12-2015

Regulation of the retinoblastoma tumor suppressor by the novel Ras effector NORE1A.

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REGULATION OF THE RETINOBLASTOMA TUMOR SUPPRESSOR BY THE NOVEL RAS EFFECTOR NORE1A

By

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B.A., University of Louisville, 2009
M.S., University of Louisville, 2013

A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

in

Biochemistry and Molecular Genetics

Department of Biochemistry and Molecular Genetics
University of Louisville
Louisville, KY

December, 2015

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A Dissertation Approved on

November 9, 2015

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DEDICATION

This dissertation is dedicated to my mother, Laurence Chanu Barnoud, the most beautiful person the world has seen. I love you, forever and always.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank all of those who played a role in the progression of this project as well as my growth and improvement as a scientist. First and foremost, I want to thank Dr. Geoffrey Clark for his efforts and perseverance throughout my entire graduate career. More importantly, I cannot thank him enough for his persistent efforts in helping me achieve my ultimate goal of obtaining a high caliber post-doctoral position. This was something I refused to compromise on, and Dr. Clark was pivotal in making that happen. I want to thank Dr. Howard Donninger, whose list for reasons why I am so grateful would most likely exceed the size of this dissertation. It must be noted that it was because of his vigorous drive and perseverance that I have developed the work ethic that I have today. In addition, he has engraved in me the valuable moral of always being humble, and that will be something that I carry with me throughout my scientific career. I must thank Dr. David Samuelson, Dr. Marcie Cole, and Dr. Christine Schaner Tooley for their support and encouragement, especially in 2015. I can honestly say that if it were not for these five people, I would have quit graduate school. It is only because of them that I was able to survive during the darkest days of my life, and come out on the other side a better, stronger man.

I must thank all of the current lab members of the Geoffrey Clark lab, including Dr. Lee Schmidt and Jessica Mezzanotte Sharpe. Dr. Schmidt provided much needed guidance from the start of my graduate studies, but more importantly continuously

provided guidance throughout the preparation of this dissertation, which allowed me to spend additional time in the laboratory, trying to accomplish my goal of publishing my work in a high impact factor journal. To Jessica: thank you for playing a pivotal role in maintaining laboratory homeostasis. More importantly, you have allowed me to enjoy one of the greatest rock and roll bands of all time, Queen.

I must also thank my family for everything that they have instilled in me and for making me the person who I am today. To my brother, Benoit Barnoud, who taught me that the most important thing in life is family. To my father, Philippe Barnoud, who has told me over and over again that we must never, never give up. To my uncle, François Chanu, and my cousins Pauline, Alexandre, and Hadrien, who have provided me the courage and strength to fight the toughest battles life brings. And to the most important person of all, my mother, Laurence Chanu Barnoud, the most genuine, selfless, giving, loving, and caring person in my life. May everyone realize that the world is better place because of you. Mom, it's never goodbye…it's see you later.

I must also thank in his own section the living legend Rex Ecarma, head coach of the University of Louisville Men's Tennis team. Without a doubt, Coach Ecarma played the most pivotal role in molding me into the man that I have become today. He taught me the importance of respect, honesty, trust, perseverance, integrity, patience, courage, and loyalty. Coach became a father to me and words cannot describe how important he was in my growth as a tennis player and as a person. Don't wish for it, work for it.

Last but not least, I must thank the amazing Dr. Bill Dean. If it wasn't for him, I would have never started graduate school. Though I have the tendency to fall off the face of the earth, I will never forget that my career as scientist started thanks to you.

ABSTRACT

REGULATION OF THE RETINOBLASTOMA TUMOR SUPPRESSOR BY THE NOVEL RAS EFFECTOR NORE1A

Thibaut François Barnoud

November 9, 2015

Ras is the most frequently mutated oncogene in human cancers. It acts as a critical branch point in signal transduction, regulating numerous downstream effectors involved in cell growth and differentiation. While Ras has the capacity to activate many growth promoting pathways, it can paradoxically regulate growth inhibitory pathways leading to apoptosis and cell cycle arrest. One of the ways Ras can inhibit the growth of cells is via a family of effectors called the RASSF proteins. One of the members of this family, RASSF5, also known as Novel Ras Effector 1A (NORE1A), is a tumor suppressor that is frequently inactivated in human tumors by epigenetic mechanisms. NORE1A binds directly to Ras, and it promotes the growth inhibitory properties of Ras by activating a tumor suppressive function called oncogene induced senescence. We have recently shown in the laboratory that NORE1A serves as a senescence effector of Ras by activating the p53 tumor suppressor. However, knockdown of p53 using siRNA technology did not completely abrogate the ability of NORE1A to induce senescence,

suggesting that there may be additional mechanisms by which NORE1A promotes senescence. The other well characterized pathway involved in Ras-induced senescence is the Rb pathway. Using immunoprecipitation techniques, we show that NORE1A forms an endogenous, Ras regulated complex with the Rb phosphatase PP1A, and that Ras/NORE1A promote the stability of the PP1A protein. Furthermore, our results show that NORE1A scaffolds PP1A to Rb in a Ras dependent manner, in turn promoting the dephosphorylation of Rb, a pro-senescent event. Using western blot analysis, we found that NORE1A can also regulate non-phosphorylation post-translational modifications of Rb, including its acetylation and SUMOylation. Using commercially available senescence detection kits, we found that loss of Rb significantly suppressed the ability of NORE1A to induce senescence in both murine and human systems. Collectively, our work strongly suggests that NORE1A is a double barreled Ras senescence node, explaining why Ras driven tumors often show loss of NORE1A expression.

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CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1 – Overview:

Cancer is the second leading cause of death among Americans, only after heart disease (1). More importantly, one of every four deaths in the United States is due to cancer (2). This disease is projected to reach healthcare costs of over 200 billion dollars by 2020 (3), highlighting the urgency not only for improved cancer therapies, but discovering novel and innovative treatments for currently undruggable cancers. Ultimately, the solution may lie in unraveling the complexities of cancer progression and gaining a better understanding of tumor development.

It is widely believed that in order for a normal cell to transform into a cancer cell, genes that regulate growth and differentiation must be altered. Cancer can be caused by alterations in oncogenes, tumor suppressor genes, and microRNAs (4). It was hypothesized over thirty years ago that cells contain proto-oncogenes, genes that are involved with normal aspects of behavior, and that such proto-oncogenes might be mutated to give rise to oncogenes. In turn, these oncogenes have the ability to convert normal cells into cancer cells (5).

Some of the most significant and well-characterized proto-oncogenes in human cancer are the three Ras proto-oncogenes. Mutations in one of these three genes can give rise to a constitutively active Ras protein. Such mutations can be a critical step in tumor initiation and development, because aberrant Ras signaling will continuously activate downstream effector pathways involved in growth, differentiation, and cell survival (6). Recent advances in genetic technology have enabled researchers to examine the true significance of Ras in cancer: over one third of all human cancers contain activating point mutations in the Ras gene (7). From an oncogenic standpoint, Ras can regulate numerous growth promoting pathways, including the classic trio of Ras effector pathways Raf, phosphoinositide 3-kinase (PI3K), and ral guanine nucleotide dissociation stimulator (RalGDS) (7-9). Furthermore, functional studies have shown that at least six Ras effector signaling pathways are involved in either the initiation or the maintenance of Ras driven cancers (10).

However, counterintuitive evidence dating as far back as the early 1980s pointed to the notion that Ras does not exclusively promote the growth of cells (11). It has now become clear that while oncogenic Ras can promote transformation by regulating numerous growth promoting pathways, Ras can paradoxically promote apoptosis and senescence (12). However, the mechanisms by which Ras can inhibit the growth of cells are poorly understood. More recent evidence has implicated the RASSF family of tumor suppressors as negative effectors of Ras (13). RASSF5, or Novel Ras Effector 1A (which from this point forward will be referred to as NORE1A), was the first identified member of the RASSF family, and has been shown to bind directly to Ras (14) in order to activate Ras mediated growth inhibitory pathways. More importantly, loss of NORE1A

expression due to epigenetic mechanisms can be found in a variety of different tumors (15), supporting its role as a tumor suppressor.

Initial evidence of NORE1A acting as a tumor suppressor was quite strong. First, transient over-expression of NORE1A can lead to apoptosis (16). Second, restoring NORE1A expression in a NORE1A negative cell line was shown to inhibit its transforming phenotype (17). And in some cases, restoring NORE1A expression in tumors that have lost NORE1A expression can significantly diminish the tumorigenic phenotype (16). This work, along with data showing that the dysregulation of NORE1A is implicated in a rare familial cancer syndrome (18), confirms that NORE1A is a *bona fide* tumor suppressor. While NORE1A has now been shown to have powerful tumor suppressive functions, in part by regulating the p53 and HIPPO tumor suppressor pathways (12,19,20), the significance of NORE1A as a negative effector of Ras is only partially understood.

Recent evidence showed that NORE1A is critical senescence effector of Ras, in part by activating the pro-senescent post-translational modifications of p53 (20). But, in addition to p53, Ras can promote senescence by activating the Retinoblastoma (Rb) protein, a tumor suppressor believed to be inactivated directly or indirectly in virtually all human cancers (21). Rb is primarily activated by dephosphorylation, and phosphatases such as PP1A promote the activation of Rb by dephosphorylation (22). Interestingly, PP1A was detected in complex with NORE1A in a yeast two-hybrid screen (23). Using state of the art biological techniques, including immunoprecipitations, western blot analysis, luciferase assays, and senescence assays, we sought out to determine if Ras could regulate Rb via its effector NORE1A. The collective work and data depicted in this

dissertation highlights the discovery of a novel signaling pathway by which Ras and NORE1A cooperate to suppress growth by activating the tumor suppressive functions of Rb, in turn promoting the onset of cellular senescence. Here, we will show that NORE1A can bind to and stabilize PP1A. This interaction is significantly enhanced by Ras, in turn allowing PP1A to activate Rb by promoting its dephosphorylation, a pro-senescent event (24). Furthermore, we show that NORE1A requires Rb in order to fully promote its senescent function.

This work identified a further mechanism by which NORE1A can promote oncogene induced senescence caused by activated Ras. After characterizing the role of NORE1A mediated dephosphorylation of Rb, we decided to investigate the potential for NORE1A to alter additional post-translational modifications of Rb. Initial experimental work found that Ras/NORE1A enhanced the total levels of Rb acetylation, currently thought to be an activating event, in part by maintaining Rb in its active, de-phosphorylated state. We go on to show that Ras/NORE1A can also modulate the SUMOylation of Rb. While the biological significance of this Rb modification remains unclear, this is the first line of evidence showing that Ras can regulate the SUMOylation of downstream targets using effector proteins such as NORE1A.

We also present evidence that, in addition to affecting the post-translational modifications of Rb, NORE1A also seems to stabilize Rb. Thus, we identify a novel function for NORE1A, acting as a Ras regulated scaffolding molecule to enhance PP1A binding to Rb to promote its activation, in turn promoting the induction of senescence.

This finding could have a significant impact, based on the notion that, depending on its regulatory molecules, PP1 can form stable complexes with as many as 650

mammalian proteins (25). Furthermore, we have provided evidence suggesting that Ras can regulate both the acetylation as well as the SUMOylation machinery, in addition to its proto-typical ability to affect protein function by phosphorylation and de-phosphorylation events. These additional findings could have major ramifications as to how Ras can regulate protein function on multiple levels, and that the code of post-translational modifications regulated by Ras could have adverse effects on protein localization, stabilization, and activity.

1.2 – The Ras Oncogene:

The Ras genes may be the most well characterized oncogenes in human cancer (26). High frequency of Ras mutations occurs in a wide spectrum of human cancers, including three of the four most deadly cancers in the US (lung, colon, and pancreatic cancer) (27), highlights the importance of Ras as an integral component of tumorigenesis. Here, we provide a chronology of the most important discoveries regarding Ras protein structure and function, the activation and regulation of Ras, the mechanisms of Ras signal transduction, both growth promoting and tumor suppressive, and its implications in cancer.

1.2.1 – The Discovery of Ras:

The identification of Ras emerged during the extensive study of acutely transforming retroviruses from mice, rats, cats, and chickens (28). The path to the

discovery of Ras began in 1964, when Jennifer Harvey discovered that preparing a murine leukemia virus, obtained from a leukemic rat, and injecting it into newborn rodents would induce sarcomas (29). The Kirsten-MSV (murine sarcoma virus) was identified in 1967 by serial passaging of murine leukemia viruses through Wister-Furth rats (30). It was not until 1975 that Scolnick et al. showed that these viral strains were recombinant viruses that carry sequences derived from the rat genome. These findings, work that many at the time thought to be irrelevant to human cancer, provided the first line of evidence of what were termed oncogenic genetic elements (28). Unfortunately, the realization that human cancers are not initiated by transforming retroviruses dampened enthusiasm.

These genes, termed Ras for rat sarcoma virus, were identified in the human genome in 1982. Three groups made the simultaneous discovery that transforming genes introduced by DNA transfection assay into NIH/3T3 cells were the same Ras genes identified earlier in the Kirsten and Harvey sarcoma viruses (31-33), and that the transforming effects were caused by a missense mutation in the Ras gene (34-37). Furthermore, the identification of mutant Ras genes in patient tumors but not in normal tissues was an important validation that the Ras mutations identified in cell culture were not just artifacts of cell passaging *in vitro* (28). While these discoveries were critical for unraveling the concept that mutations can turn proto-oncogenes into oncogenes and in turn play a significant role in tumor development, they fail to address how Ras functions, how it can be regulated, or the mechanism of signal transduction from Ras to downstream effectors.

1.2.2 – Ras and Cancer:

As the most commonly mutated oncogene in cancer, Ras has been thought of as the "Holy Grail" of cancer drug development (38). Early experimental work was slow and complicated due to the fact that 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. Initial investigations to understand the roles of activated Ras showed that oncogenic alleles contained a very specific set of point mutations.

Mammalian Ras genes acquire transformation-inducing properties by single point mutations with their coding sequences. Mutations in naturally occurring Ras oncogenes have been localized in codons 12, 13, 59, and 61 (37). In addition, *in vitro* mutagenesis studies have shown that mutations in codons 63, 116, and 119 can also confer transforming properties to Ras genes (39-41). Interestingly, the presence of a glycine residue at position 12 appears to be necessary for the normal function of Ras proteins, as it has been shown that substitution of Gly-12 by any other amino acid (with the exception of proline) results in the oncogenic activation of Ras (42). Second, substitution of Gly-13 also has transforming consequences for the harboring cells, though not all substitutions appear to have the same activating effect (39,43). Third, substitution of Gln-61 by any other amino acid residue, except Pro-61 and Glu-61, yields oncogenic Ras (44). The effects of other point mutations of Ras are less understood, though have been shown to turn Ras into an oncogene. Unlike the retroviral Ras oncogenes, which exhibit two mutations, all cellular Ras oncogenes only carry a single activating mutation (45). By

1983, all three members of the Ras family had been identified (46). Similar to H-Ras and K-Ras, the newly identified N-Ras was also found to contain activating point mutations in certain human tumors (47).

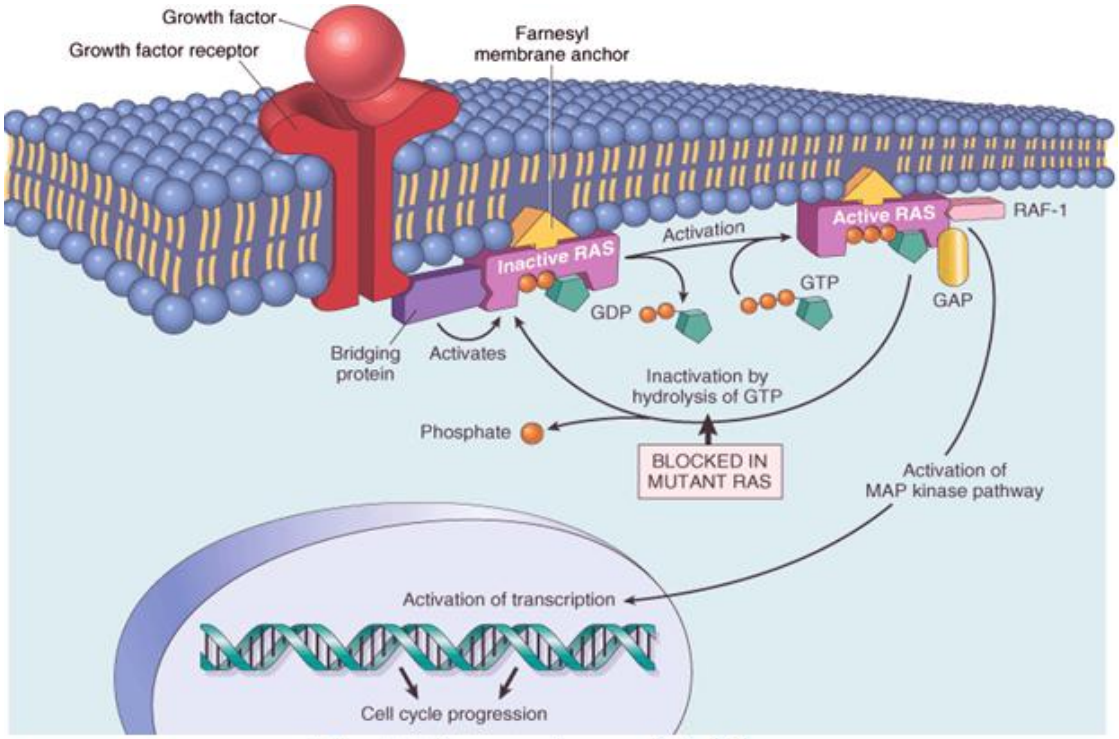
After the initial discovery of the Ras oncogene, reports began to surface noting that certain types of human cancers were specifically associated with a small number of specific mutations in the reading frame of the Ras gene. The most frequent Ras mutations are found in K-Ras, and they are frequently observed in pancreatic (48) and colon cancers (49). Second, N-Ras mutations were primarily associated with lymphoid malignancies (43,50-53) and melanomas (54). Lastly, H-Ras mutations were mostly associated with bladder carcinomas (55), though such mutations are found much less frequently compared to K- and N-Ras mutations. It was later found that mutations in Ras occur in approximately one third of all human tumors, with the highest incidence in the pancreas (90%), colon (50%), thyroid (50%), lung (30%) and leukemia (30%) (56).

