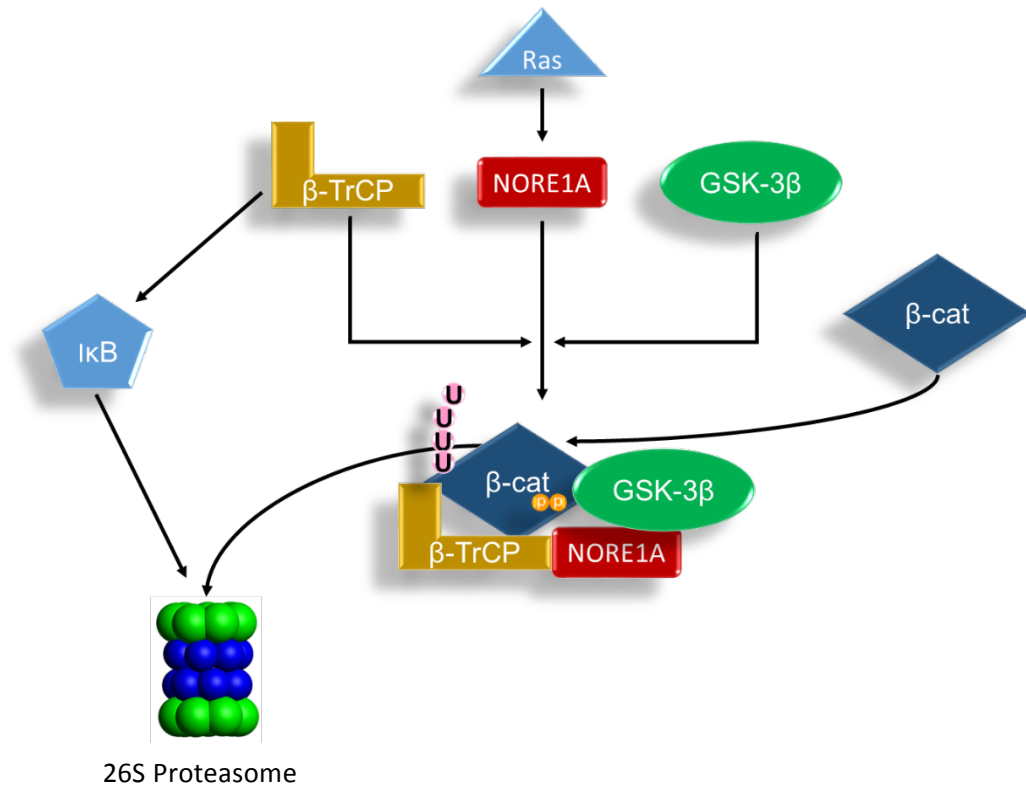


Figure 32. Enhanced Diagram of the Proposed NORE1A/GSK-3β/β-TrCP Signaling Complex - This schematic, depicting a novel NORE1A mediated protein regulatory complex, demonstrates how I hypothesize NORE1A to function as a scaffolding molecule in this protein regulatory complex. NORE1A binds and enhances both GSK-3β and β-TrCP activity on the specific substrate, β-catenin. Perhaps the reason for this lies in the potential for NORE1A to be scaffolding the individual elements of this complex together. Bringing the kinase and ubiquitin ligase in close proximity may determine the speed and success of target substrate regulation.

CHAPTER V

NORE1A SPECIFICIALLY REGULATES TAZ INDEPENDENTLY OF HIPPO BY SCAFFOLDING GSK-3 β TO β -TRCP

5.1 - Introduction

The Hippo signaling pathway is an evolutionarily conserved signaling mechanism that regulates organ size by controlling the cell cycle, regulating cell proliferation, and activating apoptosis [223, 224]. In tissue growth and development, the cell requires strict coordination of signal transduction pathways. The Hippo pathway was first believed to be important in human cancers when observations made in *Drosophila melanogaster* tissues containing Hippo mutations are transformed [225]. More recent experiments using mice and studies involving the screening of human cancer genomes have validated this belief by identifying that mutations in Hippo signaling are indeed oncogenic [226, 227]. Despite recent progress, full understanding of the Hippo pathway remains incomplete [228]. While the conserved canonical Hippo signaling pathway has been established, potentially important variations on this cascade await characterization in mammals [228]. Furthermore, additional levels of Hippo regulation potentially exist as it is likely that several upstream inputs remain to be discovered and defined [228].

The Hippo pathway consists of a signaling cascade of proteins that cooperate in controlling growth and development stimuli. Most of the key pathway regulation occurs

in a small core component of the canonical Hippo pathway. These components include the protein kinases LATS1/2 and MST1/2, which function as members of a kinase cascade [229]. These kinases work to phosphorylate and degrade the terminal elements of the Hippo pathway, the transcriptional co-activators YAP and TAZ [230, 231]. YAP and TAZ bind to and co-activate transcription factors including TEAD and β -catenin to activate transcription of genes required for cell survival and proliferation [207, 232, 233]. In recent years, the Hippo signaling pathway has gained increasing attention as mutations in the upstream regulatory components leading to the ineffective regulation of YAP/TAZ have been identified in human cancers [234]. Both YAP [235, 236] and TAZ [237, 238] exhibit oncogenic properties and elevated TAZ expression can be found in a wide variety of aggressive cancers [239].

The Hippo pathway cross-talks with several other signaling pathways such as Notch, Wnt, Sonic hedgehog (Shh) [240]. However, there is another pathway that can regulate Hippo and has some of the most powerful impacts on cellular oncogenesis, and that pathway is the Ras signaling pathway. This establishes an interesting paradigm where you have two molecular pathways, one that causes cancer and one that protects against it, signaling through each other. Ras has been shown to stimulate both oncogenic transformation and apoptosis through activation of a variety of effectors [241]. While the pro-transformation elements of Ras signaling are fairly well characterized, the pro-apoptotic mechanisms that protect the cell from hyper-active Ras stimulation are far less understood. A key breakthrough occurred when it was discovered that Ras modulates pro-apoptotic MST signaling activity via the RASSF family of proteins, canonically activating the Hippo pathway [241].

RASSF1A, a member of the RASSF family, was shown to bind MST and activate the Hippo apoptotic pathway [133, 135]. Under Ras stimulation, RASSF1A stimulates

MST kinase activity as well as binding to its substrate, LATS [242, 243]. Activation of the LATS kinase in turn leads to the phosphorylation of YAP [241]. LATS has been shown to phosphorylate YAP on multiple residues, each having different regulatory functions [244]; however, one of the key residues in apoptotic signaling involves phosphorylation of YAP on S127, which leads to its cytosolic sequestration and subsequent degradation [244].

Several other members of the RASSF family also have the potential bind MST using a special conserved domain termed the SARAH (Salvador/RASSF/Hippo) domain [133]. Thus, it was not surprising when NORE1A (RASSF5) was found to bind the MST kinases [10]. Initially, it was believed that NORE1A, much like RASSF1A, activated the MST kinases [10]. However, upon further study, this initial finding has become somewhat controversial [135]. The aforementioned study showed that while NORE1A indeed binds, MST, there was no evidence found linking NORE1A to the activation of its kinase activity [135]. Furthermore, a mutant of NORE1A that fails to complex with MST still powerfully functions as a tumor suppressor [129]. Therefore, in contrast to RASSF1A, NORE1A likely fulfills its function as a tumor suppressor independently of canonical Hippo signaling.

In canonical Hippo signaling, YAP and TAZ are usually considered to be co-regulated. When the upstream kinase module of MST1/2 and LATS1/2 is activated, YAP and TAZ are equally phosphorylated leading to their inhibition of transcriptional activity through 14-3-3 mediated cytoplasmic sequestration and proteasomal degradation [225]. However, the regulation of the two terminal Hippo pathway executors is not identical. While YAP and TAZ are co-regulated canonically by Hippo activated LATS-dependent phosphorylation, it was later discovered that TAZ has multiple phosphorylation sites recognized by a variety of other cellular kinases that can result in

different mechanisms of regulation [205]. While LATS phosphorylation of TAZ at S89 results in cytoplasmic sequestration, phosphorylation by the same kinase at S311 results in recognition and ubiquitination by the SCF- β -TrCP ubiquitin ligase complex leading to its degradation in the 26S proteasome [205]. Furthermore, TAZ contains another N-terminal phosphodegron that is unique and not found in YAP [245]. This phosphodegron at S58/62 is recognized by the GSK-3 β protein kinase, activating an additional recognition site for β -TrCP ubiquitination [18]. While the same SCF E3 component is involved in recognition of both the C-terminal LATS phosphodegron and the N-terminal GSK-3 β phosphodegron, these mechanisms are distinctly different and either one is found to be sufficient to induce TAZ degradation [18].