1.2.3 – Regulation of Ras Activation:

Once the significance of Ras in cancer was discovered, work began to shift towards understanding how Ras functions to promote cancer and how it can be regulated. Ras proteins have since then been shown to bind guanine nucleotides and possess intrinsic GTPase activity (57). The biochemical properties of Ras proteins closely resemble those of the G proteins involved in the modulation of signal transduction through transmembrane signaling systems (58). The bound guanine moiety determines the activity of G proteins: when a G protein is bound to a GTP molecule, the protein is

typically in the active conformation. However, heterotrimeric G proteins were known to possess an intrinsic GTP hydrolysis activity (which from this point forward will be referred to as GTPase activity) (**Figure 1**). Thus, in order to regulate its activity, the G protein will hydrolyze GTP into GDP, in turn shuttling itself into an inactive conformation. This on/off switch is critical for the proper regulation of G proteins such as Ras, and reactivation occurs when the GFP is exchanged for a GTP molecule, which is typically more abundant in the cell (57).

Further evidence showed that mutations of the Ras gene can cause the Ras oncoprotein to behave differently than their normal counterparts (59-61). The observation that many oncogenic mutants of Ras are reduced in GTPase activity led to the initial belief that Ras mediated transformation occurred as a result of the impaired intrinsic GTPase activity of Ras (59-62); however, this theory would eventually be proven wrong. Analysis of GTPase activities associated with a large number of Ras mutants failed to reveal a quantitative relationship between GTPase activity and transforming potential (44,63-65). The notion that mutant Ras was only about 8-fold less active than wild-type Ras had many speculating if this was sufficient to account for its transforming power (66). Work performed by Trahey and McCormick went on to show that a GTPase activating protein (GAP) allows normal Ras proteins to turn themselves off efficiently by greatly enhancing the GTPase activity of Ras, whereas oncogenic Ras proteins stay locked in the active, GTP-bound form (67). Contrary to the initial hypothesis, it is the resistance to GAPs that leaves Ras proteins permanently GTP bound. Thus, the resistance to GAP activity is far more critical for Ras transformation than its own intrinsic GTPase activity.



Model for action of RAS gene

<http://medicinembbs.blogspot.com/2011/02/essential-alterations-for-malignant.html>

Figure 1: Model for Ras Activation. Mitogenic stimuli, such as growth factors, can regulate the activity of Ras, which is predominantly found on the inner leaflet of the plasma membrane. Ras can act as a critical branch-point in signal transduction: the GTP-bound form is active while the GDP-bound form is inactive. GTP loading caused by guanine nucleotide exchange factors (GEFs) induces a conformational change in Ras that allows it to bind effectors via their Ras-binding domains. In its GTP-bound form, Ras interacts with and regulates numerous downstream effectors, such as MAPK. Ras can be inactivated by its intrinsic GTPase activity, which can be accelerated by the binding of GTPase activating proteins (GAPs). This converts GTP back to GDP, returning Ras to the off conformation.

1.2.4 – Post-Translational Modifications of Ras:

Extensive work using deletion mutants of mammalian Ras genes has led to the identification of the carboxyl terminal sequence Cys^{186}-A-A-A-X-COOH (68). Interestingly, it was shown around that time that Ras proteins have been localized to the inner side of the plasma membrane (69). More importantly, mutants lacking Cys-186 code for proteins that remain in the cytosol and cannot induce transformation of NIH3T3 cells, suggesting that this post-translational modification is required for the proper biological function of Ras (70). Interestingly, blocking C-terminal modifications of human H-Ras using C-terminal antibodies impaired normal GTP-binding function (71), validating the notion that the C-terminal processing and subsequent recruitment of Ras to the membrane is required for proper Ras function.

Even though the Ras family members show some differences in sequence homology in the C-terminus, it has been shown that they all contain a consensus CAAX sequence at the C-terminus (72-74), where C is a cysteine residue, A is aliphatic, and X is any amino acid (75). This C-terminal domain acts as a key regulator of Ras function, in part by playing a role in proper localization of Ras to the inner leaflet of the plasma membrane (76). The mechanism and sequence of events for Ras localization to the cell membrane was elucidated in the early 1990s. First, a farnesyl pyrophosphate group is covalently attached to a cytosolic Ras protein by an enzyme called farnesyltransferase (77-79). Second, Ras-converting enzyme 1 (RCE1) causes a proteolytic cleavage of the last three amino acids of Ras. Third, isoprenylcysteine carboxyl methyltransferase 1 (ICMT1) methylates the last Cysteine residue. And lastly, an enzyme called

palmitoyltransferase (PTase) transfers an additional palmitoyl group to the Cysteine residue upstream of the C-terminus, in turn allowing for proper insertion of Ras into the inner cell membrane (80).

As was the case with the complexity of GTPase regulation of Ras activity, the post-translational modifications of Ras proteins involved in recruitment of Ras to the cell membrane were also not straight-forward. Further evidence pointed to additional mechanisms for full recruitment of Ras to the cell membrane (80). Some isoforms of Ras (including H-Ras, N-Ras, and K-Ras 4A) required additional palmitoyl groups to be attached to the C-terminal cysteine residue before being able to be attached to the cell membrane; however, K-Ras-4B does not have any palmitoylation at all, but rather contains a string of positively charged Lysine residues (known as the polybasic region) found upstream of the C-terminus that are sufficient to attach Ras to the membrane (81).

1.2.5 – Growth Promoting Pathways of Ras:

The discovery of Ras behaving as a critical oncogene to drive cancer development dates back to the early 1980s. This led to an onslaught of research to try and understand the biological processes that are regulated by Ras. Studies done in 1984 showed that treatment with epidermal growth factor (EGF) led to an increase in the GTPase activity of H-Ras (82). This was the first line of evidence pointing to Ras being involved in regulation of growth pathways. These findings, along with other work implicating Ras regulation by other growth factors and cytokines, suggest that just like prototypical G proteins, Ras can behave as a signal transduction molecule (83-86). The GTP-bound form

of Ras is in the active form by virtue of its increased affinity for effectors; more importantly, there are at least eleven distinct classes of Ras effectors that have so far been identified (10), with the majority of these effectors possessing either Ras-binding domains (RBD) or Ras-association (RA) domains (87). Association with Ras-GTP promotes effector activation by increasing the concentration of effectors at the plasma membrane, where additional events facilitate activation or enhance catalytic activity (10).

The role of Ras in signal transduction was largely undefined in the early to mid 1980s, though it had clearly been established as a regulator of cell growth and differentiation. However, work done by Bar-Sagi and colleagues in 1986 showed that microinjection of Ras protein into rat embryonic fibroblasts led to a significant increase in the activation of phospholipase A (88), an enzyme known to hydrolyze phospholipids into fatty acids and is involved in the production of arachinodic acid. Further work led to the concept of Ras effector signaling pathways: evidence shown in 1988 by Fukami et al. demonstrated that inhibition of phosphatidylinositol 4,5-bisphosphate (PIP2) caused reversible and dose-dependent decrease in proliferation and reversion of cell morphology of Ras-transformed cells (89). Furthermore, work performed by Feramisco and colleagues implicated the kinase Raf as a downstream effector of Ras, though the connection between Ras and Raf was not defined (82). Due to the development of yeast two-hybrid technology, it was later shown that the interaction between Ras and Raf was direct (90). This was the first true line of evidence implicating Ras in signal transduction, suggesting that Ras is directly interacting with its effector proteins to regulate downstream signaling cascades. As a whole, Ras regulates numerous growth promoting pathways, including the Raf/MEK/Erk, PI3K/AKT, and the Ral-GDS pathways

(collectively known as the classical Ras pathways). The Ras effector signaling pathways are critical for Ras biology, and ongoing efforts over the past thirty years have begun to decipher the molecular web of Ras signaling.

1.2.5.1 – Raf/MEK/Erk Pathway:

The first Ras effector pathway to be identified was the RAF-MEK-Erk pathway (90-93). Activation of the pathway begins with Ras activation. Classically, an extracellular signal binds to a protein tyrosine kinase receptor, such as epidermal growth factor receptor (EGFR) or the platelet-derived growth factor receptor (PDGFR), in turn inducing the oligomerization of the receptor (94). This leads to the allosteric activation of the intracellular tyrosine kinase (95,96), resulting in the cross-phosphorylation of the receptors. The phospho-tyrosines in the intracellular domain can now serve as docking sites for adaptor proteins (97). Adaptor proteins such as Grb2 can now recognize SH2 domains and recruit guanine-nucleotide exchange factors (GEFs), such as Sos, to the membrane (98). The GEF is able to activate membrane-associated Ras by converting it from its inactive GDP-bound form to its active GTP-bound form. GTP binding promotes a conformational change permitting Ras to associate with downstream effectors. Raf is then recruited to the cell membrane and is activated via its interaction with Ras (99).

Raf is the best characterized Ras effector and is considered an oncogene in its own right. Raf is a member of a family of serine/threonine kinases that include Raf-1, A-Raf and B-Raf. Raf activation by Ras stimulates a kinase cascade by phosphorylation of MAPKK (also called MEK in mammalian systems), in turn activating downstream

proteins Erk1 and Erk2 (MAPK). Activation of kinases Erk1 and Erk2 by MEK can phosphorylate and activate a number of nuclear transcription factors, other kinases, among many other proteins. Interestingly, many of the MEK/Erk targets have been implicated in Ras-induced cellular transformation (100).

For many years, the Raf oncogenes were not thought to be frequently mutated in human cancer, as aberrant activation of the Raf pathway was dedicated to Ras mutations, which can regulate both the Raf and the PI3K pathways (101). More extensive studies have now shown that it is not necessary to have mutated Ras in order to have aberrant Raf signaling. Recent work has shown that B-Raf is reported to be mutated in approximately 7% of all cancers, and is commonly found in melanoma (~30 to 70%), thyroid cancer (~35 to 50%), colorectal cancer (~5 to 20%), and ovarian cancer (~30%) (102).

While it is clear that Raf is a critical effector of Ras and is involved in Ras mediated cellular transformation via MEK/Erk, activation of the Raf proteins is very complex as there are many phosphorylation sites on Raf, and Raf activation/inactivation depends in large part on its phosphorylation status (101). While the kinases involved in the regulation of the Raf/MEK/Erk pathway have been extensively studied, the knowledge of phosphatases involved in these regulatory events remains poorly understood. In addition, it has now been shown that Raf has roles outside of the canonical MEK/Erk downstream effectors, some of which involve the prevention of apoptosis. To make matters more complicated, it has been shown that the Raf/MEK/Erk pathway can paradoxically effect cell cycle arrest differentiation, and senescence (103,104). Thus, the Raf/MEK/Erk pathway seems to have some opposing functions, adding to the complexity

of its regulation. What is clear is that Raf is a critical downstream effector of Ras by binding directly to Ras; furthermore, Raf preferentially binds to the active, GTP bound form of Ras, further validating the role of Raf as a *bona fide* Ras effector (93).

1.2.5.2 – PI3K/AKT Pathway:

One of the best characterized Ras effector families is the PI3K family, of which the members play important roles as mediators of Ras mediated cell survival and proliferation (105,106). The PI3K family of enzymes is organized into 3 main classes (class I, II, and III). Interestingly, Ras is able to interact with the different isoforms of class I PI3Ks, which is the most well characterized family and the one most clearly implicated in human cancer (107). PI3-kinase catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3), which binds the pleckstrin homology (PH) domain of Akt, stimulating its kinase activity (108). Akt activation controls signaling pathways regulating different cellular processes such as cell survival, glucose uptake, and glycogen metabolism. Specifically, Akt promotes cell survival primarily by inhibiting the pro-apoptotic Bcl-2 family members BAD and BAX (109,110). Second, Akt can promote an increase in transcription of anti-apoptotic and pro-survival genes by negatively regulating the transcription factor NF-κB (111). Third, Akt can phosphorylate Mdm2, an event that antagonizes p53-mediated apoptosis (108). In addition to Akt activation, PI3K can also activate the Rac GTPase, an important mediator of oncogenic Ras transformation (112).

Although the importance of Ras proteins in developmental processes is clear, the oncogenic potential of constitutively activated Ras proteins and its implication in cancer has drawn far more attention. Interestingly, aberrant PI3K signaling is one of the most frequent occurrences in human cancer (110,113). In addition, it has been shown that PI3K signaling is indispensable to maintain transformed growth in Ras mutant cell lines both *in vitro* and in xenografts in mice. Although multiple Ras effectors are essential to initiate tumor formation, only signaling through the PI3K/Akt pathway seems to be necessary to maintain tumor growth (114). By increasing the ability for growth and by decreasing the capacity for apoptosis, PI3K signaling supports tumorigenesis. More importantly, mice with mutations in the PI3K catalytic subunit p110α, which blocks its ability to interact with Ras, are highly resistant to endogenous oncogenic K-Ras-induced tumorigenesis and H-Ras-induced skin carcinogenesis (106,108).

The Ras/PI3K pathway has also been implicated in cancer metastasis, the leading cause of death in cancer patients. Ras activates Rac via PI3K, and Rac regulation of actin reorganization and membrane ruffling can promote increased cell motility and contribute to tumor cell invasion and metastasis (115). Furthermore, oncogenic Ras and PI3K can promote the loss of anchorage-dependent growth, which is a key feature needed for tumor cells to form metastases (116-118).

1.2.5.3 – Ral-GDS Pathway:

The third classical Ras effector pathway involves the Ral-GEF proteins. RalGEF members (RalGDS, RGL, RGL2, and RGL3) link Ras proteins to activation of the RalA

and RalB small GTPases (119). Activation of Ral allows it to interact with its downstream effectors, including Sec5, RalBP1, and ZONAB, though it is likely that there are additional protein partners not yet identified. Through these interactions, Ral proteins can regulate a wide array of cellular functions, such as proliferation, migration, and vesicle sorting (120). Ral can also control gene expression through transcription factors like fos, jun, AFX, and ZONAB.

RalGDS was discovered in 1994 in a yeast two-hybrid screen performed in multiple labs (121). RalGDS was found to catalyze nucleotide exchange on both RalA and RalB, but not on other small GTPases such as the Ras, Rho, and Rab families (122). Therefore, activation of RalGDS by Ras ultimately results in the activation of Ral. Activated Ral can in turn bind to several downstream effectors involved in critical biological processes, including actin organization, cytokinesis, autophagy, gene transcription, cell proliferation, cell survival, and secondary messenger production (122).

The biological function of RalGDS, as well as the other Ral-GEF family members, is not fully understood, although there is evidence that they play an important role in Ras mediated transformation *in vivo* (123,124). Work performed by Gonzalez-Garcia and colleagues showed that mice lacking RalGDS show reduced tumor incidence, size, and progression in a skin cancer model where H-Ras was the primary driver (124). They went on to show that RalGDS was required for skin tumor formation through mediation of JNK/SAPK pathway.

Collectively, the initial evidence pointed to the idea that the activation of the Ral pathway alone has a weak oncogenic effect, but can complement the roles of other Ras effectors in promoting cell transformation in tissue culture (124). However, it has

recently been argued that the role of the Ral pathway in Ras dependent transformation could be more critical in human cells compared to rodent fibroblasts. When work performed by Counter and colleagues explored the role of Ral in Ras mediated growth transformation of immortalized human astrocytes, fibroblasts, or epithelial cells, a more prominent role for Ral GTPases as effectors of Ras in human cancer was observed (125). More importantly, work done by Hamad and colleagues made the striking observation that of the three best studied Ras effectors – Raf, PI3K, and RalGEF – that RalGEF was the most important in inducing human cells to proliferate in suspension (125). This result was unexpected because previous experiments in mouse cells had identified Raf as the most oncogenic (120). Ultimately, the evidence points to the RalGDS pathway as playing a far more significant role in human systems. While a lot of uncertainty remains in how RalGDS directly functions in Ras mediated transformation, Ral GTPase signaling has now emerged as being critical in both normal and neoplastic cell physiology (122).

1.2.5.4 – Additional Ras Pathways:

Apart from the above-mentioned Ras effectors, an increasing number of molecules that specifically interact with Ras have been described (108). Some of the additional Ras effectors identified include phospholipase C, T-Cell lymphoma invasion and metastasis 1 (TIAM1), and Ras interaction/interference 1 (RIN1), all of which add complexity and significance for Ras in signal transduction and cellular transformation.

The ground-breaking work of Illenberger and colleagues was the first line of evidence implicating small GTPases in inositol lipid signaling (126,127). Additional

work went on to show that there are at least three different PLC isozymes (PLC-β2, -γ2, and -ε) that are robustly activated by Ras (128), though it seems that PLCε may be the one most implicated in Ras mediated transformation. PLCε is a key mediator of calcium signaling, in part via the activation of Protein Kinase C (PKC) which allows for the release of calcium from the endoplasmic reticulum (128). Interestingly, recent evidence has provided a link between PLCε and tumor development. It has been shown that PLCε deficient mice are resistant to skin tumor formation in a chemical carcinogen-induced model (129). Additional evidence also points to PLCε increasing tumor formation by increasing important inflammatory responses in dermal fibroblasts (130). In addition to its link with tumorigenesis, there is now evidence showing that PLCε contains a CDC25 domain, also referred to as a RasGEF domain, theoretically placing PLCε upstream of Ras, suggesting a potential positive feedback loop for PLCε signaling (131). As a whole, the work involving the Ras/PLCε pathway has shown its importance in signal transduction and tumor development. While PLCε has been shown to contain two C-terminal RA domains and binds directly to Ras (132), many questions still remain unanswered as to the potential mechanisms by which it directly affects Ras mediated transformation.

T-Cell lymphoma invasion and metastasis-inducing 1 (TIAM1), a Rac-specific guanine nucleotide exchange factor, was first identified for invasiveness in mouse leukemia cells (133). It mediates a broad range of cellular processes, including cellular migration and adhesion (134). It has been reported that over-expression of TIAM1 is correlated with poor prognosis in patients with hepatocellular carcinoma (135), and additional reports have now shown that alterations in TIAM1 expression/function may

contribute to tumorigenesis and carcinoma progression of common types of cancer (136-139). Interestingly, TIAM1 preferentially associates with activated GTP-bound Ras through a Ras-binding domain; this was shown to promote the activation of Rac in a PI3K-independent manner (140). Just as with the Ras/PLCε pathway, the Ras/TIAM1 is not fully understood, though it is likely that Ras mediated activation of Rac via TIAM1 will have important ramifications on a number of biological processes, such as motility, growth, and invasion.

The Ras interaction/interference 1 (RIN1) protein was initially identified as a Ras effector in 1991 (141), and was later shown to bind directly to Ras (142,143). Recent work done by Wang and colleagues has shown that RIN1 has high-affinity for binding to Ras and also demonstrated that this binding directly competes with Raf1 (144). Most striking was RIN1 could inhibit cellular transformation by activated mutant Ras, thereby distinguishing RIN1 from other Ras effectors that enhance transformation (144). In addition to competing with Raf for binding to Ras, it has been proposed that RIN1 also functions by promoting endocytosis of Ras-stimulating growth factor receptors, such as EGFR (145). Thus, it seems that RIN1 behaves as a tumor suppressor by negatively regulating Ras function. This hypothesis was validated by work showing that loss of RIN1 can be found in breast tumor cell lines and that restoring RIN1 expression blocked anchorage-independent growth *in vitro* and tumor formation *in vivo* (146). Collectively, this was a critical discovery, suggesting that RIN1 was the first identified Ras binding partner that could antagonize Ras mediated transformation (142). This was the first line of evidence suggesting that effector signaling pathways regulated by Ras do not exclusively promote cellular growth and transformation.

1.2.6 – Growth Inhibitory Pathways:

Recently, there has been an appreciation that Ras, and other oncogenes, paradoxically induce both pro- and anti-apoptotic signaling (147), and that the outcome of these contradictory signals greatly depends on the cell type and context (148). The balance of positive and negative signals depends on a number of different parameters, including kinetics, stoichiometry, availability of different binding partners, and activation of other similar or countervailing forces (148) (**Figure 2**). Currently, it is believed that in normal cells, a constitutively high level of activated Ras induces a protective, pro-apoptotic response to prevent oncogenesis (148). However, oncogenic Ras is likely to promote growth and differentiation in cells that have lost functional growth inhibitory pathways. Thus, Ras activates contradictory intracellular pathways, depending on cell context, that modulate cell viability (149).

It has been known for quite some time that powerful, innate mechanisms exist that restrain oncogenic potential (147). The first line of evidence in favor of this theory, at least for Ras, was published in 1983, when Newbold and Overell showed that in normal cells, oncogenic Ras triggered a profound growth arrest resembling replicative senescence (11). However, Ras could transform these fibroblasts if the cells had been newly immortalized by carcinogens, validating the claim that Ras is protective against oncogenesis in normal cells but oncogenic in nature in transformed cells. Future work has now shed some light on how Ras negatively regulates growth. Though the mechanisms remain relatively unclear, Ras can regulate at least two growth inhibitory pathways: apoptosis and senescence.

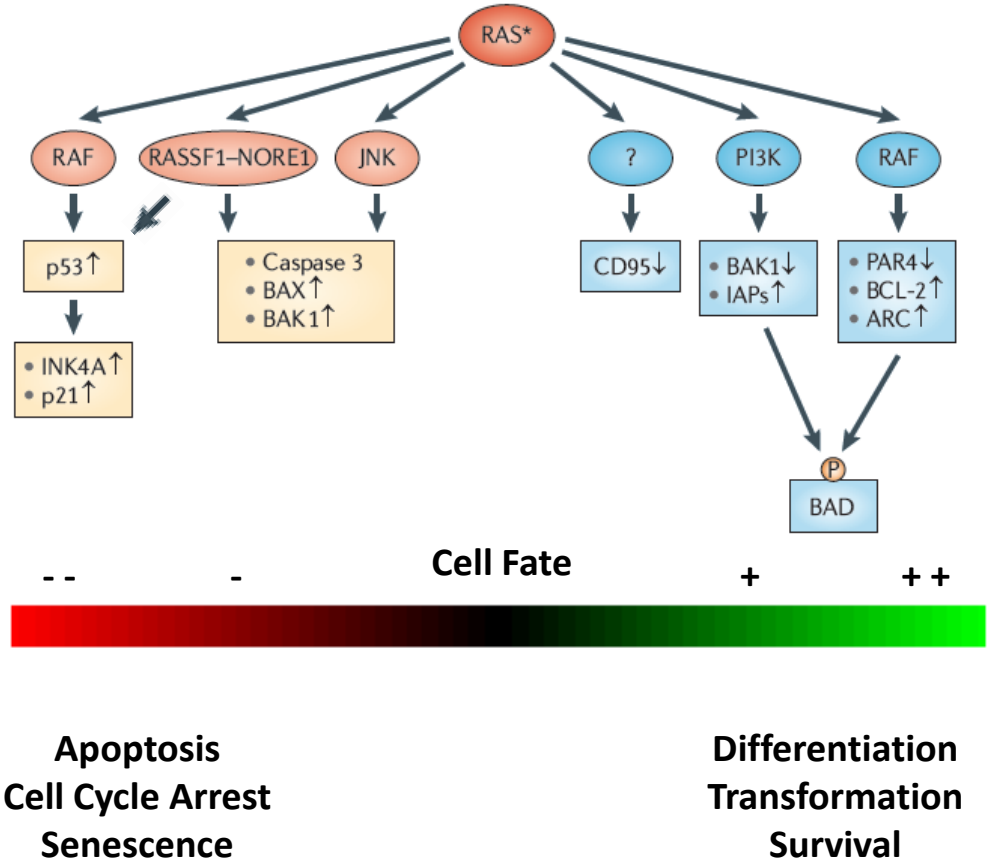


Figure 2: Ras paradoxically regulates both growth promoting and inhibitory pathways. Oncogenic Ras, depending on the cell context and cell type, can regulate both pro-growth and tumor suppressive pathways. In many cases, Ras activates cell proliferation by regulating the Raf, PI3K, and RalGDS pathways to promote growth and differentiation. However, signaling via the negative Ras effectors, including the RASSF family and MST kinases, can lead to apoptosis, cell cycle arrest, and induction of cellular senescence. It is thought that the loss of these functional growth inhibitory pathways is what drives Ras towards transformation.

1.2.6.1 – Apoptosis:

The identification of RIN1 as a potential negative effector of Ras intensified research toward the potential of Ras negatively regulating the growth of cells. It has been shown that RIN1 binds to activated Ras with a similar affinity to that seen by Raf1, and that RIN1 can inhibit cellular transformation caused by activated Ras (144). Additional findings in the 1990s validated the notion that Ras may able to regulate growth inhibitory properties of cells. First, constitutive expression of oncogenic Ras in a number of cell lines, including Jurkat (human T lymphoblastoid cell line) and murine fibroblasts, rendered cells susceptible to apoptosis following suppression of protein kinase C activity (150,151). Second, di Jeso and colleagues showed that serum withdrawal induces apoptotic cell death in K-Ras transformed cells, but not in normal differentiated thyroid cells (152), suggesting that oncogenic Ras was directly involved in serum-depleted induced apoptosis. In addition, cells over-expressing Crk-II, an adapter protein that regulates Ras activation, undergo apoptosis after serum-deprivation in a Ras dependent manner (153). Therefore, induction of apoptosis by Ras may be an important factor in limiting the expansion of somatic cells that sustain oncogenic Ras mutations (149).

While several independent studies had clearly highlighted a novel role for Ras in the regulation of apoptosis, the molecular mechanisms by which Ras could activate apoptotic signals were not completely understood, though it was clear that the ultimate outcome of pro-apoptotic Ras signals depends greatly on the cell type and context (148). This claim was based upon the notion that the Raf pathway, initially shown to promote growth, can actually be either anti- or pro-apoptotic depending on the circumstances

(148). However, this remains somewhat controversial, and it is still quite unclear exactly how the Raf pathway can differentiate between pro and anti-apoptotic signals. Thus, the focus of Ras mediated apoptosis switched toward potential negative effectors of Ras, especially after the discovery of RIN1. Interestingly, not long after the identification of RIN1, another family of negative Ras effectors was discovered that was believed to be the missing link between Ras and growth inhibition: the RASSF family.

1.2.6.2 – Ras-Induced Senescence:

Cellular senescence was first described over forty years ago by Hayflick and colleagues, who showed that normal cells had a limited ability to proliferate in culture (154). They described that the remaining cells, over many cell doublings, remained viable for many weeks, but failed to grow despite the presence of ample space, nutrients, and growth factors in the medium (155). A senescent cell is a cell that has exited the cell cycle permanently and is incapable of resuming proliferation, even upon mitogenic stimuli (156,157). Some of the key characteristics of senescent cells include a typical flat and enlarged morphology, many (but not all) cell types acquire resistance to certain apoptotic signals, altered gene expression, and other senescence markers, such as senescence-associated β-galactosidase positivity (155,158). An enormous breakthrough occurred in 1997, when Serrano and colleagues showed that ectopic expression of oncogenic Ras in normal fibroblasts resulted in a permanent cell cycle arrest and a drastic increase in senescent cells (159). This work eventually led to the concept that has since been named “oncogene induced senescence.” While oncogene induced senescence was

first seen upon ectopic expression of activated Ras, it was later shown that ectopic expression of other oncogenes, such as E2F1, Raf, mos, cdc6, and cyclin E had very similar effects (160-162). Collectively, the evidence strongly suggests that aberrant mitogenic signaling in primary cells could lead to permanent cell cycle arrest and cellular senescence; more importantly, recent studies have now clearly established the role of cellular senescence as a potential tumor suppressor mechanism *in vivo* (158). Thus, oncogene induced senescence is widely believed to be a critical fail-safe mechanism that has been developed to suppress cell proliferation caused by aberrant activation of onco-proteins in normal cells (163).

The mechanisms by which oncogenic Ras can promote the induction of senescence are slowly being discovered, but are quite complex and remain unclear. Initially, one of the hallmarks of cells undergoing oncogene induced senescence was the involvement of the p53 and the Rb pathways (164) (**Figure 3**). Oncogenic Ras alone has been shown to transform primary mouse fibroblasts that are deficient for either functional p53 or Rb pathways (165,166). However, induction of senescence in human cells seems more complex: evidence suggests that activation of both the p53 and the Rb pathways is essential for induction of senescence in a variety of human cell strains (164), and this may be due in part because of potential for cross-talk between the two pathways in human cells. While both pathways have consistently been shown to be vital for the proper activation of the senescent phenotype, it is worth noting that recent work performed by Wei and colleagues has shown that Rb is *clearly required* in human cells for Ras to induce senescence (167,168). Ultimately, Ras-induced senescence is circumvented by inactivating the p53 and Rb pathways.

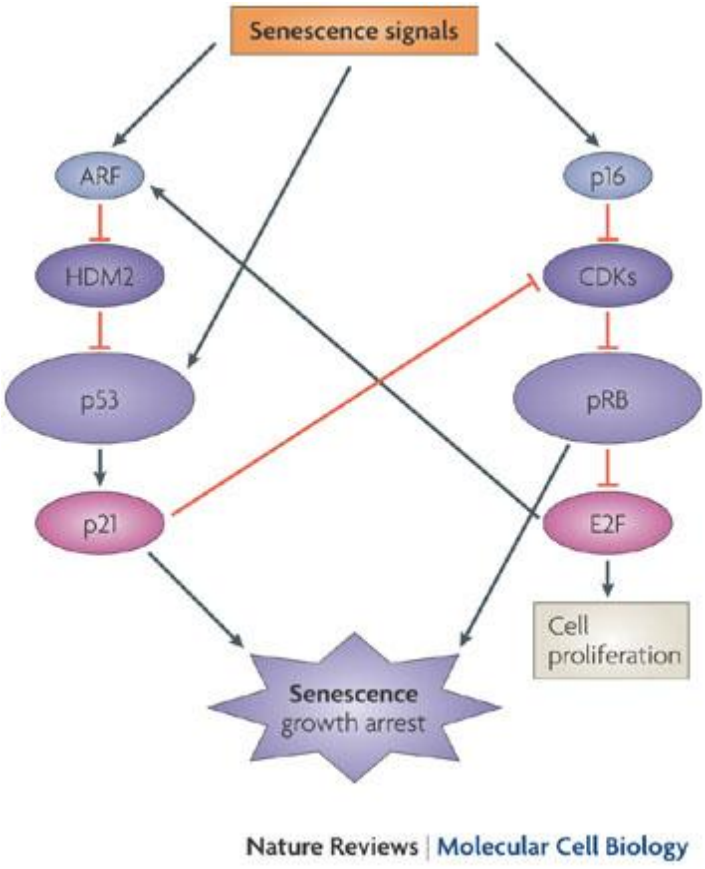


Figure 3: Pathways involved in oncogene induced senescence. Ras-induced senescence is thought to function as a critical barrier against aberrant Ras signaling that would normally promote uncontrolled cell growth. While the mechanisms involved in Ras-induced senescence are not fully understood, it is clear that Ras activates senescence via the p53 and Rb tumor suppressor pathways. Loss of these functional pathways allows Ras to bypass senescence induction and in turn promote transformation.

While the understanding of the significance of oncogene induced senescence as a critical barrier for tumor development grew, the mechanisms by which it occurs remains elusive. Additional evidence collectively showed that Ras-induced senescence truly involved an intricate web of multiple signaling mechanisms, including ATM, Arf, p16, p38, and FOXO (169). However, all of these mechanisms still ended up merging toward the two classical senescence pathways, p53 and Rb. Even though there was compelling evidence that Ras was promoting the induction of senescence through the p53 and Rb pathways, there was very little evidence linking Ras directly to these pathways. The hypothesis was that, similar to Ras mediated apoptosis, Ras was able to regulate senescence induction through its potential negative effectors. This hypothesis remained hanging in the balance, until the discovery of the RASSF family of tumor suppressors.

1.3 – The RASSF Family of Tumor Suppressors:

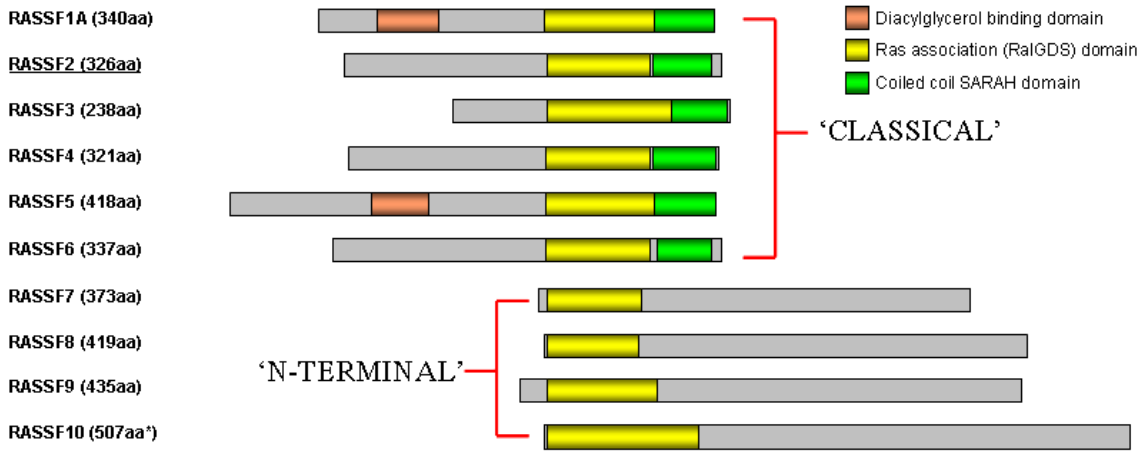
Evidence highlighting growth inhibitory functions of Ras pointed to the possibility that Ras may have unidentified negative effectors that regulate such functions. One of the ways this question was addressed was by performing large yeast two-hybrid screens as an attempt to discover novel binding partners of Ras. Ras contains several well-characterized binding domains, including the RBD and the RA domain. As a result, such two-hybrid screens would specifically focus on individual binding sites of Ras. In 1998, a potential Ras effector was newly identified by Vavvas and colleagues, which they termed NORE1 for novel Ras effector 1 (14). Two years later, a large scale data base search led to a second potential Ras effector, called RASSF1 (170). Over time, the

RASSF family of tumor suppressors would emerge as critical negative effectors of Ras, with some of the members far better characterized than others.

1.3.1 – Overview of the RASSF Family:

The RASSF (Ras-Association Domain Family) proteins belong to a family of tumor suppressor proteins that is comprised of ten members (RASSF1-10), each with multiple splice variants, with the exception of RASSF9 and 10 (171,172) (**Figure 4**). Currently, the direct association with oncogenic Ras has been observed for RASSF1A, 5A, and 6 (12,173-175). RASSF5A (NORE1A) was the first member of the RASSF protein to be cloned (14), hence the name NORE1 for Novel Ras Effector 1. The race to characterize the functional role of this family officially began once they were shown to behave as tumor suppressors.

The Ras Association (RA) domain is a functional domain found in several crucial Ras effectors, such as RalGDS and AF-6 (176). This is one of the most important consensus binding sites for all of the RASSF family members. In addition to the RA domain, RASSF proteins have several functional domains that regulate association with other proteins. The Salvador-RASSF-Hippo (SARAH) domain plays a role in specific protein-protein interactions as well as the homo- and heterodimerization of RASSF isoforms (177). Some of the key associations of RASSF proteins via the SARAH domain involve downstream kinases MST1 and MST2 as well as the tumor suppressor Salvador in order to promote apoptosis (178). As a whole, these various domains allow for



<http://atlasgeneticsoncology.org/Genes/GC_RASSF2.html>

Figure 4: Schematic representation of the 10 human members of the RASSF family of tumor suppressors. Highlighted are three functional domains: the diacylglycerol binding (DAG) domain, the Ras Association (RA) domain, and the Salvador-RASSF-Hippo (SARAH) domain. These proteins do not have any apparent enzymatic activity and are thought to primarily act as scaffolding molecules to promote the formation of critical tumor suppressor complexes. Direct association with oncogenic Ras has only been observed for RASSF1A, 5A (NORE1A), and 6. A majority of these tumor suppressors are inactivated in a variety of different human cancers, primarily by promoter methylation. Figure adapted from the Atlas of Genetics and Cytogenetics in Oncology and Hematology.

associations with numerous molecules and determine the involvement of RASSF proteins in several biological pathways in order to carry out tumor suppressor functions (177).

Perhaps the most well characterized RASSF family member is RASSF1A. It was shown in 2000 that the 3p21.3 region of the human genome contained one or more tumor suppressors, because this region frequently suffers loss of heterozygosity in lung cancer (179). That same year, Damman and colleagues cloned a gene in this region that they called RASSF1 because it contained a putative RA domain (180). Differential promoter activity and alternative splicing can lead to seven different transcripts (RASSF1A-G), though the two major isoforms are RASSF1A and RASSF1C (180). Of note, RASSF1A is considered a *bona fide* tumor suppressor, as knockout of RASSF1A in mice promotes an enhanced rate of cancer development (181). Additional work showed that RASSF1A is frequently inactivated by epigenetic mechanisms in human tumor cells (13,15,182). Furthermore, RASSF1A can also contain point mutations in up to 15% of primary tumors (183). Thus, RASSF1A may be one of the most frequently inactivated tumor suppressors identified in human cancer to date (15).