In Chapter III, I identified and characterized a direct interaction between NORE1A and β -TrCP, the E3 component of a SCF ubiquitin ligase complex [212]. This Ras mediated complex effectively regulated β -catenin protein stability and provided insight on how NORE1A functions as an effective scaffolding protein [212]. Accordingly, loss of NORE1A expression resulted in an increase in steady state levels of β -catenin and resistance to β -TrCP mediated growth inhibitory effects [212]. During my studies, I noticed that NORE1A expression appeared to specifically suppress the stability of TAZ rather than YAP. As TAZ, and not YAP, is specifically regulated by a GSK-3 β phosphodegron/SCF- β -TrCP ubiquitination process, I hypothesized that NORE1A might act as a Ras regulated scaffold to modulate the action of GSK-3 β and SCF- β -TrCP on TAZ, as well as β -catenin.

Here I now show NORE1A regulates TAZ outside of canonical Hippo signaling. My data shows that NORE1A is found in an endogenous complex with GSK-3 β and acts as a Ras regulated scaffold for GSK-3 β and β -TrCP. This allows NORE1A to promote the GSK-3 β dependent degradation of TAZ independently of the canonical Hippo

pathway. Moreover, this property is an essential component of NORE1A tumor suppressing activity, as a point mutant of NORE1A that cannot interact with GSK-3 β is defective for suppression of TAZ, and defective for the ability to promote senescence [14].

Thus I identify a novel mechanism by which Ras can control TAZ levels independently of the canonical Hippo pathway. Moreover, as the role of GSK-3 β in cancer development is now known to be extensive [246], the identification of a Ras/NORE1A/GSK-3 β signaling pathway may reveal a novel function of Ras.

5.2 - Results

NORE1A Differentially Regulates Hippo pathway endpoints YAP and TAZ

NORE1A was originally thought to regulate cellular apoptosis by binding and activating the MST1/2 kinases, resulting in an induction of Hippo apoptotic signaling [10]. However, another study would go on to show that NORE1A does not activate MST1/2 kinase cascade [135]. In response to these opposing reports, I sought to determine if NORE1A had any effect on the stability of YAP and TAZ, thereby modulating Hippo pathway signaling. To investigate this, HEK-293 cells were transfected with mammalian expression plasmids expressing YAP or TAZ paired with either NORE1A or an empty vector control. Here I observed YAP stability was not affected by the presence of NORE1A (**Figure 33**). However, TAZ protein levels were powerfully suppressed in the presence of NORE1A demonstrating that NORE1A differentially regulates YAP and TAZ (**Figure 33**).

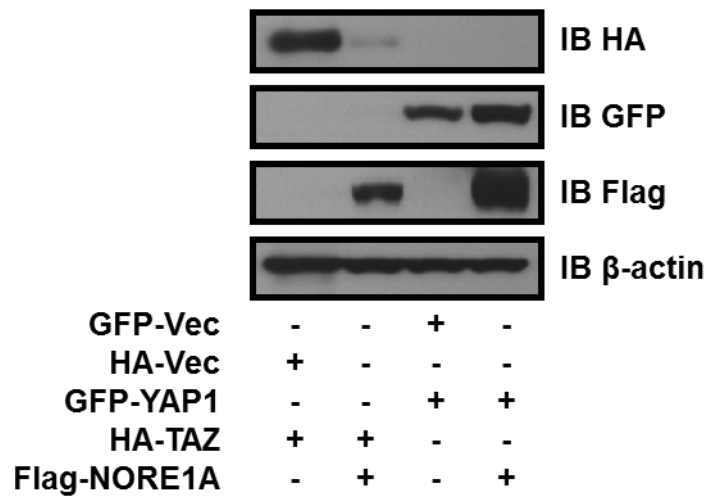


Figure 33. NORE1A Differentially Regulates YAP and TAZ - NORE1A differentially regulates YAP and TAZ. To determine whether NORE1A regulated YAP and TAZ in similar manners or with similar specificity, I took HEK293 cells and transfected them with expression constructs expressing YAP or TAZ paired with an empty vector or a construct expressing NORE1A. 24 hours post-transfection, the cells were lysed and the total levels of YAP and TAZ were examined by Western blot. NORE1A strongly downregulated TAZ protein levels, yet did not have a negative effect on the stability of YAP.

Knockdown of NORE1A expression leads to enhanced steady state levels of TAZ

To confirm the result from figure 33 where NORE1A suppresses TAZ protein levels, I tested whether knocking down NORE1A had the reverse effect on endogenous steady state TAZ. To achieve this, HEK293 cells were transfected with two different shRNA constructs against NORE1A [14, 212]. 48-hours post-transfection, the cells were lysed and analyzed on a Western blot for levels of endogenous TAZ. In the cells where NORE1A was suppressed, there was a substantial increase in steady state levels of TAZ (**Figure 34**).

Lithium Chloride treatment blocks NORE1A mediated down-regulation of TAZ

In addition to GSK-3 β , NORE1A has been reported to interact with the MST1/2 kinases [133]. These kinases can also mark TAZ for degradation. So I sought to determine whether NORE1A was working through GSK-3 β by using a specific GSK-3 β inhibitor, LiCl₂ [218, 247]. HEK-293 cells were transfected with a construct expressing TAZ paired with either an empty vector or a construct expressing NORE1A. 24 hours post-transfection, the transfected cells were split and treated with either LiCl₂ or water, which served as the carrier solution. 24 hours after treatment with the GSK-3 β inhibitor, the cells were lysed and the cell lysates were examined by Western blot. As expected, in the absence of LiCl₂, NORE1A effectively suppresses TAZ protein levels. However, in the presence of LiCl₂, the ability of NORE1A to suppress TAZ was markedly reduced suggesting that NORE1A is indeed working via GSK-3 β to suppress TAZ protein levels (**Figure 35**).

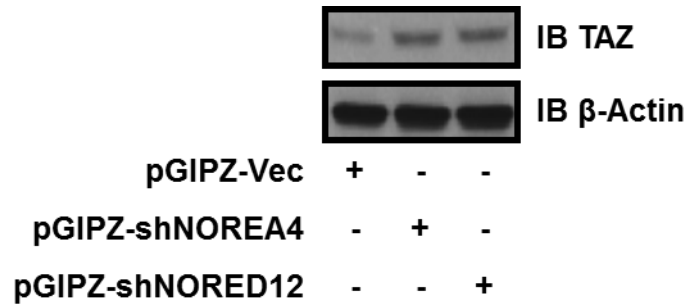


Figure 34. Knockdown of NORE1A Results in Increased Steady-State TAZ Levels -

To confirm the over-expression data where NORE1A suppressed TAZ protein levels, I sought to determine whether knockdown of NORE1A would impair the cell's ability to properly regulate the growth and survival regulator, TAZ. HEK293 cells were transfected with shRNA constructs against NORE1A and lysed 48-hours post-transfection, and the cell lysates were examined by Western blot. In the cells where NORE1A was knocked down, endogenous levels of TAZ are elevated.