The RASSF1A protein regulates a broad range of biological processes that are essential for normal growth control. Lacking enzymatic activity (15), RASSF1A seems to act as a scaffolding molecule in order to modulate tumor suppressor pathways. RASSF1A plays a critical role in microtubule stability by interacting with several micro-tubule associated proteins (MAPs), in turn forming a complex with microtubules and regulating mitosis and cell cycle progression (15). One of the ways RASSF1A is thought to regulate the cell cycle is by inhibiting the accumulation of cyclin D1 and blocking the cell cycle at the G1/S phase (184,185). Furthermore, there is some evidence suggesting that

RASSF1A mediated stabilization of microtubules may have a role in the regulation of autophagy (186). RASSF1A has also clearly been implicated in promoting apoptosis by associating with pro-apoptotic kinases MST1/2 to modulate their kinase activity, in turn activating the HIPPO pathway and inducing cell death (178). More recently, it has been shown that RASSF1A is also directly involved in DNA repair by regulating the acetylation of the DNA repair protein XPA (187). This finding shows that, in addition to acting as a scaffolding molecule, RASSF1A can also regulate protein function by modulating their post-translational modifications. However, this function has been defined solely for RASSF1A, the best characterized member of the family, and it remains to be seen if the other members of the RASSF family can perform such a powerful function. While the functions of the majority of the other RASSF family members remain unclear, more evidence has come to light suggesting that another RASSF member may be just as potent a tumor suppressor: RASSF5, or NORE1A.

1.3.2 – The Tumor Suppressor NORE1A (RASSF5):

NORE1 was the first member of the RASSF family of proteins to be cloned (14). While differential promoter usage and alternative splicing mechanisms leads to the expression of three transcripts (A-C), NORE1A is the longest and most common isoform. NORE1A has been shown to have approximately 50% sequence homology to the relatively well characterized RASSF1A tumor suppressor (188). As in the case with RASSF1A, the initial evidence suggested that NORE1A behaves as a tumor suppressor. NORE1A is frequently down-regulated in human tumors by promoter methylation, and in

some primary tumors the NORE1A locus undergoes loss of heterozygosity (15,189). In addition, a genetic translocation event leading to loss of NORE1A results in a familial human cancer syndrome (18). Several *in vitro* studies have shown that exogenous expression of NORE1A can promote apoptosis and in some cases cell cycle arrest (12,17,19). Additionally, restoring endogenous levels of NORE1A expression to a NORE1A-negative tumor cell line suppressed the tumorigenic phenotype (12). While the evidence clearly implicates NORE1A as a tumor suppressor, how NORE1A functions in this role remains relatively unclear.

Interestingly, NORE1A has been shown to associate with oncogenic Ras via its RA domain in a GTP-dependent manner (19), with a similar affinity compared to that of other known Ras effectors (190). Because NORE1A was shown to form an endogenous complex with Ras in cells (19), NORE1A is now considered a *bona fide* Ras effector (191). Since activated forms of Ras are known to paradoxically induce growth inhibitory pathways in addition to classical growth promoting ones, it was thought that NORE1A could behave as a critical negative effector of Ras.

One of the first mechanisms of NORE1A mediated tumor suppression investigated was the ability of NORE1A to bind to the kinases MST1/2, key components that feed into the HIPPO tumor suppressor pathway (192). Briefly, the core of the HIPPO pathway consists of a kinase cascade, whereby MST1/2 phosphorylate and activate the kinases LATS1/2, in turn phosphorylating the transcriptional co-activators YAP and TAZ (193). Phosphorylation of YAP and TAZ will either retain them in the cytoplasm or promote their degradation, in turn promoting the induction of apoptosis (193). Initial work published by Avruch and colleagues suggested that Ras/NORE1A can drive

apoptosis by binding to MST1/2 (192). However, their findings failed to show an increase in the kinase activity of MST1/2 as well as not showing any effects on YAP/TAZ phosphorylation. Interestingly, a report by Aoyama et al. showed that mutants of NORE1A lacking the MST-binding domain can inhibit tumor cell growth independently of the MST1/2 kinases (17). Thus, the role of NORE1A in regulating the HIPPO pathway is quite controversial, though it seems that by some currently unknown mechanism NORE1A may modulate effectors of the HIPPO pathway, either directly via MST1/2 or non-canonically.

Recently, work performed by Schmidt and colleagues has characterized a novel Ras-NORE1A axis in the regulation of the Wnt pathway. They showed that NORE1A forms a direct, endogenous, Ras-regulated complex with the E3 ubiquitin ligase β-TrCP, ultimately promoting the degradation of the terminal executor of Wnt signaling, β-catenin (194). β-catenin serves as both a nuclear transcriptional regulator and a key component of adherens junctions (195). Thus, by recruiting β-TrCP and promoting the ubiquitination and subsequent degradation of β-catenin, NORE1A can regulate the stability and turnover of important proteins. Furthermore, the work identifies a novel Ras regulated pathway of growth inhibition. However, Ras is known to negatively affect growth not only by regulating apoptotic pathways, but also by promoting senescence. Once it was established that NORE1A seemed to play a role in cell cycle arrest (191), it raised the intriguing possibility that NORE1A could be a senescence effector of Ras.

1.3.3 – The Link Between Ras-Induced Senescence and NORE1A:

It is widely believed that the senescence response is a crucial fail-safe mechanism that protects the cells from tumorigenic transformation, such as aberrant Ras signaling (196). Ras-induced senescence came to light in 1997, when it was shown to promote G1 arrest and cause an increase in the senescent proteins p53, p21, and p16 (159). While the mechanisms are not fully defined, it is widely accepted that Ras-induced senescence primarily involves the p53 and Rb pathways (169). The link between Ras and the activation of these two powerful senescence pathways is still missing, though the hypothesis remains that it must be regulating these pathways through one of its negative effectors.

NORE1A promotes apoptosis when over-expressed or in the presence of activated Ras, but lower, more physiological levels of NORE1A seem to promote G1 cell cycle arrest (17). With the exception of the MST kinases, the signaling pathways involved in NORE1A mediated tumor suppression remain poorly defined. In order to try and identify novel mechanisms of action of NORE1A, Calvisi and colleagues performed a microarray analysis of kidney cells induced to express NORE1A at physiological levels, and determine alterations in gene expression by qRT-PCR. Of particular interest was the induction of the cyclin-dependent kinase (cdk) inhibitor $p21^{CIP1}$ (191). Similar to NORE1A, over-expression of $p21^{CIP1}$ induces G1 arrest (197). The study showed that NORE1A over-expression up-regulates $p21^{CIP1}$, but more importantly, loss of NORE1A expression led to the reduction in $p21^{CIP1}$ levels (191). One of the best characterized activators of $p21^{CIP1}$ is p53 (198). Interestingly, the work done by Calvisi et al. showed

that the activation of $p21^{CIP1}$ by NORE1A is p53 dependent, and that by some unknown mechanism, NORE1A promoted the nuclear translocation of p53 (191). This links NORE1A to the $p21^{CIP1}$/p53 pathway, suggesting that NORE1A might be involved in Ras mediated activation of p53.

As Ras senescence pathways are known to operate, in part, via p53 (159), Donninger et al. examined the potential role of NORE1A in Ras-induced senescence and p53 activation. Their findings showed that NORE1A is a potent inducer of p53 dependent senescence, and that knockdown of NORE1A impaired Ras-induced senescence and enhanced its transforming ability (20). Specifically, NORE1A forms an endogenous, Ras regulated complex with the kinase HIPK2, and that this interaction is essential for Ras/NORE1A induced senescence (20). Collectively, they showed that NORE1A positively regulates the pro-senescent post-translational modifications of p53 while also negatively regulating its pro-apoptotic modifications, which occur as a result of Ras mediated scaffolding of p53 and HIPK2 via NORE1A. While this work clearly implicates NORE1A as a senescence effector of Ras by activating p53, the loss of p53 did not completely abrogate the ability of NORE1A to induce senescence, suggesting that there might be additional mechanisms by which NORE1A can promote senescence. In addition to the p53 pathway, Ras can promote the induction of senescence by activating another tumor suppressor: the retinoblastoma protein, Rb.

1.4 – The Retinoblastoma Tumor Suppressor:

The retinoblastoma susceptibility gene (Rb-1) was the first tumor suppressor to be cloned, and was originally identified in pediatric malignant tumors of the retina known as

retinoblastoma (199). Rb has now been identified as a crucial tumor suppressor and is believed to be directly or indirectly inactivated in nearly all human cancers (21). The Retinoblastoma tumor suppressor belongs to a cellular pathway that plays a crucial role in restricting the G1-S transition of the cell cycle (200). However, it is now evident that Rb plays multiple tumor suppressive functions in addition to its proto-typical role of cell cycle regulation. Interestingly, Rb has been shown to bind to over one hundred protein partners and has been shown to mediate transcriptional regulation of hundreds of target genes. These protein partners and transcriptional targets are thought to mediate the numerous cellular functions of Rb, including temporary and permanent cell cycle arrest, genomic stability, and differentiation (21). For a long time, Rb has been viewed as "just" a regulator of cell cycle progression; however, recent observations indicate that Rb functions in multiple pathways and biological processes that are dysregulated during tumor initiation and progression (200).

1.4.1 – The Discovery of Rb:

Retinoblastoma (Rb) is the most common intra-ocular malignant tumor in childhood, with an incidence of 1 in 15,000 live births (201). The most important studies to investigate the pathogenesis of retinoblastoma began with work published by Knudson in 1971, which concluded that retinoblastoma could be inherited and formulated the "two-hit theory" in order to explain its pathogenesis (202). He believed that the tumor phenotype is not apparent unless both copies of the gene are damaged. The cloning of the Rb gene and the identification of biallelic Rb mutations in retinoblastoma tumors confirm

the hypothesis that such gene products exert the action of a tumor suppressor. Several human tumors show mutations and deletions of the Rb gene, and inherited allelic loss of Rb confers increased susceptibility to cancer formation (203).

1.4.2 – Rb and Cancer:

Mutations in the Rb pathway are almost universal in cancer, but different components of this pathway are selectively affected in distinct cancer types (200). Originally, it was shown that the Rb-1 gene is a frequent target for direct mutation only in small cell lung carcinoma and retinoblastoma (204,205). However, multiple reports have gone on to show that mutations affecting the retinoblastoma gene are actually frequently encountered in other cancers, including osteosarcoma, prostate, and breast cancer (206). As a further indication of its fundamental role in tumor suppression, Rb can be functionally inactivated by constitutive hyperphosphorylation in tumors that do not have mutations in the retinoblastoma gene (207). Furthermore, loss of heterozygosity at the Rb-1 locus has been reported in many different sporadic cancers, suggesting that it is directly mutated outside of the lung and retina, but on a less frequent basis (208). Lastly, DNA tumor viruses that express oncoproteins, such as adenovirus E1A, SV40 large tumor antigen, and human papillomavirus (HPV) E7, bind and inactivate Rb (209-211). These proteins have all been shown to be required in order for the viruses to transform cells. However, the retinoblastoma protein is most frequently inactivated in human cancer by the negative regulatory activity of cyclin dependent kinases (212). Collectively, it is the dysfunction of the Rb pathway that is considered to be critical for the development

for the majority of human cancers, in large part because of the loss of functional tumor suppressive functions of Rb.

1.4.3 – Tumor Suppressive Functions of Rb:

Since the late 1980s, it has been shown that Rb behaves as a tumor suppressor. Over the years, the mechanisms by which Rb can promote its tumor suppressive functions have been elucidated. It is now believed that the presence of Rb may prevent tumor formation by inducing differentiation, controlling-cell cycle arrest, maintaining genomic stability, and inducing senescence in response to oncogenic stresses (21). Furthermore, the absence of Rb has been associated with increased angiogenesis (213) and metastasis (214), although the mediators of these functions are less well understood. While copious amounts of research has been done to characterize the mechanisms of actions of Rb, the fundamental aspects of Rb mediated tumor suppression focus on two critical components: regulation of the cell cycle and induction of cellular senescence.

1.4.3.1 – Rb and Cell Cycle Progression:

Two of the most important proteins involved in the cell cycle machinery are cyclin-dependent kinases (Cdks) and cyclins. Cyclin/Cdk complexes are formed during distinct phases of the cell cycle, and are specifically involved in the phosphorylation of a distinct set of target proteins, including Rb. The fluctuation in cyclin expression and the resultant oscillation in Cdk activity forms the basis of a coordinated cell cycle

progression. Cdks activated in response to mitogenic stimuli phosphorylate and inactivate Rb, allowing for progression through the cell-cycle (215).

One of the critical steps in cell cycle progression is the G1 to S transition phase. At this particular point, the cell may decide whether to continue its advance and complete the cell cycle. This point is referred to as the "restriction point," or the R point, and is thought to be a critical event in normal cellular proliferation control (216). It is perceived that Rb is the key mediator that serves as the R point switch. Rb is hypophosphorylated in resting G0 cells, is increasingly phosphorylated during progression through G1 and is maintained in a hyperphosphorylated state until late mitosis (217-219).

When in its actively growth-suppressing hypophosphorylated state, Rb physically associates with E2F factors and blocks their ability to activate expression of genes that encode products necessary for S-phase progression (**Figure 5**). The Rb proteins repress gene transcription required for transition from G1 to S phase by directly binding to the trans-activation domain of E2F and by binding to the promoter of these genes as a complex with E2F (220). The importance of the Rb-E2F interaction in cell growth control is demonstrated by the finding that all naturally occurring Rb mutants isolated from human tumors lack the ability to bind and negatively regulate E2F (221). However, Rb can regulate G1-S transition through E2F independent mechanisms. Rb has been shown to inhibit Cdk activity and G1-S progression by increasing the expression of p27; furthermore, Rb can stabilize the p27 protein by binding the Skp2 protein and interfering with the Skp2-p27 complex, thus avoiding p27 ubiquitination (222).

In addition to directly binding and blocking the activation of E2F transcription factors, Rb can repress transcription by remodeling chromatin structure through its

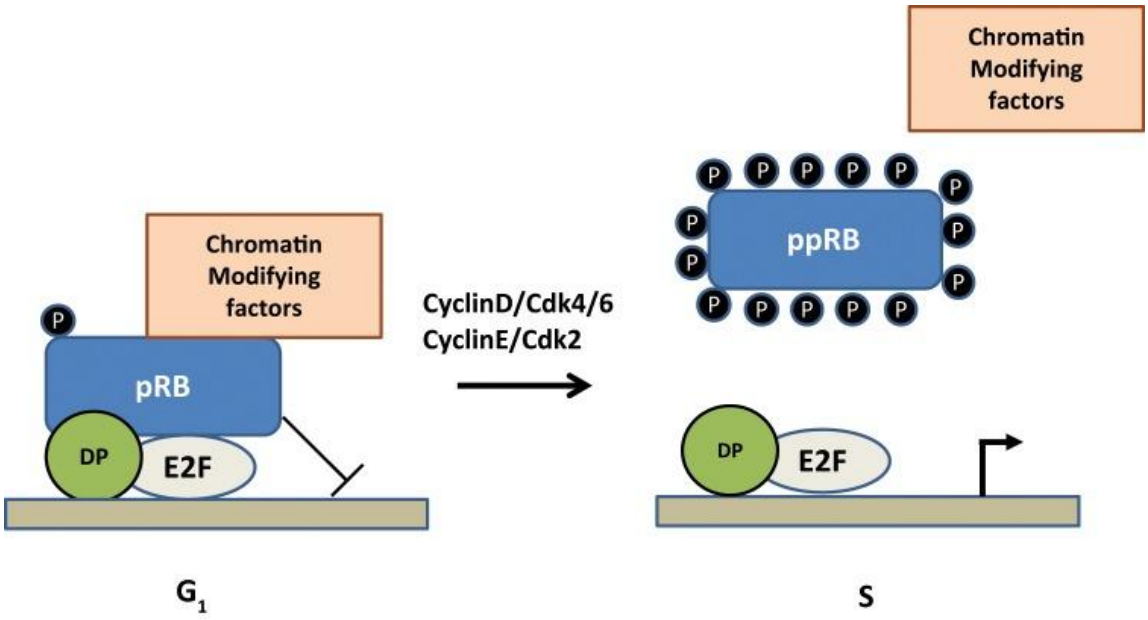


Figure 5: A model for cell cycle regulation by Rb. The Rb tumor suppressor is primarily regulated by its phosphorylation status. When Rb is hypophosphorylated, it will bind to and inhibit the activity of E2F transcription factors, as well as recruit chromatin modifying enzymes to repress the transcription of E2F responsive genes. However, cyclin/Cdk complexes can be activated in response to mitogenic stimuli and can phosphorylate and inactivate Rb, allowing for progression through the cell cycle. While the mechanisms involved in Rb phosphorylation are well characterized, the activation of Rb by dephosphorylation remains poorly defined.

interaction with proteins such as hBRM, BRG1, HDAC1, and SUV39H1 (223) which are involved in nucleosome remodeling, histone acetylation/deacetylation, and methylation, respectively. Of particular interest is the ability of Rb to recruit HDAC1 to E2F complexes to repress gene expression, such as cyclin E (224). Work done by Zhang et al in 2000 showed that histone deacetylase is involved in modulating the repressive activity of Rb on E2F gene promoters (225). In conclusion, Rb has now been shown to have multiple mechanisms involving the regulation of cell cycle. Interestingly, prolonged cell-cycle arrest can lead to the induction of senescence.

1.4.3.2 – Rb and Senescence:

Multiple reports have shown that Rb is required for oncogene induced senescence in mouse cells (226,227), and it is widely believed that Rb can induce the senescence phenotype in part by promoting a permanent cell cycle arrest. However, in human cells both the p53 and the Rb pathways have to be inactivated in order for Ras to bypass senescence (159), suggesting that both pathways are required in human systems. These differences in the senescence mechanism may provide a logical explanation as to why it is easier to transform rodent cells than human cells in experimental systems (228). While it is clear that Rb is required for Ras-induced senescence, the mechanisms linking Ras to the Rb pathway remain unknown.

One mechanism by which Rb can promote this irreversible cell cycle arrest is by promoting the formation of heterochromatin around genes required for cell proliferation, such as E2F target genes. Such heterochromatin structures are referred to as SAHF

(senescence-associated heterochromatin foci). While these structures are critical for the induction of senescence, it does not explain how Rb is activated to promote such a phenotype. In addition, work performed by Chicas and colleagues showed that, in addition to E2F repression, Rb can repress the replication machinery of senescent cells in part by repressing the expression of cyclin E1 (215).

During Ras-induced senescence, Rb can be activated by the cyclin-dependent kinase inhibitors (CKIs) which block the Cdks that normally inactivate the Rb protein by phosphorylation (229). Two of the most important CKIs are p21 and p16, both of which are simultaneously required for Ras-induced senescence in human cells (230). Interestingly, inhibition of Cdks is not the only means to inhibit Rb phosphorylation. While the mechanisms that lead to the phosphorylation of Rb are well documented, the knowledge of the process that counteracts Rb phosphorylation is still quite limited (231). Recent work has now shown that two phosphatases, PP1 and PP2, play pleotropoic roles in the mammalian cell cycle, particularly in mitosis. Further support for a major role for PP1 in Rb dephosphorylation came from a yeast two-hybrid screen that reported a direct association between Rb and the catalytic subunit of PP1. It was later shown through *in vitro* binding assays that isoform PP1A preferentially binds the hypophosphorylated form of Rb and that this association occurs in early G1. It must be noted that subsequent work showed that Rb actually binds all PP1 isoforms, but that PP1A is widely considered to be one of the major cellular phosphatases involved in Rb dephosphorylation (232-236). Interestingly, it has been recently reported that PP1A enzymatic activity is regulated by Ras (237) and additional reports have shown a correlation between Ras activation and PP1/PP2A activation (238). More importantly, work performed by Castro and colleagues

showed that PP1A could behave as a tumor suppressor in human cells through its contribution to Ras-induced senescence (22). This highlights the importance of Rb activation by dephosphorylation in order to promote Ras-induced senescence. However, the mechanism by which Ras regulates PP1A remains unknown.

1.5 – A Novel Signaling Pathway for NORE1A Induced Senescence:

Increasing evidence has implicated Ras-induced senescence as a potent barrier against malignant transformation, and it is widely believed that loss of functional senescence pathways allows Ras to bypass senescence induction and drive transformation. The exact mechanisms by which Ras induces senescence are not fully understood, but center around two powerful senescence effectors: p53 and Rb. Ras has been shown to promote senescence by activating both of these tumor suppressors. And while p53 and Rb are required to promote Ras-induced senescence, the mechanisms linking Ras to p53 and Rb remain unknown.

Recently, NORE1A has been identified as a *bona fide* tumor suppressor, and has now been shown to be a negative effector of Ras. While initial findings depicted a novel role for NORE1A as a mediator of Ras-induced apoptosis, the work revolved around artificial, over-expression studies, and thus may have masked the true tumor suppressive functions of NORE1A. In 2009, work performed using more physiological levels of NORE1A expression strongly suggested that NORE1A may be far more important in cell cycle arrest, bringing up the intriguing possibility that NORE1A might be playing a role in oncogene induced senescence. Recent work has now clearly implicated NORE1A as a

direct, potent senescence effector of Ras. These groundbreaking findings by Donninger et al. highlight that NORE1A promotes Ras-induced senescence by scaffolding HIPK2 to p53 in a Ras dependent manner, in turn modulating the pro-senescent post-translational modifications of p53. Although NORE1A acts through p53 to promote senescence, some of the pro-senescent phenotype of NORE1A still remains even after all p53 has been removed. This suggests that NORE1A induced senescence may not be promoted exclusively by p53, pointing to the possibility that NORE1A may have additional, previously unidentified mechanisms to activate Ras-induced senescence. Bearing in mind that the onset of senescence in human cells requires both p53 and Rb, we hypothesized that NORE1A might also act through the Rb pathway. Furthermore, PP1A was detected in complex with NORE1A in a yeast two-hybrid screen (23), suggesting that these two proteins may interact and potentially provide the missing link that allows Ras to regulate Rb in order to promote senescence.

Work presented in this dissertation demonstrates a novel mechanism, in addition to p53, by which NORE1A can promote senescence induction. We will show evidence that NORE1A can activate the powerful Rb tumor suppressor. Through the formation of an endogenous, Ras regulated complex with the phosphatase PP1A, NORE1A can scaffold PP1A onto Rb in turn promoting its dephosphorylation, a pro-senescent event. Additionally, we show that NORE1A requires Rb to promote senescence in mouse embryonic fibroblasts, and that loss of Rb significantly suppresses NORE1A induced senescence in human cells. These findings explain why NORE1A is such a powerful tumor suppressor. Collectively, NORE1A seems to behave as a critical senescence effector of Ras, by linking Ras to both the p53 and Rb pathways to fully promote the

senescent phenotype. In addition to regulating Rb dephosphorylation, we also provide evidence that NORE1A can regulate some of the non-phosphorylation post-translational modifications of Rb, including the acetylation and the SUMOylation, both of which are currently believed to activate the tumor suppressive phenotype of Rb.

CHAPTER 2

MATERIALS AND METHODS

2.1 – Vectors and Plasmids:

pCDNA3-HA and pCDNA3-FLAG – pCDNA3 expression plasmid vector was purchased from Invitrogen (Carlsbad, CA). It contains a CMV promoter upstream of a multiple cloning site; however, it does not express an epitope tag, making it inconvenient for basic biological studies. Therefore, we generated pCDNA3 expression vectors containing fused HA and FLAG epitope tags, which were performed by Dr. Geoffrey Clark. Ultimately, the expression of inserted genes results in either a fused HA of FLAG tag on the N-terminus of the protein.

pEGFP-C1 – This vector construct was purchased from Clontech (Mountain View, CA). It is driven by a CMV promoter and allows for a Green Fluorescent Protein (GFP) tag to be fused to cDNAs that can be inserted into the multiple cloning site. This is a very useful tool that can be used to track protein localization within the cell by fluorescent microscopy as well as for protein-protein interactions via coimmunoprecipitations (described in Section 2.8.1).

pmKATE2-C – This vector construct was obtained from Evrogen (Moscow, Russia). Nearly identical to pEGFP-C1, this construct expresses a Red Fluorescent Protein (RFP) tag that can be fused to cDNAs inserted into the multiple cloning site, an can be used in a similar manner for biological assays.

NORE1A – NORE1A is the centerpiece of this dissertation project. The full length human NORE1A was purchased from Origene (Rockville, MD) and was sub-cloned into the relevant expression constructs by Dr. M. Lee Schmidt. Briefly, the NORE1A cDNA clone was PCR amplified with primers "hNore1a5" with the sequence 5' – GCAGATCTATGGCCATG GCGTCCCCGGCCATC – 3' and "hNore1a3'" with the sequence 5' – GCGAATTCTTACCCA GGTTTGCCCTGGGATTC – 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 sub-cloning 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 mammalian expression construct containing an in-frame 5'-HA tag and 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 KATE (pmKATE2-C) digested with BglII and EcoRI.

H-Ras-12V and K-Ras-12V – pCGN-HA-H-Ras12v was created by cloning the full-length H-Ras12v cDNA as a BamHI fragment into pCGN vector under the control of a CMV promoter (239). KATE-tagged H- and K-Ras12V were generated by subcloning a BamHI fragment from pCGN-H- and K-Ras12V into pmKATE2-C.

shNORE1A – shRNAs for human NORE1A (#1: 5′-TATATATAGCTATATGCCT-3′; #2: 5′-AGCTTTGTGCTAAAGGAGA-3′; Scrambled: 5′ATCTCGCTTGGGCGAGAGT AAG-3′; RHS4531-EG83593) were obtained from Open Biosystems (Rockford, IL) (20).

shRb – pLKO-Rb1-shRNA-19 and 63 were purchased from Addgene (Plasmid #25640 and 25641). Briefly, unique short hairpin RNAs (shRNA) to the RB1 mRNA in the pLKO.1-Puro lentiviral expression vector were obtained from Open Biosystems. Clones 19 and 63 were originally shown to have >99% knockdown of Rb expression relative to the empty pLKO.1-infected clone (240).

siPP1A – Control (sc-37007) and siPP1A siRNA (sc-36299) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). While the sequences for the siPP1A siRNA constructs were not disclosed by Santa Cruz, it is noted that this PP1A siRNA is a pool of three target-specific 19-25 nucleotide siRNAs designed to knock down the gene expression of human PP1A.

Rb – Human Rb expression construct (pGS5L-HA-Rb) was obtained from Addgene (Plasmid #10720) and digested with BamHI/NheI and NheI/EcoRI in two separate digests to generate 1.9 Kb and 0.8 Kb fragments, respectively. The two fragments were cloned into pEGFP-C1 (Clontech) digested with BglII/EcoR1 to generate a GFP-tagged full length Rb expression construct. To maintain consistency of plasmid backbones, an identical double digest was performed in order to generate a pcDNA3.1-HA-Rb construct. Briefly, following a double digest of pGS5L-HA-Rb to generate the 1.9 Kb and

0.8 Kb fragments, we set up a ligation using both fragments along with a pcDNA3.1-HA fragment from HA-XpA-2KQ that had been digested BamHI/EcoRI.

Ubiquitin – We obtained the full length, wild-type HA-tagged Ubiquitin expression construct, pRK5-HA-Ubiquitin-WT, from Addgene (Plasmid #17608).

PP1A – We purchased the full length, wild-type GFP-tagged PP1A expression construct, pEGFP(C1)-PP1A, from Addgene (Plasmid #44224).

SUMO1 – We purchased the full length, wild-type HA-tagged SUMO1 expression construct, pcDNA3-HA-SUMO1-WT, from Addgene (Plasmid #48966).

PCAF – We obtained the full length, wild-type FLAG-tagged PCAF expression construct, pCI-FLAG-PCAF, from Addgene (Plasmid #8941).

Pc2 – pEGFP-Pc2 (wild-type) was generously provided by Dr. Andrew Sharrocks, from the University of Manchester (Manchester, United Kingdom).

IL-6-PROM-01-Luc – A luciferase reporter construct containing an IL-6 promoter was purchased from Active Motif (Carlsbad, CA).

2.2 – Antibodies:

Anti-GFP – The primary antibody detecting the GFP-epitope tag was purchased from Santa Cruz Biotechnology (sc-9996). For western blot analysis and the detection of proteins containing a GFP tag, the GFP-antibody was diluted at 1:500 in a 5% milk solution of 1X TBST (TBS-Tween: 50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween-20). The blots were incubated in antibody solution at 4°C overnight. After incubation with primary antibody, blots were exposed to a mouse-IgG-HRP secondary (Cat. # NA931-1ML) purchased from Amersham (acquired by GE Healthcare, Uppsala U.K.) at a dilution of 1:10,000 in 1X TBST for 1 hour at room temperature, followed by detection by West Pico Enhanced Chemi-Luminescence purchased from Thermo Fisher Scientific (Rockford, IL).

Anti-HA – The primary antibody detecting the HA-epitope tag (Cat# MMS-101P) was purchased from Covance Inc. (Princeton, NJ). For detection of HA-tagged proteins on a western blot, the antibody was generally diluted at 1:10,000 in a 5% milk solution of 1X TBST. However, for particular instances such as detections of weaker protein-protein interactions, the HA primary antibody was used at a dilution of 1:5,000. Blots were incubated in primary antibody solutions overnight at 4°C. Following the primary antibody incubation, western blots were exposed to a mouse-IgG-HRP secondary at a dilution of 1:10,000 in 1X TBST for 1 hour at room temperature, and ultimately detected by West Pico ECL.

Anti-FLAG – The primary antibody used for detection of proteins with FLAG epitope tags was purchase from Sigma (Cat# F1804). The antibody was diluted at 1:1,000 in a 5% milk solution of 1X TBST solution, and blots were incubated overnight at 4°C. After the incubation with primary antibody, blots were exposed to a mouse-IgG-HRP secondary antibody at a dilution of 1:10,000 in 1X TBST for 1 hour at room temperature and detected by West Pico ECL.

Anti-tRFP – The primary antibody used for detecting the KATE epitope tag was purchased from Evrogen (Cat#AB233). While this primary antibody is generated to detect the RFP tag, it is also able to detect the mKate2 tag, allowing it to be effective for our studies. Western blots were incubated in the anti-RFP antibody at a dilution of 1:1,000 in a 5% BSA (bovine serum albumin) (Sigma Cat. #A3059-100G) solution in 1X TBST solution at 4°C overnight. Following incubation in primary antibody, blots were exposed to a Goat anti-Rabbit-HRP secondary antibody from KPL Inc. (Gaithersburg, MD) (Cat. #374-1506) at a dilution of 1:10,000 in 1X TBST for 1 hour at room temperature, and detected using West Pico ECL.

Anti-NORE1A - Rabbit polyclonal NORE1A antibodies were raised against the synthetic human NORE1A peptide: KYDKFRQKLEEALRESQGKPG by ProSci (Poway, CA). Two different rabbit polyclonal antibodies were generated by ProSci: PAS #17071 (0.73mg/mLx2.75mL=2.0mg) and PAS #17072 (1.17mg/mLx2.0mL=2.34mg), both in PBS with 0.02% NaN3. The date of generation was October 3rd, 2014. These antibodies were used for detection of NORE1A on western blots and also for immunoprecipitations

(IP). After performing test IPs using over-expression studies, we concluded that PAS #17071 was better suited for IPs, and PAS #17072 would be used for western blot analysis. For IPs, whole cell lysates were incubated with 10 µL (equaling approximately 7.3 µg) of PAS #17071 antibody in 1 mL final volume of modified RIPA buffer overnight, following by a 4 hour immunoprecipitation using rabbit conjugated agarose beads purchased from eBiosciences Inc. (acquired by Rockland Inc., Pottstown PA) (Cat. #00-8800-25). For full IP protocol, see Section 2.8.1.2) – immunoprecipitations. For western blot analysis, the PAS #17072 antibody was added to a 5% milk in 1XTBST solution at a dilution of 1:500 and incubated at 4°C overnight. The blots were then probed with a Rabbit-IgG-HRP (KPL) secondary antibody at a dilution of 1:10,000 in 1X TBST for 1 hour at room temperature, followed by detection using a West Pico ECL.

Anti-Phospho-Rb-Ser795 – When western blotting for phosphorylated levels of endogenous Rb protein, an anti-Rb antibody that specifically recognized phosphorylated Rb at Serine 795 was purchase from Cell Signaling Technology (Beverly, MA) (Cat. #9301). This antibody was diluted at 1:1,000 in a 5% BSA/1XTBST solution and blots were incubated overnight at 4°C. Following incubation in primary antibody, blots were exposed to a goat anti-Rabbit-HRP secondary antibody at a dilution of 1:10,000 in 1XTBST solution for 1 hour at room temperature, followed by detection of protein using West Pico ECL.

Anti-Rb [4H1] – Endogenous levels of total Rb protein was detected on a western blot using an antibody purchased from Cell Signaling (Cat. #9309) diluted at a concentration

of 1:1,000 in 5% milk in 1X TBST. Blots were incubated in primary antibody at 4°C overnight. Following the primary antibody incubation, blots were probed with a mouse-IgG-HRP secondary antibody diluted at 1:10,000 in 1X TBST solution for 1 hour at room temperature, followed by detection using West Pico ECL.

Anti-PP1A – When western blotting for endogenous levels of PP1A protein, an anti-PP1A antibody (C-19) purchased from Santa Cruz (sc-6104), and is a goat polyclonal IgG provided at 200 µg/mL, and its epitope maps at the C-terminus of PP1A of human origin. This antibody was diluted at 1:200 in a 5% milk in 1X TBST solution; blots were incubated in PP1A primary antibody overnight at 4°C. Following the overnight incubation, blots were probed with a rabbit anti-Goat IgG-HRP (purchased from Santa Cruz, sc-2768) diluted at 1:10,000 in 5% milk in 1X TBST solution for 1 hour at room temperatures, followed by protein detection using West Pico ECL.

Mouse and Rabbit-HRP TrueBlot Secondary Antibody – Instances occurred when experiments required agarose conjugated beads that are bound with mouse or rabbit secondary antibodies. This can cause the heavy and light chains of antibodies to be detected by the respective mouse/rabbit secondary antibodies during western blot analysis. Fortunately, a TrueBlot® product line of secondary antibodies generated by Rockland Inc. do not detect heavy and light chain IgG bands. Thus, there were some cases were this secondary antibody was used rather than the standard mouse-HRP secondary antibody previously mentioned. Western blots were incubated with the TrueBlot Mouse antibody (Cat. #18-8817-33) was diluted at a dilution of 1:2,000 in a 5%

milk in 1X TBST solution for approximately 1 hour, followed by detection using West Pico ECL. Similarly, the TrueBlot Rabbit-HRP secondary antibody does not detect the heavy and light chains of antibodies conjugated to agarose beads. When necessary, the TrueBlot Rabbit secondary (Cat. #18-8814-33) was used in exactly the same manner as the TrueBlot Mouse secondary antibody.

2.3 – Cell Lines:

HEK 293 – Human Embryonic Kidney 293 cells (HEK 293) are a specific cell line originally derived from human embryonic kidney cells. These embryonic kidney cells were transformed by exposing cells to sheared fragments of adenovirus type 5 DNA (241). HEK 293 cells are straightforward to grow in culture, but more importantly transfect very readily and have been widely used in cell biology as hosts for gene expression. These cells were purchased from ATCC (Manassas, VA) and were widely used for our studies. HEK 293 cells are grown in DMEM supplemented with 10% FBS.

HEK 293T – An important variant of the HEK 293 cell line is the HEK-293T cell line, which have been modified to express the SV40 Large T-antigen, which allows for increased protein production by allowing replication of transfected plasmids containing the SV40 origin of replication. This cell line was primarily used for coimmuno-precipitation experiments. This cell line was also obtained from ATCC and are cultured in DMEM supplemented with 10% FBS.

COS-7 – The COS-7 cell line (an abbreviation for CV-1 in Origin with SV40 genes) is a fibroblast-like cell line derived from the kidney cells of the African green monkey (*Cercopithecus aethiops*). COS-7 cells were developed by Yakov Gluzman by immortalizing CV-1 cells with a mutant version of the SV40 virus that can produce the Large-T antigen but has a defect in genomic replication (242). While not being human in origin, COS-7 cells are a valuable tool to study potential co-localization of fluorescently labeled proteins, as their flat morphology allow the option to do high resolution, live-cell imaging microscopy as it reduces background fluorescence. This cell line was purchased from ATCC and is maintained in DMEM supplemented with 10% FBS.

A549 – Purchased from ATCC, A549s are human lung adenocarcinoma epithelial cells. This cell line was initiated by D.J. Giard and colleagues through explants culture of lung carcinomatous tissue from a 58-year old Caucasian male (derivation information courtesy of ATCC). This cell line harbors the K-Ras (G12S) mutation, giving these cells a constitutively activated Ras protein. In addition, these cells are null for NORE1A expression, and it has been recently shown that expressing NORE1A in A549 cells leads to the induction of cellular senescence (20). A549s also express wild-type p53 and Rb. These cells were cultured in DMEM supplemented with 10% FBS.

NCI-H1299 – This cell line, obtained from ATCC, is a human non-small cell lung carcinoma derived from the lymph node. There are two significant reasons why these cells are so highly transformed. First, H1299 cells have a point mutation in N-Ras (N-Ras-Q61K), rendering it constitutively active. Second, they do not express p53 (243) as a

result of a homozygous partial deletion within the p53 gene. These cells are cultured in RPMI medium supplemented with 10% FBS.