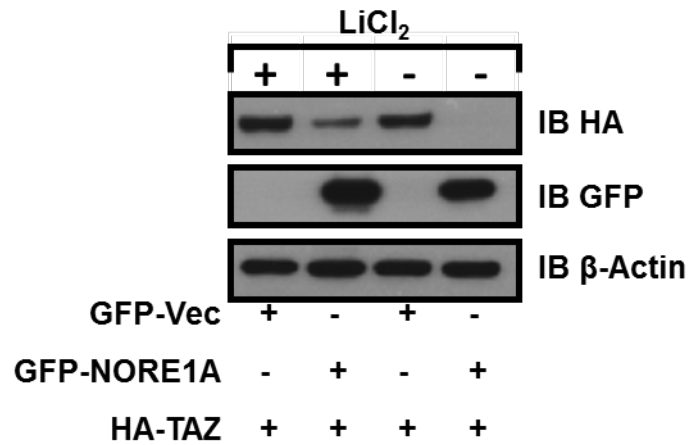
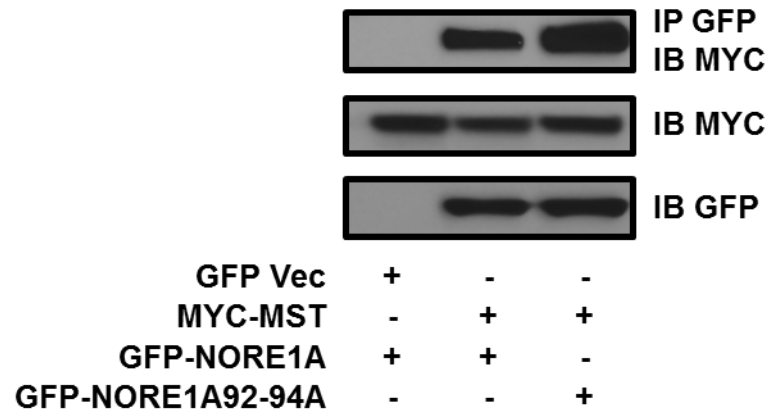


Figure 35. Lithium Chloride Impairs the Ability of NORE1A to Regulate TAZ - HEK-293 cells were transfected with expression constructs expressing NORE1A and TAZ. 24 hours post-transfection, the cells were treated with LiCl₂ at a final concentration of 10mM for another 16 hours. LiCl₂ is known to be an effective inhibitor of GSK-3β kinase activity. In the cells treated with LiCl₂, NORE1A mediated downregulation of TAZ is impeded. This results suggests that NORE1A is regulating TAZ protein levels in coordination with GSK-3β.

Disruption of the NORE1A/GSK-3 β interaction suppresses the ability of NORE1A to down-regulate TAZ independently of Hippo (MST)

To confirm the Lithium Chloride data suggesting that NORE1A is regulating TAZ independently of canonical Hippo signaling (MST), I went back and employed the mutant of NORE1A that lost the ability to bind GSK-3 β (NORE1A92-94A mutant). HEK-293T cells were transfected with expression constructs for GSK-3 β paired with empty vector, NORE1A, and the triple point mutant, NORE1A92-94A mutant. GSK-3 β was found to complex strongly with NORE1A; however, GSK-3 β failed to complex with the mutant NORE1A (**Figure 30**). This mutant does not, however, disrupt binding to MST, allowing for us to specifically isolate NORE1A/GSK-3 β functions (**Figure 36**) [14].

Moving further, in addition to being regulated by GSK-3 β , TAZ is canonically regulated by Hippo signaling where the cellular levels of TAZ protein are suppressed by activation the MST/LATS kinase cascade. Like other RASSF family members, NORE1A has the ability to bind the MST1/2 kinases [133]. However, this may not result in their activation [135]. Thus, I sought to more conclusively determine whether NORE1A is working through MST1/2 kinases (Hippo) or through the GSK-3 β kinase, or perhaps both MST1/2 and GSK-3 β to regulate TAZ. To eliminate Hippo components, I used a NORE1A deletion mutant lacking the SARAH motif which cannot bind MST kinases [9]. To confirm that the deletion of the SARAH motif disrupted MST1/2 binding to NORE1A, HEK-293T cells were transfected with expression constructs expressing Myc-MST along with an empty vector, GFP-NORE1A, or the truncation mutant GFP-NORE1A Δ SARAH. Cells were lysed 24 hours post-transfection and immunoprecipitated against the GFP epitope tag. MST was found in a complex with NORE1A, however, when NORE1A loses the SARAH domain, binding to MST is ablated (**Figure 37**). After it was determined that this truncation mutant failed to bind MST, I wanted to see if it retained



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Figure 36. Wild-Type NORE1A and the 92-94A Mutant Complex with MST - TAZ can be regulated by both GSK-3 β and MST kinases. Figure 30 showed that the NORE1A92-94A mutant failed to bind GSK-3 β and I needed to know whether this mutation also disrupted the ability of NORE1A to bind MST. HEK-293T cells were transfected with expression constructs expressing NORE1A, the 92-94A mutant, and MST. Whole cell lysates were immunoprecipitated by GFP-agarose beads and the experiment shows that NORE1A and the 92-94A mutant both bind MST.

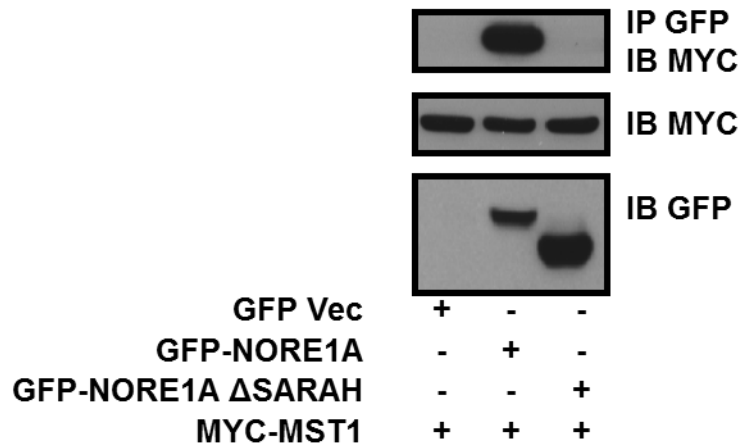


Figure 37. Removal of the NORE1A-SARAH Domain Disrupts NORE1A/MST Binding - To isolate the effects of the NORE1A/MST interaction, I sought to develop a mutant of NORE1A that fails to complex with MST. NORE1A is known to associate with MST via the SARAH (Salvador/RASSF/Hippo) domain. I removed this domain from the protein and performed a co-immunoprecipitation to confirm that removal of the SARAH domain disrupted the ability of NORE1A and MST to bind. HEK-293T cells were transfected with expression constructs expressing NORE1A, the ΔSARAH, and MST1. Cells were immunoprecipitated after 24 hours and the IP was analyzed by Western blot. I confirmed that removal of the SARAH domain disrupted the ability of NORE1A to form a protein complex with MST.

binding to GSK-3 β . HEK-293T cells were transfected with expression constructs expressing HA-GSK-3 β paired with an empty vector, GFP-NORE1A, and GFP-NORE1A Δ SARAH. The cells were lysed 24 hours post-transfection and immunoprecipitated. While the removal of the SARAH domain disrupted NORE1A binding to MST, it did not affect its binding to GSK-3 β (**Figure 38**).

These mutants of NORE1A were then employed in an experimental system allowing investigation of the relative importance of the interactions between NORE1A, MST, and GSK-3 β to down-regulate TAZ. HEK-293 cells were transfected with expression constructs expressing HA-TAZ paired with a GFP-vector, GFP-NORE1A, GFP-NORE1A Δ 92-94A, or GFP-NORE1A Δ SARAH. The cells were lysed 24 hours post-transfection and levels of TAZ were analyzed by Western blot. Agreeing with previous results, the wild-type NORE1A strongly suppressed TAZ protein levels and the mutant of NORE1A that failed to complex with GSK-3 β also failed to down-regulate TAZ protein levels (**Figure 39**). Furthermore, the mutant of NORE1A that retains binding to GSK-3 β and not MST still effectively regulates TAZ protein levels. Thus, NORE1A can regulate TAZ outside of canonical Hippo signaling operating through GSK-3 β instead of MST1/2 (**Figure 39**).

NORE1A suppresses TAZ transcriptional co-activation via GSK-3 β

Data presented thus far shows that NORE1A powerfully suppresses TAZ protein stability. Thus, I sought to determine whether this suppression also resulted in a suppression of TAZ mediated TEAD promoter activity. To answer this, I performed a series of luciferase assays using a TEAD-promoter reporter construct (TEAD-Luc). HEK-293 cells were transfected with expression constructs expressing HA-TAZ and the TEAD-Luc reporter along with an empty vector, NORE1A, NORE1A Δ 92-94A, and

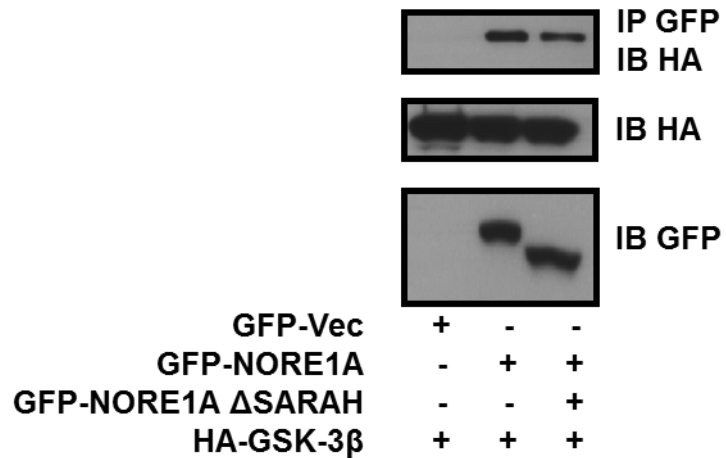


Figure 38. NORE1A-ΔSARAH Mutant Retains Binding with GSK-3β - HEK-293T cells were transfected with expression constructs expressing wild-type NORE1A and the truncation mutant lacking the SARAH domain with GSK-3β. While the SARAH-less NORE1A failed to bind MST (Figure 37), data shown here shows that it still complexes with GSK-3β similarly to wild-type NORE1A

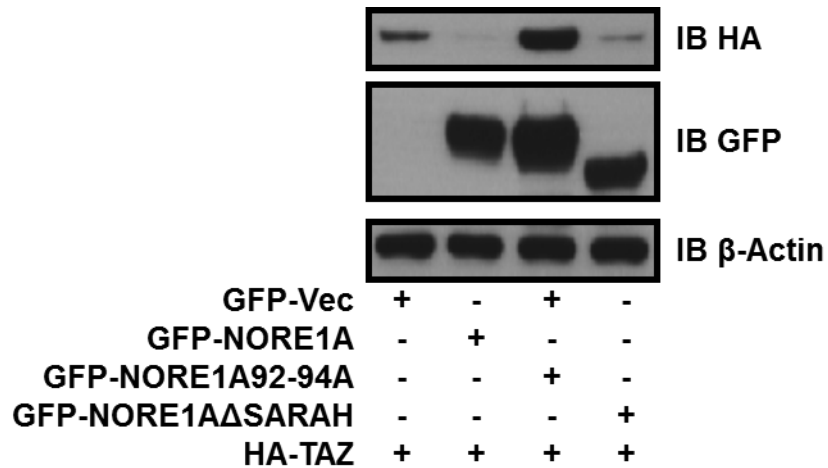


Figure 39. NORE1A Regulates TAZ Independently of Hippo (MST) Via GSK-3 β -

Now that I have developed two mutants of NORE1A that each bind only one of the kinases that regulate TAZ, I sought to see if I could identify whether NORE1A was regulating TAZ via GSK-3 β , MST, or both. HEK293 cells were transfected with an expression construct expressing TAZ paired with wild-type NORE1A, the 92-94A mutant (fails to bind GSK-3 β), or the Δ SARAH (fails to bind MST). I found that wild-type NORE1A and the mutant that does not complex with MST both strongly suppressed TAZ protein levels. However, the mutant of NORE1A that fails to complex with GSK-3 β also failed to suppress TAZ. Taken together, this supports my hypothesis that NORE1A regulates TAZ specifically via GSK-3 β .

NORE1A Δ SARAH. Wild-type NORE1A suppressed TAZ mediated TEAD-promoter activation (**Figure 40**). Additionally, the triple point mutant of NORE1A (92-94A) that failed to bind GSK-3 β also was unable to suppress TAZ activation of the TEAD promoter (**Figure 40**). Yet, the mutant of NORE1A (Δ SARAH) that fails to bind MST, but still binds GSK-3 β , was still able to suppress TAZ mediated TEAD activation (**Figure 40**). These observations correlate with my protein stability observations and show that NORE1A mediated TAZ suppression indeed affects transcription of TEAD.

NORE1A coordinates with β -TrCP to control TAZ protein levels

I recently reported that NORE1A can form a direct, Ras regulated complex with the E3 ubiquitin ligase, β -TrCP [212]. TAZ is known to be ubiquitinated by β -TrCP, and it is entirely plausible that NORE1A may also be regulating TAZ by coordinating GSK-3 β with β -TrCP. To test this hypothesis, HEK-293 cells were transfected with expression constructs expressing TAZ along with an empty vector, NORE1A, and β -TrCP. TAZ protein levels were suppressed in the presence of NORE1A and β -TrCP, and the effect was amplified when both NORE1A and β -TrCP were co-expressed with TAZ (**Figure 41**). However, when a dominant negative β -TrCP (Δ FBOX) was transfected into this experimental system, it disrupted the ability of NORE1A to effectively down-regulate TAZ (**Figure 41**), showing that NORE1A is also using β -TrCP to regulate TAZ.

NORE1A scaffolds β -TrCP and GSK-3 β to form a protein regulatory complex

I have shown that NORE1A can complex with key elements of an ubiquitin ligase mechanism that control phosphorylation and subsequent ubiquitination of substrates. NORE1A has no apparent enzymatic activity and it is hypothesized to function as an