HBEC-3KT – This is an immortalized, non-transformed human bronchial epithelial cell line, which was provided courtesy of Jerry Shay (UT South Western, Dallas, TX). This cell line was established by infecting primary human bronchial epithelial cell culture with human telomerase (hTERT) and mouse cyclin dependent kinase 4 (CDK4) expressing retrovirus constructs and selecting with Puromycin and G418 (244). Because they were immortalized without the use of any viral oncogenes, such cells can almost be considered primary cells. These cells were cultured in keratinocyte serum-free medium containing bovine pituitary extract and recombinant epidermal growth factor (Invitrogen).

HepG2 – This is a human hepatocellular carcinoma cell line derived from the liver tissue of a 15-year old Caucasian male who had well-differentiated hepatocellular carcinoma, which is the fifth most common cancer worldwide. HepG2 cells contain a mutant Ras (N-Ras-Q61L) but have wild-type p53 and Rb (245). More importantly, NORE1A and PP1A protein can be detected in this cell line via western blot analysis, which is the predominant reason for which we performed endogenous coimmunoprecipitations in this cell line. HepG2 cells are maintained in DMEM supplemented with 10% FBS.

MEFs – Mouse embryonic fibroblasts (MEFs) are primary murine cells with the senescence machinery intact that can be isolated from mouse embryos and can be expanded in culture. Interestingly, such cells undergo significant induction of senescence

in the presence of activated Ras, making these cells useful tools to study Ras effectors and their role in senescence. Wild-type and $Rb^{-/-}$ MEFs were generously provided by Dr. Brian Clem (University of Louisville, Louisville, KY) and were used to study the potential role of Rb in NORE1A mediated senescence. $Rb^{-/-}$ MEFs were generated and validated as previously described (226). MEFs were grown in DMEM supplemented with 10% FBS.

293FT – The 293FT cell line is a fast-growing, highly transfectable clonal isolate derived from human embryonic kidney cells transformed with the SV40 large T antigen. The 293FT cell line is ideal for generating high-titer lentivirus production. This cell line was used to generate lentivirus carrying shRNAs against Rb in order to address the role of Rb in NORE1A mediated senescence. A detailed explanation of the generation of lentiviral particles is discussed in section 2.7.3. 293FT cells were grown in DMEM supplemented with 10% FBS, 1% Pen-Strep, 0.1mM MEM non-essential amino acids, 6mM L-Glutamine, 1mM MEM sodium pyruvate and 500μg/ml G418 sulfate (Corning Cellgro Cat. #30-234-CR).

2.4 – Cell Culture Materials:

Growth Media – Different cell lines grow optimally under different conditions, including the growth medium. Thus, several specific growth media were obtained. Dulbecco's Modified Eagle's Medium (DMEM) 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 a 1% solution of Penicillin/Streptomycin antibiotic (Corning Cellgro Cat. #30-002-CI). 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). HBEC-3KT cells require a special growth media formulation and we 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).

Trypsin – Trypsin EDTA (0.25%) was purchased from Corning Cellgro (Cat. #25-053-CI) and used to remove adherent cells from culture dishes and flasks. Media was removed by aspiration from cell culture dishes and washed once with 1X PBS, which was purchased from Corning Cellgro (Cat. #21-040-CV and #46-013-CM). 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.

Cell Culture Flasks and Dishes – All cells were passaged in either T-25 or T-75 culture flasks purchased from TPP. When plating cells for biological experiments, cells were plated in 60 mm or 100mm cell culture dishes as well as 6, 12, and 24 well plates, all of which were purchased from Greiner Bio-One. In particular instances, 96-well plates were purchased from TPP.

Lysis Buffer and Chemicals for Cell Treatment – For biological assays where protein-protein interactions were not involved, we lysed cells in RIPA buffer purchased from Sigma (Cat. #R0278), which contains the following: 150mM NaCl, 1.0%, 1.0% IGEPAL® CA630, 0.5% sodium deoxycholate, 0.1% SDS, and 50mM Tris pH 8.0. This lysis buffer allows for efficient cell lysis and is suitable for protein detection by western blot analysis. To inhibit the proteasome, cells can be treated with a chemical called MG-132, which we obtained from Sigma (Cat. #C2211-5MG). Stock solutions of MG-132 were dissolved in DMSO to a concentration of 10 mM and diluted in cell culture media to obtain a final working concentration of 10 µM. For the purposes of our studies, cells were treated with MG-132 for 6 hours prior to lysis when applicable. To study changes in protein stability, we purchased a chemical called cycloheximide from Sigma (Cat. #P8833-10MG). It works by inactivating the transferase II enzyme involved in peptide chain elongation, ultimately inhibiting protein synthesis. One can then analyze protein decay over a time course to determine the stability of a protein of interest. Stock powder of cycloheximide was dissolved in 100% ethanol (EtOH) to a concentration of 20 mg/mL, and was then added to cell culture media to obtain a final concentration of 20 µg/mL.

2.5 – Transfections:

Transfection of DNA plasmids and siRNAs into mammalian cells is a critical means by which to study gene function. There are multiple options that are commercially available to transfect cells; however, different cell lines can be transfected more or less optimally depending on the transfection reagent. Therefore, we used several different reagents in order to transfect cells as efficiently as possible. These include: JetPRIME, Lipofectamine 3000, and DharmaFECT.

2.5.1 - JetPRIME™ Transfection:

The JetPRIME™ DNA transfection reagent (VWR Cat# 89137-972) can be purchased from Polyplus-Transfection Inc. (Illkirch, France). It is generally described as a cationic polymer-based reagent to ensure high DNA transfection efficiency by forming positively charged complexes with DNA that can enter the cell via endocytosis, in turn releasing plasmid DNA into the cytoplasm, eventually reaching the nucleus. For most of our transfection procedures, 1-2 µg of DNA plasmids of choice were mixed in 200 µL of JetPRIME™ transfection buffer provided by the manufacturer. To this mix, 2-4 µL of JetPRIME™ was added, depending on the amount of DNA (JetPRIME™ was consistently added at a ratio of 2 µL per 1µg DNA plasmid). The mix would be vortexed for 10 seconds and spun down for 10 seconds using a tale-top micro-centrifuge. The DNA/JetPRIME™ mixture was incubated for 10 minutes at room temperature and was added dropwise to the dish (in this case, 60 mm dish) and incubated for 6 to 8 hours.

After this incubation, the culture media/transfection mix you be aspirated and fresh media would be added and remain overnight. This general transfection protocol can be modified based on the size of the cell culture dish used as well as the overall amount of DNA plasmid desired. This transfection reagent was used for transfections of HEK 293, HEK-293T and COS-7 cells, and would generally yield high transfection efficiency. However, this reagent was not as efficient for transfecting other cells used in this project, such as A549 cells.

2.5.2 – Lipofectamine 3000®:

The Lipofectamine 3000® reagent was purchased from Invitrogen (Cat. #LC3000015). It is a lipid based formulation designed for cells that are difficult to transfect when using other transfection reagents such as JetPRIME™. For the purposes of this project, MEFs and A549 cells were transfected using Lipofectamine 3000® according to the manufacturer's instructions. Cells were plated in 35mm dishes or 12-well plates (in duplicate) and thus were transfected in a similar manner. First, two tubes, (name?) each containing 100 µL of Opti-MEM (Invitrogen Cat. #31985062), were prepared. In the first tube, 3.75 µl per µg of DNA was added. In the second tube, the respective DNA was added, followed by the addition of 2 µL of P3000™ reagent per 1 µg of DNA. After mixing both tubes thoroughly, the DNA/P3000™/Opti-MEM mixture from tube 2 was added to tube 1 (containing Lipofectamine 3000®/Opti-MEM), further mixed and incubated for 5 minutes at room temperature. After the incubation, the mixture was added drop-wise to the cells and was incubated for 24 hours. Fresh media would be

added to the cells the following day and were incubated for an additional 24-48 hours depending on the biological assay being performed.

2.5.3 - DharmaFECT™ siRNA Transfection:

Transfection of siRNA into cultured mammalian cells required very specific transfection reagents. We purchased the DharmaFECT™ transfection reagent from GE Healthcare. All siRNA transfections of siPP1A into A549 cells were performed using this reagent, and were always performed the day prior to transfections of DNA plasmids. First, A549 cells were plated in 35mm dishes at a concentration of 200,000 cells per dish. The next day, the siRNA transfection was performed according to the manufacturer's protocol. In tube 1, we diluted the siRNA by adding 5 µL of a 10 mM stock siRNA into 195 µL of Opti-MEM. In tube 2, we added 5 µL of DharmaFECT reagent (formulation #2) into 195 µL of Opti-MEM. We gently mixed both tubes and allowed them to incubate at room temperature for 5 minutes. Then, we added the contents of Tube 1 to Tube 2, for a total volume of 400 µL. We again mixed carefully and incubated for 20 minutes at room temperature. Following the incubation, we added 1.6 mL of antibiotic-free DMEM to the mix for a final volume of 2 mL transfection media and a final siRNA concentration of 25 nM. The transfection media was added dropwise to the cells and incubated for 24 hours. For optimal knockdown, fresh media would be added the following day and the incubation was continued for a minimum of an additional 48 hours, allowing for a total of 72 hours for proper protein knockdown.

2.6 – Molecular Biology:

In order to thoroughly address the goals involved in this project, numerous experiments were performed which required a number of different plasmids in several combinations. Thus, when identifying the functions of specific proteins, as well as effects of proteins on other protein-protein interactions, it was critical that our over-expression studies have proteins with different epitope tags. In order to address this, a number of molecular biology techniques were performed which include the following: restriction enzyme digests, DNA ligations, agarose gel electrophoresis, bacterial transformations, and plasmid preparation and purification.

2.6.1 – Restriction Enzyme Digests:

In order to generate DNA plasmids with different epitope tags, plasmids of interest must be subcloned into different expression vectors. In turn, all DNA subcloning requires restriction enzymes to shuttle DNA fragments from one vector to another. All of the enzymes used in this project were purchased from New England Biolabs® Inc. (Ipswich, MA). When performing restriction enzyme digests, reactions would consist of our DNA of interest, enzymes with their optimal buffers, and BSA when necessary. Restriction enzyme digests were incubated for approximately 2 hours in a 37°C water bath.

2.6.2 – Agarose Gel Electrophoresis:

DNA from restriction enzyme digests were run on 1% agarose gels in order to separate our linearized fragments of interested according to size. Agarose gels were made by adding 0.5 grams of SeaKem LE agarose, purchased from Lonza (Basel, Switzerland) (Cat. #50004), into 50 mL of 1X TAE buffer (Tris-Base, Acetic Acid, EDTA) purchased from MediaTech (a Corning subsidiary) (Cat. #46-010-CM), giving a final concentration of 1% gel by weight. In order to make the gel, the agarose/TAE solution had to be melted. After melting, but before casting, 2 µL of ethidium bromide (Invitrogen) was added and mixed well. The ethidium bromide will then intercalate into our linear DNA allowing for the visualization of DNA using a UV box. Gels were then loaded and ran at approximately 85 volts to allow for separation of the relevant DNA fragments. In the instance where DNA fragments needed to be re-annealed to other vectors via DNA ligations, the retrieval of DNA from the agarose gel was required. In such cases, DNA bands were cut out and purified using a GenElute™ column (purchased from Sigma, Cat. #56500) according to the manufacturer's protocol.

2.6.3 – DNA Ligation:

Following restriction enzyme digests, agarose gel electrophoresis and DNA purification, DNA ligation reactions were performed in order to recombine DNA fragments into our vectors of interest. Generally, a 3:1 molar ratio of vector backbone fragment to the DNA fragment containing our gene of interest was used in order to

achieve efficient ligations. The two DNA fragments (though the double digest of human Rb contained three fragments) were added to eppendorf tubes along with 1µL of 10X ligation buffer, 1 µL of T4 DNA ligase enzyme (both buffer and ligase were purchase from New England Biolabs®), and 5 µL of molecular grade water were added, making a total ligation mixture volume of 10µL. The ligation reaction was mixed thoroughly and was allowed to incubate overnight in a 10-15°C water bath.

2.6.4 – Bacterial Transformation:

Chemically competent DH5α were purchased from Invitrogen (Cat. #18265-017) and were used for both whole plasmid as well as complete ligated plasmid transformations. In order to transform a plasmid into these bacteria, approximately 100 ng of whole plasmid (or typically 2-5 µL of a completed ligation reaction performed as in section 2.7.3) was mixed with 30 µL of DH5α bacteria and incubated on ice for 30 minutes. The bacteria/plasmid mixture was placed into a 42°C water bath for 30 seconds in order to "heat shock" the bacteria, in turn allowing the plasmid DNA to efficiently enter the bacteria. After heat shock, the mixture was placed back on ice for an additional 2 minutes. Then, 250 µL of SOC media (Corning Cellgro Cat# 46-003-CR) was added to the mixture and incubated in a 37°C water bath for 1 hour. After this incubation the mixture was plated on LB agar plates containing the relevant antibiotic depending upon the resistance marker of the plasmid (either Ampicillin with a concentration of 100µg/mL or Kanamycin at a concentration of 50µg/mL). For whole plasmid transformations, 50 µL of the final mixture would be added to the LB agar plate; however, for transformations of

DNA ligation, the entire volume of the transformation would be added to two plates (we would add 125 µL per plate on two separate plates), as the transformation efficiency from DNA ligations would be far less than whole plasmid transformations. The plates were placed in a 37°C incubator overnight.

2.6.5 – Plasmid Purification:

Following a successful plasmid transformation, individual colonies from the LB agar plates would be selected and grown in LB broth media supplemented with the relevant antibiotic at the concentrations listed in section 2.7.6, and were incubated using a 37°C shaker at 300 RPM. The colonies were expanded to either 5 mL overnight cultures for mini-prep (small scale) purification; however, for midi-prep (large scale) purification, colonies expanded to a 5 mL culture for approximately 8 hours were then added to 75 mL of LB broth media and incubated at 37°C overnight. DNA plasmids were extracted and purified using either mini-prep (purchased from Qiagen, Cat. #27106) or midi prep kits (purchased from Sigma, Cat. #NA0200-1KT). Both mini- and midi-prep plasmid purification techniques were performed following the manufacturer's protocols. Once the plasmid purifications were complete, we obtained the plasmid concentrations using a Nano-Drop instrument (part of the DNA sequencing core, purchased from Thermo Scientific).

2.7 – Biological Assays:

Attempting to address the hypotheses of this project required the use of several state of the art biological techniques and assays. Some of the important goals involved identifying novel protein-protein interactions, studying protein stability as well as post-translational modifications, and induction of cellular senescence. The biological assays used to answer these questions included the following: immunoprecipitations, western blot analysis, luciferase assays, and senescence assays. The detailed explanations of how these assays were performed, as well as data analysis resulting from these assays, are listed below.

2.7.1 – Immunoprecipitations:

One of the most common and applicable techniques used to study protein-protein interactions is the coimmunoprecipitation assay. This technique involves agarose beads that typically have an antibody conjugated to them, which allows for the precipitation of a particular protein. By pulling down proteins of interest using such antibody-conjugated beads, one can look for the presence of other proteins that interact with it. From a theoretical standpoint, coimmunoprecipitations are considered relatively straightforward; however, such a technique can be quite difficult, especially at the endogenous, physiological level. Detailed below are procedures involved for coimmunoprecipitations that were performed, both at the over-expressed and endogenous level.

2.7.1.1 – Immunoprecipitation of Over-Expressed Proteins

Coimmunoprecipitations of over-expressed proteins were generally performed 24 hours post-transfection (as described in Section 2.6) of cells that were plated in 60mm dishes. Cells were lysed in 300µL of Modified RIPA buffer (in some cases, more or less might be used depending upon cell confluency), which consists of 50mM Tris-HCl pH7.4, 200mM NaCl, and 1% NP-40. Modified RIPA buffer was supplemented with a protease inhibitor cocktail (Sigma) and 1 mM sodium orthovanadate. Cells were harvested using a cell scraper, transferred to eppendorf tubes, and placed on a rotator for 4 hours at 4°C. After the incubation, each sample was syringed 6-8 times using a 25 gauge syringe needle, followed by a max speed centrifuge for 3 minutes at 4°C. The supernatant was removed from the insoluble pellet, placed in a new eppendorf tube, and quantified using a Bio-Rad protein quantification assay (Cat. #500-0006). Typically, 1 mg of protein was used and additional Modified RIPA buffer was added to each sample to make a total volume of 1 mL (giving us consistent samples of 1 mg/mL). Then, agarose beads with the relevant epitope tag were added according to the respective manufacturer protocol: either 5 µL of GFP (GFP-Trap® Beads from Allele Biotechnology, Cat. #ABP-NAB-GFPA100), HA (Sigma, Cat. #A2095-5ML), or FLAG (Anti-Flag M2 Affinity Gel from Sigma, Cat. #A2220-5ML) conjugated agarose beads were used per sample. In all coimmunoprecipitation experiments using such beads, lysates were rotated overnight at 4°C. The following day, the immunoprecipitations were centrifuged for 2 minutes at 3000 RPM using a refrigerated centrifuged to pellet down the beads. The supernatant was removed and the beads were washed with 500 µL of

Modified RIPA buffer. This washing procedure was repeated a total of three times. At the last wash step, the beads were re-suspended in 12 µL of RIPA buffer and 4µL of 4X NuPAGE LDS sample running buffer. The IPs, along with 50 µg of whole cell lysate per sample, were run on a SDS-PAGE gel and analyzed by western blot as described in Section 2.8.2.

2.7.1.2 – Immunoprecipitation of Endogenous Proteins:

As endogenous proteins do not have epitope tags that can be immunoprecipitated using antibody-conjugated beads, the protocol for coimmunoprecipitation of endogenous proteins varies to the one used for over-expressed proteins. Again, cells were lysed in Modified RIPA buffer; however, in this particular case 3 mg of total purified protein was placed in a final volume of 1 mL (protein lysate plus Modified RIPA buffer). First, a primary antibody against the protein of interest was added and rotated at 4°C overnight. The following day, 15 µL of an agarose conjugated secondary antibody (Rockland Cat. #00-8811-25) was added and rotated for 4 hours at 4°C as to bind to the primary antibody that is now attach to the protein of interest. The agarose beads were again washed three times with Modified RIPA buffer as previously described (Section 2.8.1.1). Following the wash steps, the beads were resuspended in 12 µL of RIPA buffer and 4 µL of 4X NuPAGE LDS sample running buffer. The immunoprecipitations, along with their relevant controls (which included lysate with no primary antibody and buffer with primary antibody) and whole cell lysate, were analyzed by western blot analysis.