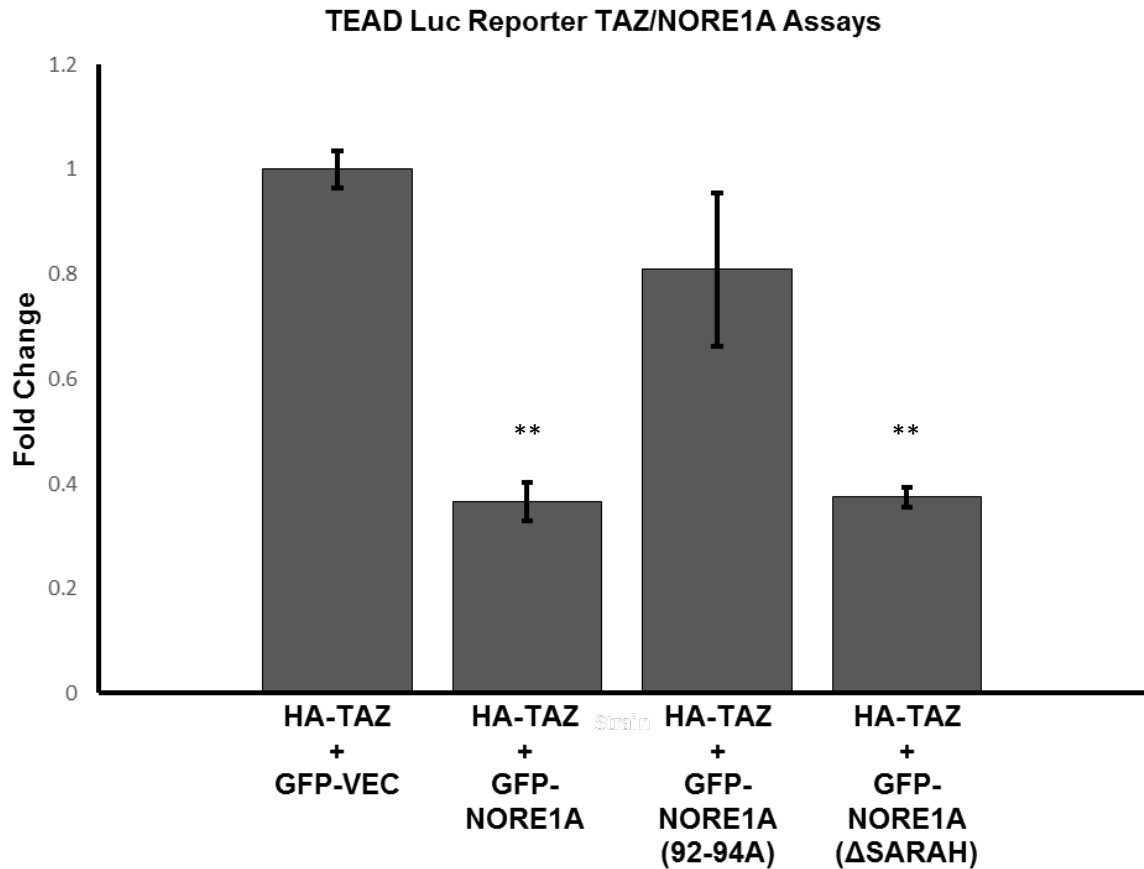


Figure 40. NORE1A Suppresses TAZ Mediated TEAD Promoter Activity - To investigate whether NORE1A mediated TAZ protein suppression (Figure 39) also resulted in a suppression of TAZ mediated TEAD transcriptional co-activation, HEK293 cells were transfected with a TEAD luciferase reporter construct along with an expression construct expressing TAZ. I then paired this system with either wild-type NORE1A, the 92-94A mutant, or the truncation mutant lacking the SARAH domain. Echoing the results from the protein stability experiment, wild type NORE1A ($p=0.001$) and the mutant that fails to bind MST (Δ SARAH) ($p=0.001$) still both strongly suppressed TAZ mediated transcriptional activity. The mutant of NORE1A that failed to bind GSK-3 β (92-94A) also failed to suppress TAZ ($p=0.25$) mediated transcriptional activity further supporting my hypothesis that NORE1A is regulating TAZ independently of Hippo signaling (MST) via GSK-3 β .

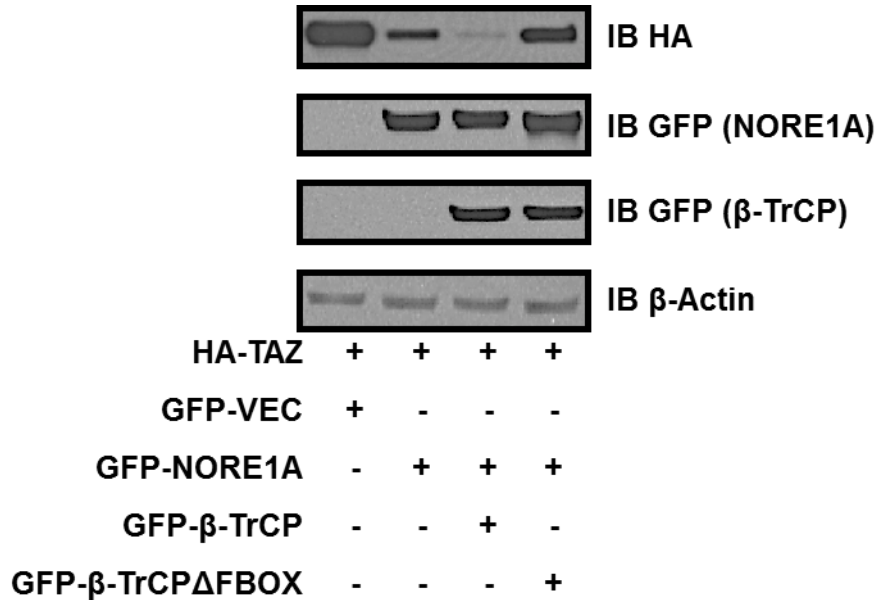


Figure 41. NORE1A Cooperates with β -TrCP to Regulate TAZ Protein Levels - Phosphorylation of TAZ results in its recognition and subsequent ubiquitination by the E3 ubiquitin ligase, β -TrCP. I previously published that NORE1A can bind and regulate β -TrCP, and I sought to see if NORE1A was also regulating TAZ via β -TrCP. HEK293 cells were transfected with expression constructs expressing TAZ along with NORE1A, NORE1A paired with β -TrCP, and NORE1A paired with a catalytically inactivate β -TrCP (Δ FBOX). NORE1A suppression of TAZ was enhanced in the presence of β -TrCP. Intriguingly, the effect was impaired when a dominant negative β -TrCP (Δ FBOX) was co-expressed with NORE1A. Taken together, this supports that NORE1A is also functioning through β -TrCP to regulate TAZ.

effective scaffolding element. Perhaps NORE1A regulates different cellular protein substrates by scaffolding GSK-3 β to β -TrCP and enhancing its mechanistic productivity. To investigate this, HEK-293T cells were transfected with a HA-GSK-3 β along with GFP-Vector or GFP- β -TrCP with and without Flag-NORE1A. Cells were lysed 24 hours post-transfection and the cell lysates were immunoprecipitated by the GFP-tag and analyzed by Western blot. I found GSK-3 β in a barely detectable complex with β -TrCP, however, the addition of NORE1A strongly stabilized the complex (**Figure 42**). Thus, this data suggests that NORE1A indeed works as a scaffolding protein stabilizing the GSK-3 β and β -TrCP into a protein complex. This observation helps explain how NORE1A functions in the regulation of TAZ as well as helps support my hypothesis from Chapter IV where NORE1A scaffolds GSK-3 β to β -TrCP to Regulate β -catenin.

NORE1A cooperates with GSK-3 β to induce cellular senescence

In primary mammalian cells, expression of oncogenes, such as activated Ras, induces premature senescence rather than transformation [248]. Ras (Oncogene) induced senescence can be bypassed by a number of different genetic events including inactivation of the p53/p21/p19ARF pathway, inactivation of the Rb family of proteins, and aberrations in p53, PML, SIR2, KLF4, and YAP/TAZ [248, 249]. Recent research has now identified GSK-3 β as a key player in the activation of cellular senescence [248, 250, 251]. These reports demonstrate that loss GSK-3 β expression is a key factor that determines whether a cell transforms or becomes senescent in the presence of constitutively active Ras [248]. Exactly how GSK-3 β functions in senescence is not exactly clear and likely occurs on multiple levels including regulation of β -catenin, p53, and potentially partial suppression of TEAD genes [248, 249, 252]. NORE1A is a

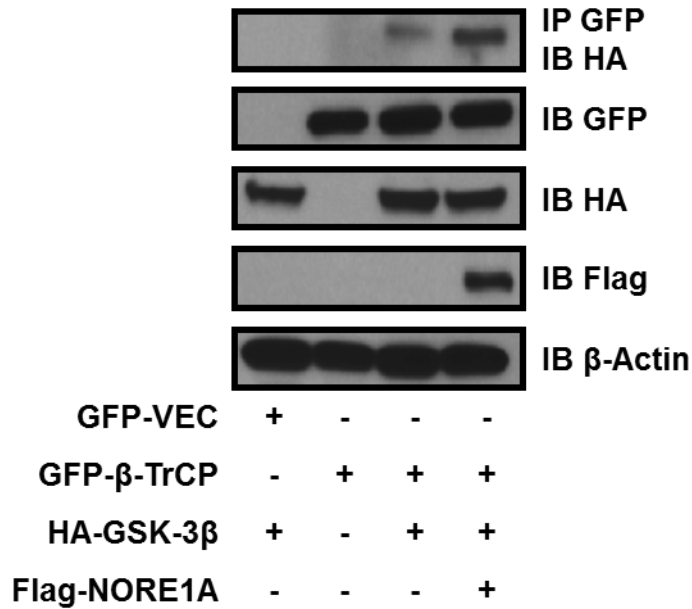


Figure 42. NORE1A Scaffolds GSK-3β to β-TrCP - NORE1A may be functioning as a scaffolding molecule stabilizing a protein degradation complex comprised of a priming kinase and associated ubiquitin ligase. Reports have shown that TAZ protein levels are regulated by GSK-3β and β-TrCP. Perhaps NORE1A functions as a scaffolding molecule and stabilizes these proteins in a complex. To test this, HEK-293T cells were transfected with expression constructs for GSK-3β, β-TrCP, and NORE1A. 24 hours post-transfection, cells were lysed and immunoprecipitated. GSK-3β and β-TrCP were detected weakly in a complex. However, in the presence of NORE1A, the association between GSK-3β and β-TrCP was greatly enhanced.

powerful senescence effector that functions through both p53 and possibly other pathways including Rb and p16 [14]. Now that I have shown that NORE1A binds and scaffolds GSK-3 β , and considering that both NORE1A and GSK-3 β are implicated in activating Ras (oncogene) induced senescence (OIS), I sought to determine whether NORE1A functions through GSK-3 β to shift cells into a senescent phenotype. To achieve this, I took A549 cells and transfected them with either an empty vector or an expression construct expressing a GFP-tagged NORE1A. 6-hours post-transfection the cells were then treated with LiCl₂ or water, the carrier solution, at a final concentration of 10mM for 72-hours. The cells were then processed using a β -gal senescence kit. The number of positive blue cells were counted and quantified. Results show that NORE1A strongly induced a senescent phenotype in the A549 cells and this effect was suppressed by the LiCl₂ suggesting that NORE1A is using GSK-3 β in part as a senescence effector (**Figure 43, 44**).

NORE1A Induced Senescence is Suppressed By Overexpression of TAZ

While GSK-3 β is known to be able to activate senescence via several cellular targets including p53 and β -catenin[250, 251], recent research has now identified that loss of TEAD transcriptional activity can also be a factor to induce cellular senescence [249]. Accordingly, the potential exists that NORE1A's regulation of TAZ via GSK-3 β functions as another arm of NORE1A senescence. Since I have shown that NORE1A indeed induces senescence by GSK-3 β , I were then curious whether this was due to its regulation of TAZ or if NORE1A and GSK-3 β were cooperating to induce senescence via another target.

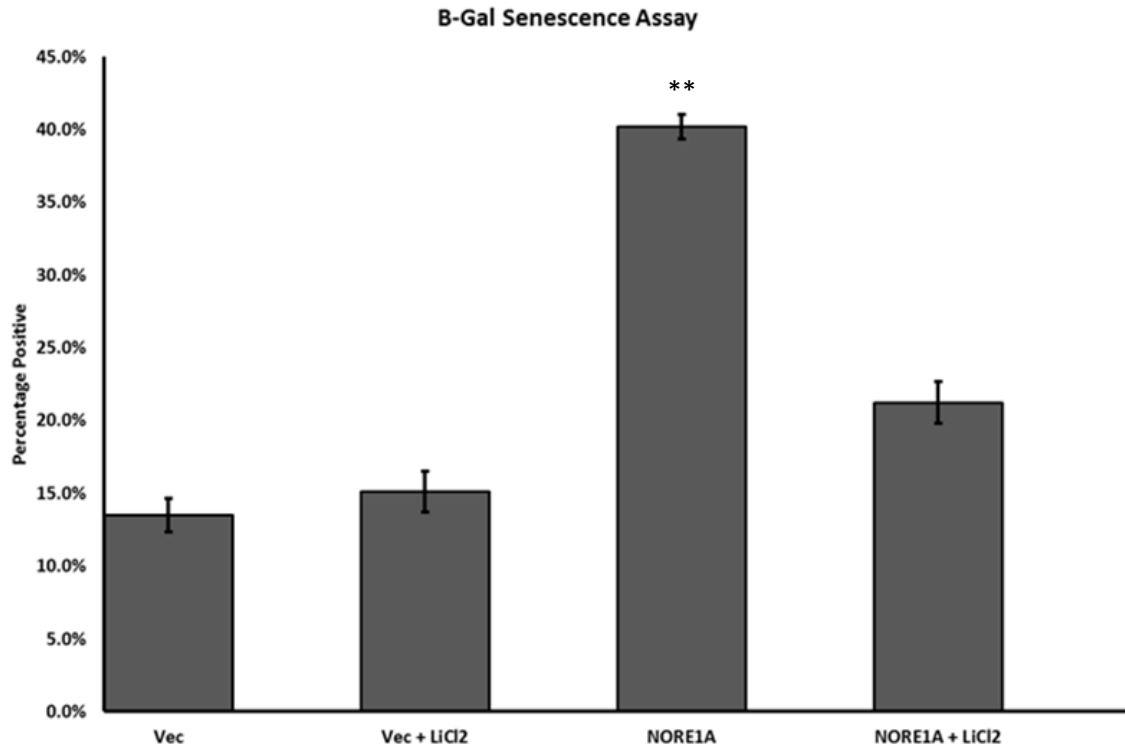


Figure 43. Lithium Chloride Blocks the Ability of NORE1A to Induce Senescence - NORE1A is a powerful inducer of senescence. While p53 is one of the better-characterized pathways in which NORE1A functions in senescence, I have data that now suggests that NORE1A can also induce senescence through alterations in the Hippo pathway endpoint, TAZ. Loss of TEAD promoter activity has been reported to shift cells into senescence [249]. So, I sought to determine whether suppression of GSK-3 β impairs the ability of NORE1A to induce cellular senescence. A549 cells were transfected with expression constructs expressing an empty vector or NORE1A. 6-hours post-transfection, the cells were treated with LiCl₂ at a final concentration of 10mM for 72 hours. The cells were then processed for β -gal activity using a senescence detection kit. The cells were then counted and the results quantified. As expected, NORE1A strongly shifted cells into senescence ($p=0.05$). This effect was impaired in the presence of LiCl₂, supporting the hypothesis that NORE1A is working with GSK-3 β to activate senescence.

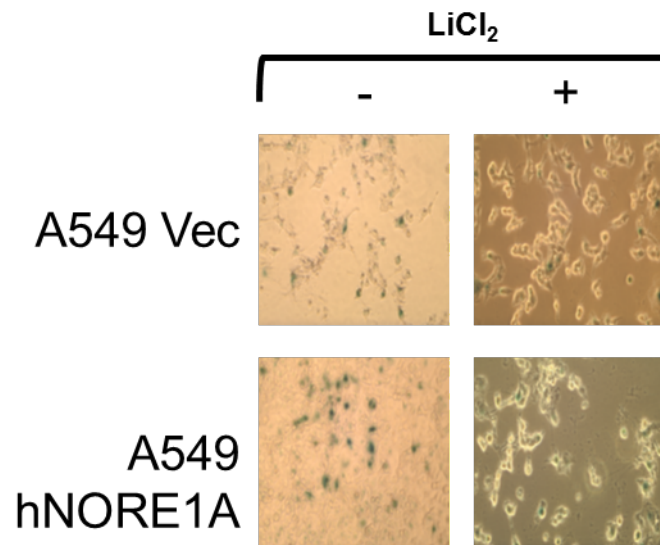


Figure 44. Representative Pictures from the NORE1A Senescence Assay - Representative photographs taken of A549 cells in the senescence assay described in Figure 10. Positive cells are easily seen with blue color, most prevalent in the A549 cells transfected with NORE1A and not treated with LiCl₂.

To test this hypothesis, I surmised that excessive over-expression of TAZ would over-come the ability of NORE1A to regulate TAZ and induce senescence, thus reversing or suppressing the ability of NORE1A to induce senescence. A549 cells were transfected with an empty, and expression construct for NORE1A, and then an expression construct for NORE1A along with an expression construct for TAZ transfected in a 5 times the amount used in previous experiments. Here I found that NORE1A on its own increased the level of cellular senescence from roughly 12% background level to roughly 42% (**Figure 45**). However, when levels of TAZ were elevated to where NORE1A could no longer successfully regulated them, the ability of NORE1A to induce senescence was suppressed and only about 20% of the cells were found to be β -gal positive (**Figure 45**). Thus, this experiment defines a novel NORE1A senescence arm and NORE1A fulfills its role as a senescence effector by the downregulation of TAZ, suppressing its ability to co-activate TEAD promoter genes.

5.3 - Discussion

The Hippo signaling pathway performs key roles in both organ size and tumorigenesis; however, components of the Hippo pathway are deregulated in various human cancers. In canonical Hippo signaling, the core mechanism employs a kinase cascade consisting of the MST1/2 and LATS1/2 kinases to phosphorylate the major functional output components YAP and TAZ, marking them for degradation [204, 253].