2.7.2 – Western Blotting:

Whole cell lysates, as well as any immunoprecipitation samples, were combined with a 4X LDS sample buffer purchased from Invitrogen (Cat. # NP0007) made in the following manner: 450 µL of LDS sample buffer plus 50 µL of β-Mercaptoethanol purchased from Sigma (Cat. # M6250-100ML). The 4X LDS sample buffer was added to sample to give a final concentration of 1X. Whole cell lysates and IPs (which were vortexed thoroughly) were heated at 95°C for 10 minutes in order for efficient denaturation of protein. Once the protein samples were prepared, they were loaded on a pre-cast 10% Bis-Tris Polyacrylamide Gel obtained from Invitrogen (Cat. #NP0302BOX) and was filled with 1X MOPS SDS Running Buffer (Cat. #NP0001). All protein gels were run at 120 Volts until the gels had run to completion (indicated by the loading dye reaching the bottom of the gel). The gel was then removed from the casting tray and prepared for the transfer phase.

Protein gels were transferred onto a 0.2 µm pore size nitrocellulose membrane using a complete immersion (wet) transfer in 1X Transfer Buffer (Cat. #NP0006-1), which was made in the following manner: 50 mL of 20X Transfer Buffer, 200 mL of methanol, and 750 mL DI water. The prepared transfer buffer was always pre-chilled in a -20°C freezer before use. All protein gels were transferred at 35 volts for approximately three hours.

Following the transfer phase, nitrocellulose membranes would be washed in 1X TBST solution for 5 minutes and then blocked in a 5% milk in 1X TBST solution for 1 hour. After blocking, the membranes (blots) would be incubated in primary and

secondary antibodies as previously described in Section 2.3. Between primary and secondary antibodies, as well as after secondary antibody removal before West PICO ECL detection, blots were washed in 1X TBST 3 times for 5 minutes and one additional time for 10 minutes. Blots were immersed in West PICO ECL solution for 2 minutes and exposed to chemi-luminescent detection film purchased from MidSci™ (Valley Park, MO) (Cat. #EBA45).

2.7.3 – Generation of Stable Rb Knockdown Cell Lines:

In order to effectively study the significance of Rb in NORE1A mediated senescence, we generated a matched pair of A549 cell lines that were either wild-type or stably knocked down for Rb expression. To generate Rb knockdown cells, 293FT cells were transfected with the pLKO-Rb knockdown constructs (described in Section 2.1) and a mixture of the viral packaging plasmids pLP1, pLP2 and pLP/VSVG (Invitrogen) at a ratio of 1:3, using JetPRIME™ transfection reagent. Approximately 48 to 72 hours following transfection, supernatant containing viral particles was harvested and clarified by centrifugation at 2000 RPM for 5 minutes. 2 mL of the clarified viral supernatant containing 4 μg/mL polybrene was added to A549 cells and the cells incubated for 12-16hr at 37°C. This viral transduction step was repeated once more. After the second transduction step, cells stably transduced with the knockdown constructs were selected in 1μg/ml of puromycin purchased from Sigma (Cat. #P8833-10MG) and used as an early passage pooled population. All aspects involving the generation of Rb knockdown cell

lines were carried out by Dr. Howard Donninger, and the validation of the matched paired cell system was performed by western blot analysis.

2.7.4 – Senescence Assays:

The main focus of this project was to elucidate the mechanisms by which NORE1A can promote the induction of senescence. While there are several established markers of senescence, the gold standard used to study cellular senescence is by an increase in β-galactosidase activity that can be detected at a sub-optimal pH (246). For the purposes of this project, we purchased a senescence detection kit from BioVision Inc. (Milpitas, CA) (Cat. #K320-250) that can be used to detect β-galactosidase activity from mammalian cells grown in culture. We performed senescence assays in MEFs (wild-type MEFs have the senescence machinery intact) and A549s (that can be induced for senescence by NORE1A transfection) (20). For MEFs and A549s, approximately 50,000 cells were seeded per well in 12-well plates. The day after plating, cells were transfected with the relevant expression constructs (as described in Section 2.5), and fresh media added every 24 hours for 72 hours post-transfection. Cells were then stained for β-galactosidase as recommended by the manufacturer's protocol. The scoring of the assay was performed by counting five random fields of view per well, doing a ratio of the number of β-galactosidase positive stained cells to the total number of cells in the field of view. This allows for a percentage of cells undergoing senescence. For senescence assays involving PP1A siRNAs, the siRNAs were transfected first and the transient transfection of NORE1A would be performed the following day. These cells were incubated for an

additional 72 hours before β-galactosidase staining. For any senescence assay that was performed in this dissertation, the relative expression of GFP-tagged NORE1A was taken 48 hours post-transfection to ensure consistent transfection efficiency throughout the experiment.

2.7.5 – Luciferase Assays:

Luciferase assays were performed using reagents using the LightSwitch™ luciferase assay kit with a corresponding luciferase reporter construct containing the IL-6 promoter generated by Active Motif (Carlsbad, CA). A549 +/- Rb cells (described in Section 2.8.2) were plated at a concentration of 5,000 cells per well in a 96-well plate. These cells were transfected with the IL-6 promoter luciferase construct in the presence or absence of GFP-NORE1A, and were incubated for 48 hours. Before performing the luciferase assay, pictures were taken to show consistent expression of GFP-vec/NORE1A throughout the experiment. Cells were lysed according to the manufacturer's protocol, and the samples were read using a Lumat LB 9507 from Berthold Technologies (Oak Ridge, TN). The data was analyzed using the Relative Luciferase Units (RLU).

2.8 – Image Acquisition, Processing, and Statistical Analysis:

A Pharos FX plus Molecular Imager from BioRad (Hercules, CA) was used to digitize images prior to quantification using Quantity One software (BioRad). Figures were compiled using Photoshop software (Adobe). All data are reported as mean ±

standard deviation. Difference between treatment groups were tested using a two-sided Student's t-test as appropriate. Data was considered statistically significant where $p<0.05$.

CHAPTER 3

RAS REGULATES RB VIA NORE1A

3.1 – Introduction:

Ras mutations are the most frequent oncogenic events in human cancer and can be found in approximately 30% of all human cancers (247). In experimental systems, activated forms of Ras can be powerfully transforming, and transgenic animal models have validated the role of Ras activation in tumorigenesis (8,248). However, despite the extensive evidence linking Ras to transformation and tumor development, activated Ras can also promote a state of irreversible cell cycle arrest called oncogene induced senescence (159,249). This tendency for deregulated Ras activity to provoke senescence can be observed in Ras driven tumors (246). It appears that senescence provides a potent barrier to suppress the development of Ras driven cancer, as malignant tumors lose the senescence phenotype (246). The exact mechanisms by which Ras can promote senescence are not completely understood, but it appears the main Ras senescence pathways involve the p53 and Rb tumor suppressors (250). Initial evidence in mouse embryonic fibroblasts (MEFs) suggests that loss of functional p53 or Rb pathways alone is sufficient for Ras to bypass senescence (159). More recent studies show that *in vivo*,

suppression of p53 function (251) or Rb (252) enhances Ras mediated transformation in murine systems. However, human systems may require inactivation of both p53 and Rb for full senescence evasion (253). Thus, inactivation of p53 and Rb senescence pathways may be essential for Ras-induced transformation.

In addition to the classic trio of growth promoting Ras effector proteins, Raf, PI-3 kinase and RalGDS, Ras also interacts with growth suppressing effector proteins, including NORE1A (RASSF5) (6,254). NORE1A is a member of the RASSF family of tumor suppressors that is frequently down-regulated during tumor development, and its inactivation has been linked to a rare familial cancer syndrome (13,15,18). NORE1A binds directly to Ras (19) and is thought to act as a scaffolding protein as it lacks any apparent enzymatic activity. NORE1A connects Ras to the pro-apoptotic Hippo pathway (19) and has apoptotic properties (12). NORE1A$^{-/-}$ MEFs are predisposed to Ras-induced transformation, unlike wild-type MEFs which require inactivation of p53 or Rb to allow transformation by Ras (255). Furthermore, up-regulation of Ras activity in primary tumors is often correlated with inactivation of NORE1A (15,256). Thus, NORE1A acts as a potent barrier against aberrant Ras signaling, and loss of NORE1A allows Ras to circumvent its own growth inhibitory properties, shifting the balance of Ras activity towards transformation (20).

We have recently shown that NORE1A is a powerful Ras senescence effector that acts via p53 (20). NORE1A forms an endogenous, Ras regulated complex with the kinase HIPK2, which can regulate both the phosphorylation and the acetylation of p53. In turn, NORE1A scaffolds HIPK2 to p53 and stimulates pro-senescent p53 acetylation. Although we found that NORE1A induced senescence is heavily dependent upon p53, we

noticed that NORE1A retained partial senescence inducing activity even when nearly all detectable p53 had been eliminated from the system (20), suggesting that additional mechanisms are required for the effects of NORE1A on senescence to fully manifest.

In addition to p53, Ras can promote senescence induction by activating the Rb pathway. The Retinoblastoma (Rb) gene was the first identified tumor suppressor gene in rare cases of pediatric tumors of the retina called retinoblastoma (199,222,257). Furthermore, alterations in the Rb gene or inactivation of the Rb protein have been identified in a variety of human cancers (258,259), and it is now widely accepted that the inactivation of the Rb protein may be one of the most frequent events in cancer (260). In addition to its important function in regulating the cell cycle, recent evidence points to Rb having critical functions in other biological processes, including chromosomal stability, regulation of apoptosis, and oncogene induced senescence (159,261,262). Inactivation of Rb *in vitro* or *in vivo* suppresses Ras-induced senescence (159,252), implicating Rb as an important effector of Ras mediated senescence. Thus, while it appears that Ras activates senescence, in part, via Rb, exactly how Ras modulates Rb activity remains unclear.

Rb regulation is complex and involves both inhibitory phosphorylation and activating dephosphorylation events. While the mechanisms of Rb phosphorylation by cyclin-dependent kinases are well characterized (231), the processes that activate Rb by dephosphorylation remain unclear. Recent reports have shown that the phosphatases PP1 and PP2A play important roles in the mammalian cell cycle (263-265). Moreover, it has been shown that PP1 phosphatases can act on Rb to promote the formation of the active, hypophosphorylated form of the protein (232,236). Intriguingly, PP1A enzymatic activity can be regulated by Ras (237). Thus, PP1A might serve as the link between Ras and Rb,

and Ras may, in part, promote senescence by activating PP1A, thereby promoting the dephosphorylation and activation of Rb (22). Exactly how Ras stimulates the activity of PP1A towards Rb remains obscure.

PP1A, a key regulator of Rb activity, was first detected in complex with NORE1A in a yeast two-hybrid system (23), and since we have recently established that NORE1A mediated Ras-induced senescence is only partly driven by p53, we sought to determine if NORE1A could also be modulating senescence by regulating Rb function. We now show that NORE1A regulates the dephosphorylation of Rb by forming an endogenous, Ras regulated, complex with PP1A, scaffolding it to Rb and enhancing the Rb/PP1A complex. Moreover, suppression of Rb suppresses NORE1A induced senescence. Thus, we now identify a powerful new mechanism by which Ras can induce senescence via regulating the phosphorylation status of Rb. Therefore, NORE1A acts as a critical node linking both p53 and Rb to Ras. This may explain why Ras driven tumors often exhibit reduced NORE1A expression (256).

3.2 – Results:

NORE1A forms an endogenous, Ras regulated complex with PP1A –NORE1A is primarily localized to the nucleus (20), and has been observed to shuttle between the cytoplasmic/nuclear cell fractions (266). However, the localization pattern of PP1A is more complex. PP1A exhibits diffuse expression in the cytoplasm and the nucleoplasm, but also accumulates in unidentified nuclear bodies (267). Since NORE1A also occurs in nuclear speckles, we determined whether NORE1A and PP1A co-localized in

mammalian cells using transient transfections of the fluorescently tagged proteins. We found that a pool of GFP-PP1A specifically co-localized with KATE-NORE1A in the nucleus (**Figure 6**). The KATE protein is a far red fluorescent protein that gives bright far-red fluorescence to easily detect proteins engineered to contain a fused KATE tag (268,269). An empty KATE vector was used as a negative control.

To determine if NORE1A and PP1A can be found in a complex, we performed coimmunoprecipitations in HEK-293T cells co-transfected with NORE1A and PP1A in the presence or absence of activated Ras. We found that NORE1A does complex with PP1A. Furthermore, the results show that the interaction of NORE1A and PP1A is significantly enhanced in the presence of activated Ras (**Figure 7A**). Further analysis confirmed that endogenous NORE1A could be coimmunoprecipitated with endogenous PP1A from the HepG2 human liver hepatocellular carcinoma cell line (**Figure 7B**), confirming that the NORE1A/PP1A interaction is physiologically relevant.

Ras/NORE1A Stabilize PP1A – While studying the effects of Ras on the NORE1A/PP1A interaction, we observed an increase in the levels of PP1A in whole cell lysates in the presence of Ras and NORE1A. We hypothesized that Ras/NORE1A could be promoting PP1A stability. To address this, we analyzed the effects of NORE1A and Ras on PP1A protein stability by using cycloheximide treatment after transient transfections in HEK-293 cells. While the results show that Ras or NORE1A individually did not seem to promote the stability of PP1A, the presence of both Ras and NORE1A together lead to a statistically significant ($P<0.05$) increase in PP1A expression, even 24 hours after cycloheximide treatment (**Figures 8, 9**). Thus, it seems that Ras and NORE1A

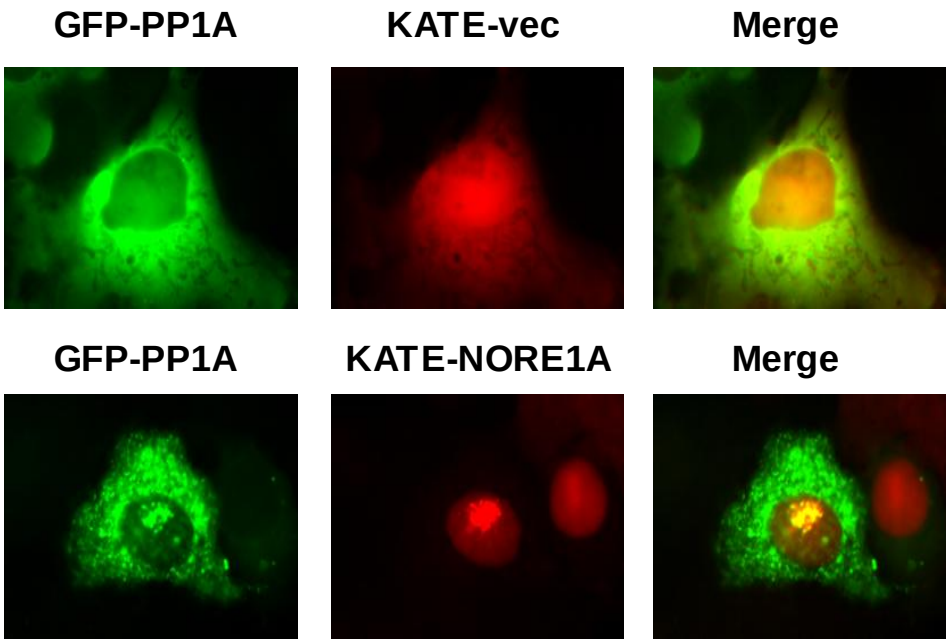


Figure 6. Exogenously expressed NORE1A and PP1A co-localize in the nucleus. COS-7 cells were transfected for 48 hours with GFP-PP1A in the presence or absence of KATE-NORE1A. While PP1A can be found throughout the cell, there is an important pool of PP1A strongly co-localizing in the nucleus with NORE1A. Representative images were taken using an IX50 inverted system microscope (Olympus) and a SPOT camera (Diagnostic Instruments Inc.).

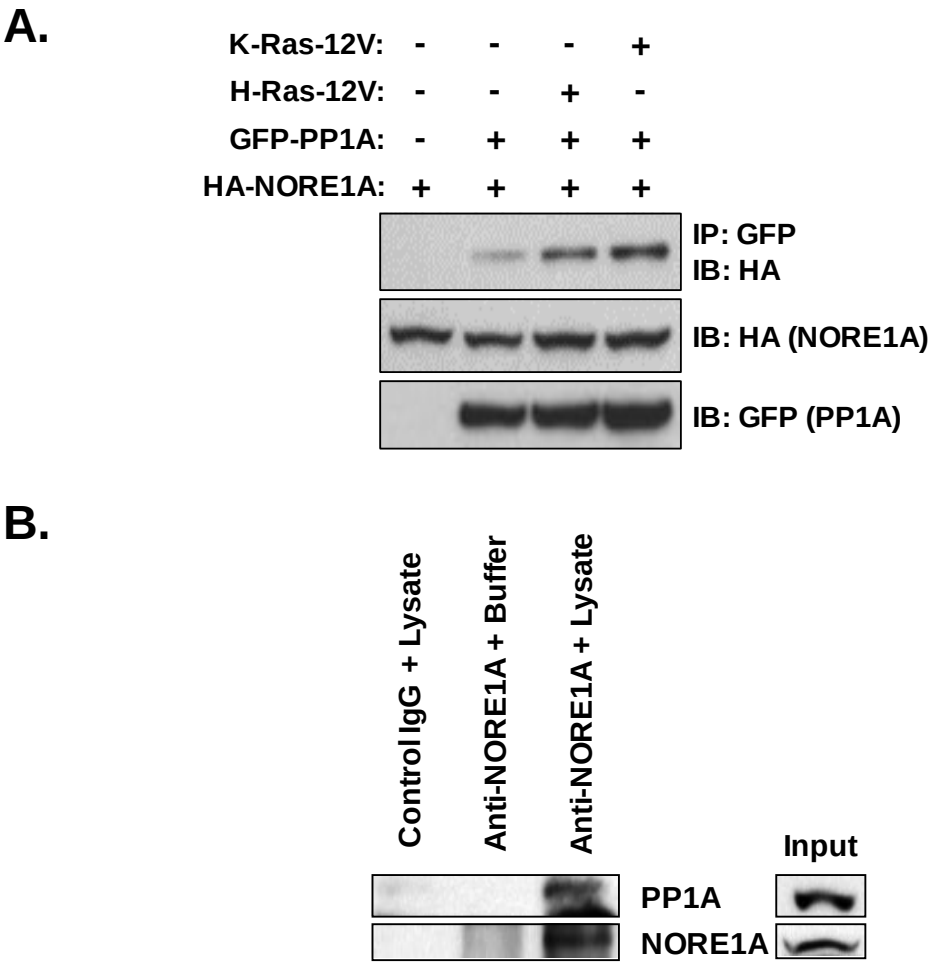


Figure 7. NORE1A forms an endogenous, Ras regulated complex with PP1A. A. *Activated Ras enhances the interaction between NORE1A and PP1A*. HEK-293T cells were co-transfected with expression constructs for PP1A, NORE1A, and activated H- or K-Ras for 24 hours. Cells were then lysed and equal amounts of protein extracts were immunoprecipitated with anti-GFP. The immunoprecipitates were analyzed by western blotting with anti-HA and anti-GFP antibodies. **B.** *NORE1A and PP1A are found in an endogenous complex*. HepG2 cells were immunoprecipitated (IP) for NORE1A and immunoblotted (IB) for PP1A. IgG incubated with HepG2 lysates and Ig/NORE1A antibody incubated with lysis buffer served as negative controls.