YAP and TAZ are transcriptional co-activators that exert powerful survival signals and also have oncogenic potential when improperly regulated. They require precise and

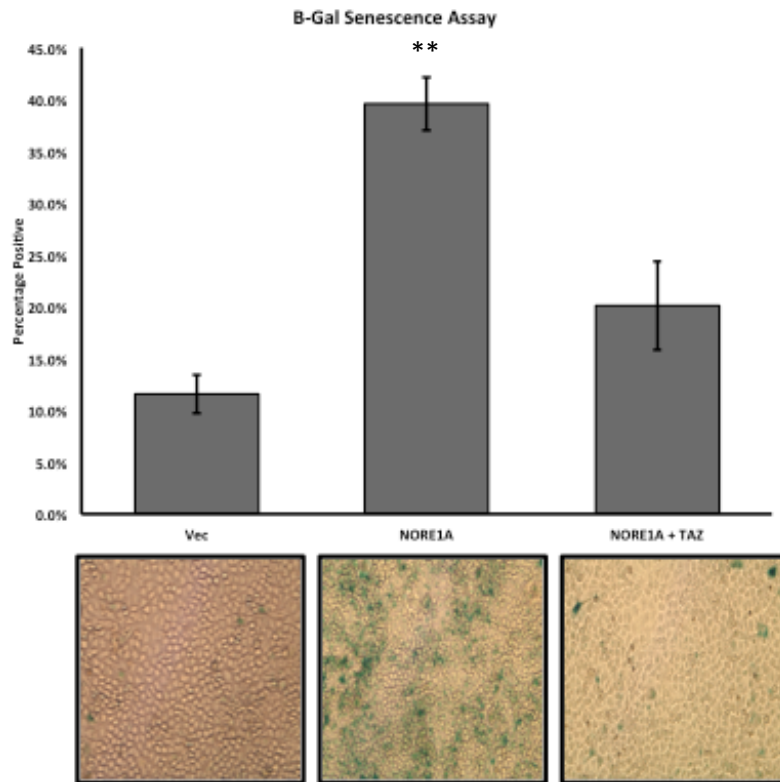


Figure 45. Identification of a Novel NORE1A Senescence Arm – NORE1A cooperates with GSK-3 β to drive senescence by suppressing TAZ mediated co-activation of TEAD. A549 cells were transfected with expression constructs expressing an empty vector, NORE1A, and NORE1A along with excessive levels of TAZ. NORE1A again powerfully induces senescence ($p=0.05$). However, this induction is over-powered when levels of TAZ rise above the ability of NORE1A to properly regulate it. Thus, NORE1A is able to induce senescence by the downregulation of TEAD genes co-activated by TAZ.

specific regulation to maintain cellular homeostasis. YAP and TAZ each share approximately 50% sequence homology, yet they contain differences including lack of an N-terminal proline-rich domain and a SH3 binding motif [204]. This would suggest YAP and TAZ potentially have both conserved and non-conserved modes of regulation [204]. YAP/TAZ are rarely found mutated in different cancers, yet improper regulation of these proteins is commonly observed in human tumors [254, 255]. Conversely, suppression of YAP and TAZ protein levels results in inhibition of cellular proliferation and a shift to apoptosis and senescence [256]. Taken together, this suggests that its not mutations in YAP and TAZ specifically, but dysfunction in their regulatory mechanisms that can drive oncogenesis.

Although YAP and TAZ are co-regulated by Hippo signaling, TAZ differs from YAP in possessing a unique, Hippo independent phospho-degron [18]. This phospho-degron is recognized by the GSK-3 β kinase and phosphorylation of TAZ by GSK-3 β promotes its degradation via the β -TrCP degradation complex. Thus, TAZ and YAP can be both co- and differentially regulated.

One of the inputs that can regulate the Hippo pathway is Ras [232, 257]. Although Ras is a potent oncoprotein and is implicated in the development of the majority of human tumors [258, 259], activated Ras is paradoxically capable of stimulating apoptosis and senescence [7, 14]. The Hippo pathway is one of the best characterized of these Ras death effector pathways. Ras is connected to Hippo pathway by its effector RASSF1A. RASSF1A is a member of the RASSF family of proteins and directly binds MST kinases [260]. The interaction of Ras with RASSF1A promotes the activation of the MST kinases and therefore Hippo pathway activation, leading to down-regulation of YAP. Many tumors exhibit loss of RASSF1A expression [126, 261], thus uncoupling Ras from Hippo and facilitating transformation.

NORE1A, like RASSF1A, is a RASSF family member that serves as a direct, pro-apoptotic Ras effector [10, 11]. NORE1A suppresses tumor cell growth, is frequently inactivated in human tumors, and genetic inactivation of NORE1A is implicated in a hereditary cancer syndrome [15, 126, 187]. Therefore, NORE1A is likely to serve as an important human tumor suppressor and its loss results in a marked cellular oncogenic shift. Like RASSF1A, NORE1A also binds the MST kinases directly, and thus has the potential to connect Ras to the Hippo pathway [10]. However, deletion mutants of NORE1A that cannot interact with MST kinases retain the ability to inhibit cell growth and suppress the tumorigenic phenotype [129]. Moreover, a thorough biochemical analysis did not support an obvious role for NORE1A in MST kinase activation [135]. Thus, unknown tumor suppressor pathways independent of the canonical Hippo signaling pathway must be modulated by NORE1A.

We recently showed that NORE1 forms a direct, endogenous complex with β -TrCP [212]. β -TrCP is the E3 substrate recognition component for the SCF- β -TrCP ubiquitin ligase complex, and is a key element in regulation of both Wnt and Hippo. During my experiments examining the role of NORE1A in Hippo signaling, I noticed that NORE1A appeared much more active towards TAZ than YAP. Bearing in mind it has been shown that NORE1A does not seem to activate MST kinases, even though it binds them [135], I wondered if NORE1A might be activating a TAZ specific/ β -TrCP non-canonical degradative pathway.

My experiments have shown that NORE1A does indeed preferentially suppress cellular TAZ protein levels via β -TrCP. However, I also found that NORE1A not only interacts with β -TrCP, but also forms an endogenous complex with the kinase responsible for TAZ phosphorylation: Glycogen synthase kinase 3 Beta (GSK-3 β).

GSK-3 β is a serine/threonine kinase that is an important component of diverse signaling pathways involved in the regulation of cell survival, protein synthesis, glycogen metabolism, cell mobility, and proliferation [220]. It appears to function to suppress tumorigenesis, yet the roles in which GSK-3 β operates as a tumor suppressor are not fully characterized [221]. One of the better-studied roles of GSK-3 β in tumor suppression is centered on its control of β -catenin, the terminal executor of the Wnt signaling pathway. Phosphorylation of β -catenin by GSK-3 β targets it for ubiquitination by the substrate recognition protein β -TrCP resulting in its subsequent proteasomal degradation.

Unlike many other protein kinases in the cell, GSK-3 β has constitutively active kinase activity [262]. Thus, control of GSK-3 β function may rely on targeting proteins that control its coupling to the appropriate substrate. NORE1A may serve as one of these targeting proteins, for when I used a specific GSK-3 β inhibitor; I blocked the ability of NORE1A to promote TAZ degradation. Thus I reveal a new function for NORE1A as a scaffolding molecule for GSK-3 β . This links NORE1A to the potential control of a broad range of targets some of which include β -catenin, Snail, Smad1, Smad3, BCL-3, p21, HIF-1 α , and Cyclin D1 [263]. However, although I found that the interaction of NORE1A with β -TrCP was Ras dependent [212], its interaction with GSK-3 β was not. Therefore, NORE1A may have Ras dependent and Ras independent effects on GSK-3 β activity.

One of the most interesting aspects of this study is the revelation that NORE1A differentially regulates Hippo pathway endpoints. Much of the research on RASSF family proteins attributed their tumor suppressive capabilities to their binding of the MST kinases, and subsequent activation of canonical Hippo signaling as a conserved function. However, closer examination of the relationship between RASSF proteins and

Hippo has revealed that their relationship is much more complicated [129, 133]. While it is well demonstrated that RASSF1A can regulate YAP stability by enhancing MST/LATS phosphorylation on S127, I have never observed NORE1A to do the same, despite the fact that I, and others, have shown that NORE1A complexes with MST. Thus, NORE1A must be performing other tasks with MST; however these tasks are not involved in the regulation of Hippo signaling and at this point remain largely uncharacterized.

NORE1A still remains connected into the Hippo signaling pathway, but unlike its close family relative, RASSF1A, NORE1A functions non-canonically and specifically regulates the Hippo pathway endpoint, TAZ. Initially, I was unsure what the implications of the specific regulation of one of the two Hippo pathway endpoints were on the cell until I found a report showing that varying degrees of alterations in TEAD transcriptional output alter whether the pathway is apoptotic or senescent [249]. Explained further, the full suppression of YAP and TAZ mediated TEAD activation by canonical Hippo signaling is indeed apoptotic, yet partial suppression of TEAD by specific regulation of YAP or TAZ by non-canonical mechanisms induces a senescent phenotype preferentially over the activation of apoptosis. This provides some of the first explanations why RASSF1A appears to be an apoptotic effector while NORE1A appears to be a senescence effector.

Thus, I identify a novel role for NORE1A in scaffolding the protein kinase GSK-3 β to ubiquitin ligase β -TrCP. NORE1A is key in the stabilization of this complex and loss of NORE1A impairs the ability of GSK-3 β and β -TrCP to properly regulate substrates such as TAZ. Furthermore, I now know that NORE1A can complex with several other kinases involved in β -TrCP phosphodegron process including HIPK2 [14], Aurora Kinase, and PLK-1 (unpublished observations). Thus, this highlights a new potential role for NORE1A in protein regulation by functioning mechanistically as a scaffolding various protein kinases to β -TrCP

CHAPTER VI

CONCLUSION

This work identifies a completely new and unanticipated signaling pathway for the Ras oncogene. It reveals that NORE1A forms a direct, physiological, Ras regulated complex with the ubiquitin ligase substrate recognition protein β -TrCP. This allows Ras to use NORE1A as an effector to regulate the stability of multiple proteins playing key roles in growth, transformation, and death. Mechanistically, it shows NORE1A acts as a scaffolding protein facilitating complex formation between GSK-3 β and β -TrCP to enhance proteasomal regulation of β -catenin and TAZ, which are the active end points of the Wnt and Hippo pathways respectively (**Figure 46**).

These results provide a mechanistic explanation for some of the contradictory reports previously published in the literature describing the relationship between Ras and Wnt. In the presence of NORE1A Ras is a negative regulator of β -catenin, but in its absence is a positive regulator. Moreover, these results also explain discrepancies in the reported role of NORE1A in the regulation of the Hippo pathway. They show that NORE1A does not activate the canonical Hippo pathway, but exhibits a potent and specific non-canonical regulation of the TAZ half the YAP/TAZ transcriptional co-activator endpoints of Hippo signaling. This allows us to explain why NORE1A is far more pro-senescent than pro-apoptotic.

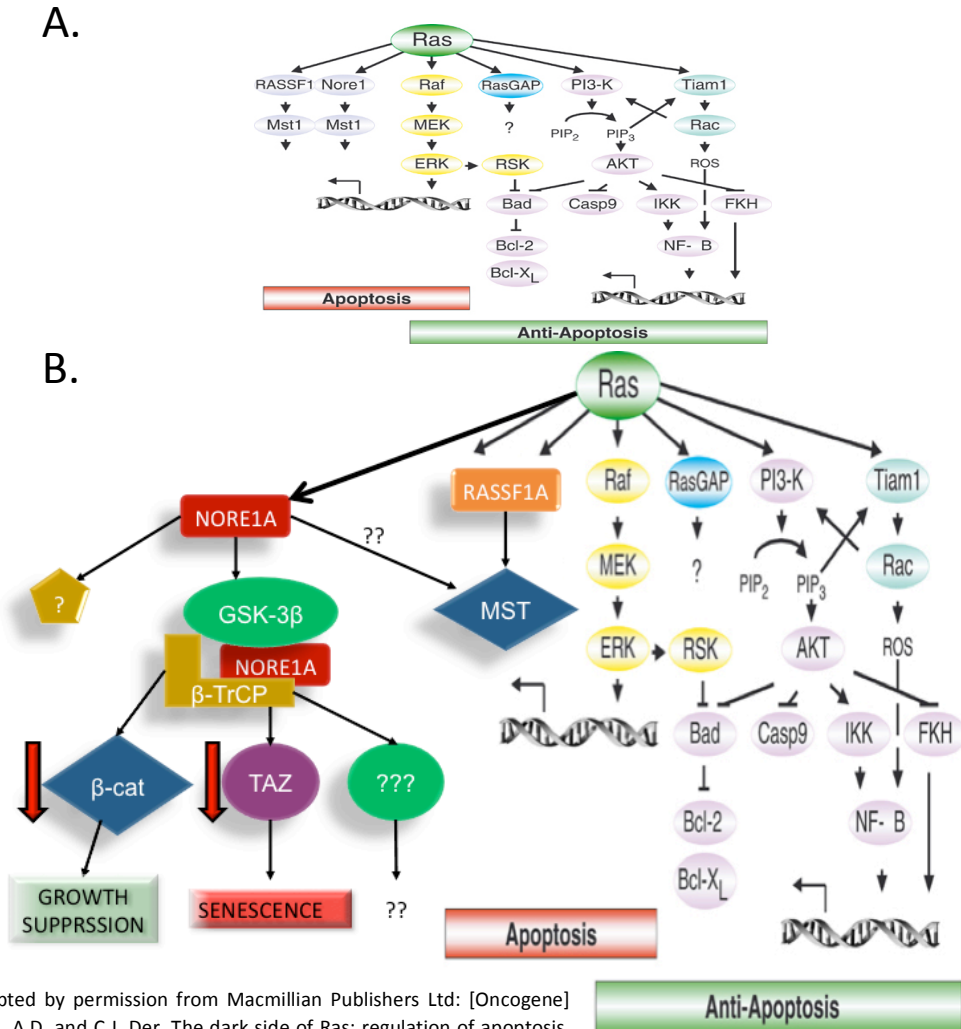


Figure 46. Diagram of the Novel Ras/NORE1A/GSK-3 β / β -TrCP Signaling Pathway

– This schematic shows the previously accepted model for NORE1A mediated tumor suppression (A) along with my Novel Ras/NORE1A signaling pathway allowing for the coordinate control of the Wnt (β -catenin) and Hippo (TAZ) signaling pathways (B). My results show that NORE1A scaffolds GSK-3 β to β -TrCP qualitatively and quantitatively enhancing their phosphorylation and ubiquitination of oncogenic substrates. Moreover, I identify a novel NORE1A senescence arm where NORE1A splits the Hippo, specifically regulating TAZ and not YAP, resulting in the partial suppression of TEAD transcriptional activity and the activation of cellular senescence.

Experiments showing that NORE1A has no effect on the β -TrCP target I κ B confirmed that the pathway is specific to a sub-set of the broad range of β -TrCP target proteins. This may help explain why β -TrCP has been reported to exhibit both oncogenic and tumor suppressive functions in different systems as levels of NORE1A present may dictate its balance of forces.

Future studies should be centered on finding other kinases that cooperate with β -TrCP in protein regulation and investigating whether NORE1A also scaffolds them into a single kinase/ubiquitin ligase complex. Key kinases worth investigating are PLK-1 (involved in Claspin regulation), HIPK2 (involved in regulation of numerous proteins), and RSK1 (involved in DEPTOR regulation). Additionally, other β -TrCP substrates should be examined to see if NORE1A is involved in their regulation as well. One that immediately comes to mind is the MDM2 ubiquitin ligase. This ubiquitin ligase controls p53 stability and since NORE1A and p53 cooperate to induce senescence, a Ras regulated mechanism where NORE1A cooperates to negatively regulate MDM2 is an intriguing possibility and warrants examination. Such an interaction might allow coordinate suppression of TAZ and activation of p53 to achieve senescence.

Finally, this project may also have ramifications for personalized medicine focused on Ras tumors, as Ras tumors lacking NORE1A expression may be quite different in the utilization of the Wnt and Hippo pathways compared to those retaining the expression of NORE1A. Therefore, patients receiving treatments for a Rasopathy should have the NORE1A status of the tumor determined to see if they require coordinate therapies targeting Wnt and Hippo as well the oncogenic form of Ras. Coordinate therapies may enhance the effectiveness of targeting mutated Ras and may finally give cancer patients a better chance for surviving cancer.

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