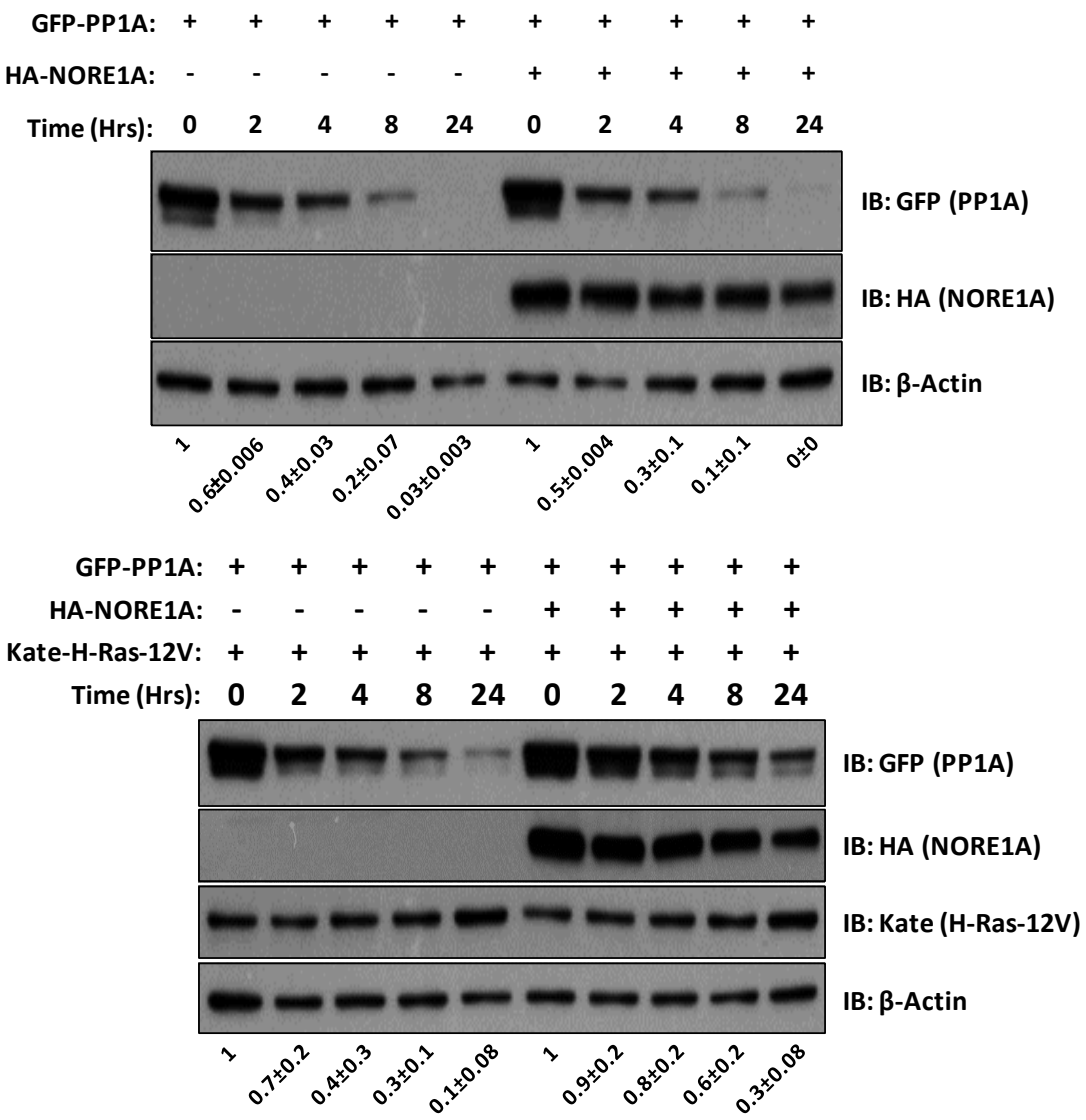


Figure 8. Ras/NORE1A stabilizes PP1A. HEK-293 cells were transfected with PP1A, NORE1A, and activated H-Ras expression constructs for 24 hours. The cells were treated with cycloheximide (20 μg/mL) and lysed at the indicated times after addition of cycloheximide. Levels of PP1A protein were measured by western blot analysis. Shown is a representative blot of three independent experiments. The density of the bands was quantitated using ImageJ software and relative PP1A expression was calculated after normalizing to β-Actin expression. In cells transfected with both NORE1A and Ras, there was a statistically significant increase in the levels of PP1A ($P<0.05$).

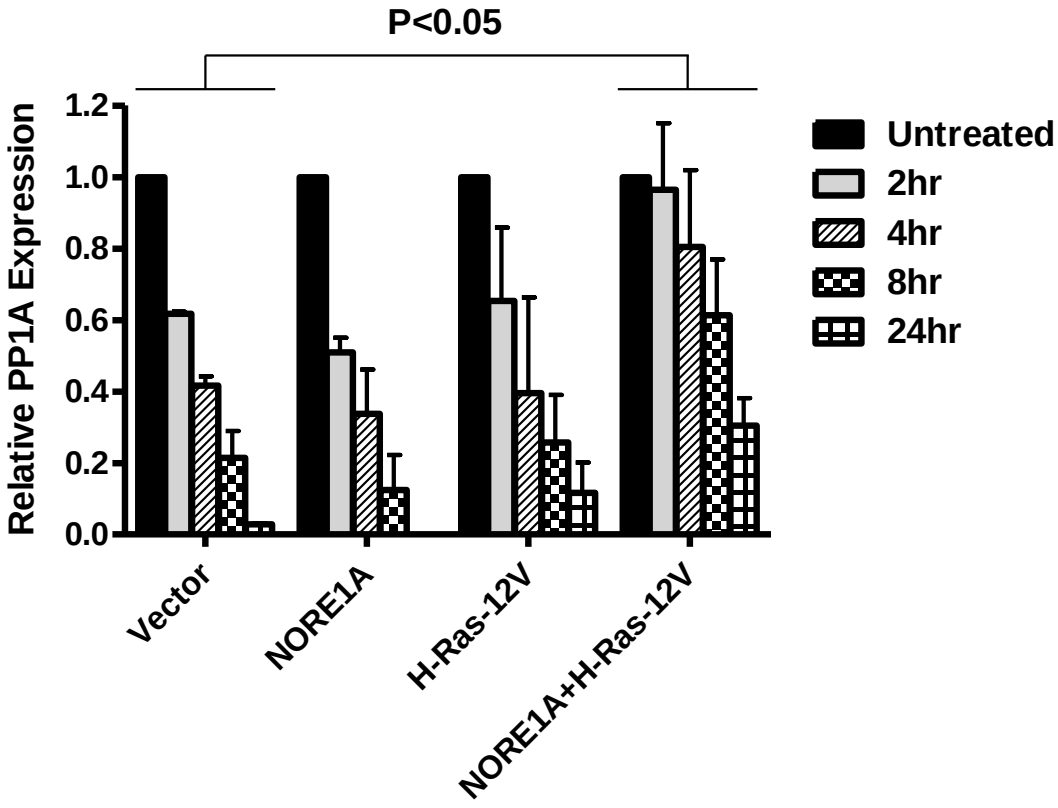


Figure 9. Quantification of the relative PP1A expression in the presence and absence of NORE1A and activated H-Ras following cycloheximide treatment. Quantification of three independent experiments was performed, which is shown as a representative image in Figure 8. Briefly, the blots were quantified densitometrically to calculate the relative amount PP1A in the presence or absence of NORE1A and H-Ras-12V following cycloheximide treatment. *, P<0.05 compared to cells transfected with GFP-PP1A alone.

cooperate to promote PP1A stability. Since NORE1A regulates the stability of other target proteins via the proteasome (194), we sought to determine whether Ras/NORE1A may be similarly regulating PP1A stability. We co-transfected HEK-293T cells with PP1A and an HA-ubiquitin-1 expression construct in the presence and absence of NORE1A and activated Ras, treated the cells with the proteasome inhibitor MG132, immunoprecipitated the PP1A and analyzed the immunoprecipitates for ubiquitinated PP1A by western blotting with an anti-HA antibody. Although the levels of ubiquitinated PP1A appear reduced in the Ras/NORE1A sample (**Figure 10**), quantification of multiple experiments showed that the apparent decrease was not statistically significant. This suggests that the stabilization effect may be independent of the proteasome system.

NORE1A forms an exogenous, Ras regulated complex with Rb – PP1A is a phosphatase that binds to Rb and modulates its activity by dephosphorylation (270). Since we have now established an endogenous interaction between NORE1A and PP1A, it is plausible that NORE1A could be found in a complex with Rb. We first wanted to determine if the proteins co-localize in the cells. To address this, we co-transfected COS-7 cells with GFP-tagged Rb in the presence of either KATE-vector (used as a negative control) or KATE-NORE1A for 48 hours. We did find strong co-localization of the two proteins within the nucleus (**Figure 11A**). To confirm that the proteins interact, we co-transfected HEK-293T cells with NORE1A and Rb in the presence and absence of activated Ras and found that NORE1A coimmunoprecipitated with over-expressed Rb, and that this interaction was enhanced in the presence of Ras (**Figure 11B**).

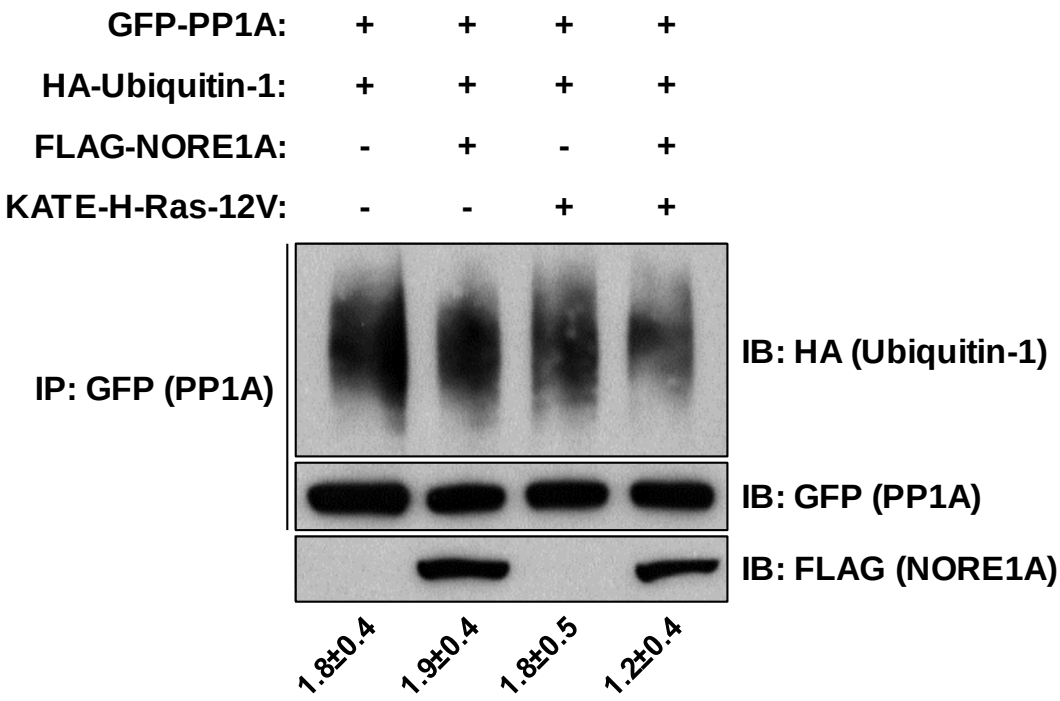


Figure 10. Ras/NORE1A do not significantly alter proteosomal degradation of PP1A. HEK-293 cells were transfected with expression constructs for PP1A, NORE1A, activated H-Ras and Ubiquitin-1 for 24 hours. The cells were then treated with the proteosome inhibitor MG132 for 4 hours, lysed and equal amounts of protein immunoprecipitated with anti-GFP. The immunoprecipitates were analyzed by western blotting with anti-HA, anti-GFP and anti-FLAG antibodies. The density of the bands was quantitated using ImageJ software and the relative amount of ubiquitinated PP1A was calculated after normalizing to the total amount of PP1A immunoprecipitated. Although there appeared to be less ubiquitinated PP1A in the presence of both NORE1A and activated Ras compared to the vector control, this was not statistically significantly different.

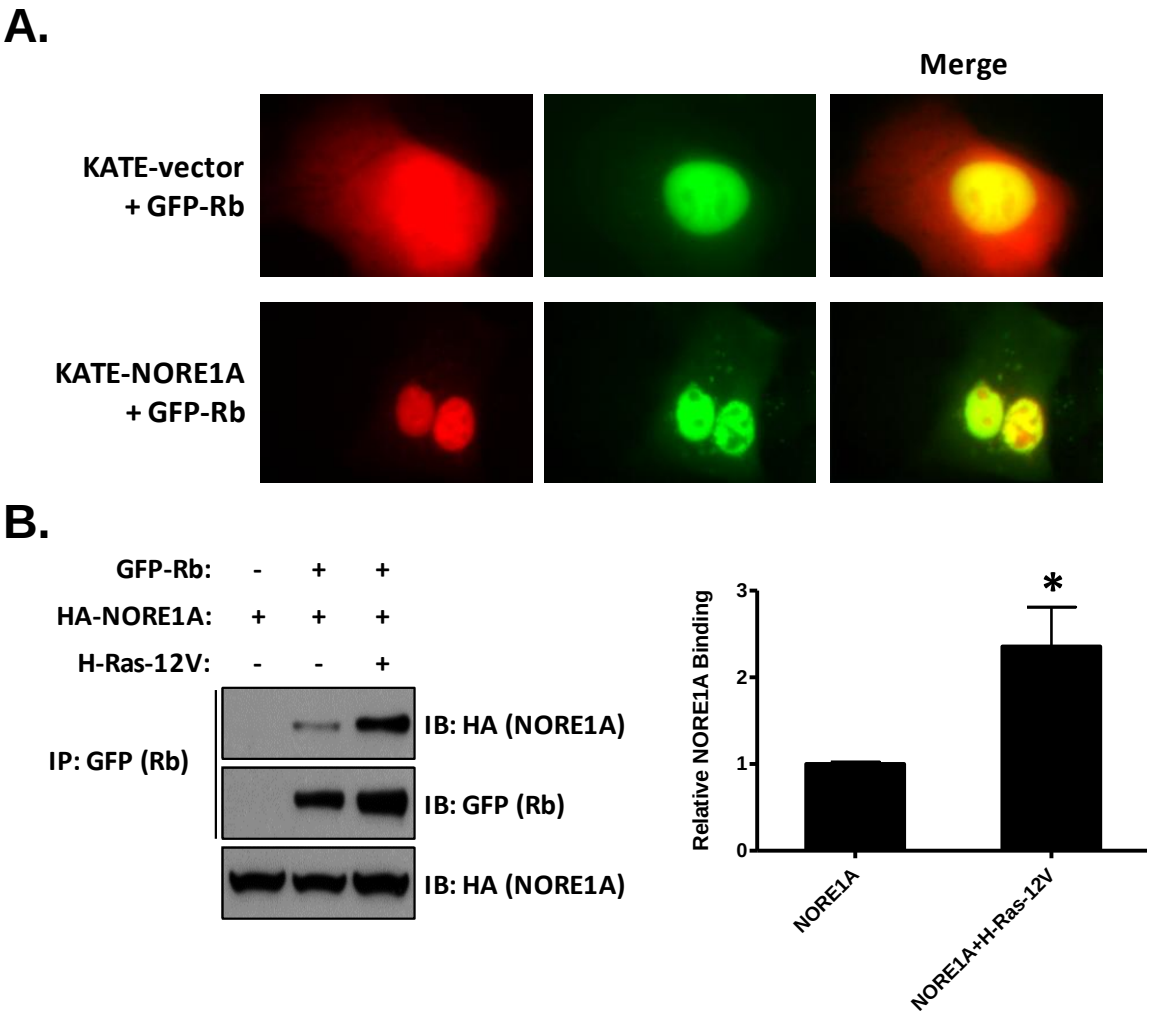


Figure 11. NORE1A complexes with Rb. A. *Exogenously expressed NORE1A and Rb co-localize in the nucleus*. COS-7 cells were transfected for 48 hours with GFP-Rb in the presence or absence of KATE-NORE1A. **B.** *NORE1A forms a Ras regulated complex with Rb*. HEK-293T cells were co-transfected with expression constructs for Rb, NORE1A, and activated H-Ras for 24 hours. The cells were then lysed and immuno-precipitated with anti-GFP. The immunoprecipitates were analyzed by western blotting with anti-HA and anti-GFP antibodies. *, $P<0.05$ compared to cells transfected with NORE1A alone (quantification of three independent experiments).

NORE1A Cooperates with Ras to Scaffold PP1A to Rb – NORE1A is a tumor suppressor that is thought to act primarily as a scaffolding molecule, as it has no apparent enzymatic activity (15,191,194). We have recently identified a Ras mediated mechanism by which NORE1A scaffolds HIPK2 to p53 in order to promote the pro-senescent functions of p53 (20). Thus, a similar mechanism may be occurring here where NORE1A acts to scaffold PP1A onto Rb in a Ras dependent manner. To test this, we co-transfected HEK-293T cells with Rb, PP1A, and NORE1A expression constructs in the presence or absence of activated Ras, immunoprecipitated the NORE1A and immunoblotted for the presence of both Rb and PP1A. We found that both PP1A and Rb could be found in a complex with NORE1A simultaneously, and the levels of both PP1A and Rb in the complex with NORE1A increased in the presence of activated Ras (**Figure 12**). To confirm that NORE1A was scaffolding PP1A to Rb, we transfected HEK-293T cells with Rb and PP1A, in the presence and absence of NORE1A and activated Ras, and examined the effects of Ras and NORE1A on the complex formation between PP1A and Rb. The results show that indeed, the Rb/PP1A complex is enhanced by NORE1A, and that this effect is further increased in the presence of activated Ras. A representative blot is shown in **Figure 13** and quantification of two independent experiments is shown in **Figure 14**. Exactly how Ras facilitates the interaction between NORE1A and PP1A and Rb is not entirely clear. However, binding of Ras to NORE1A induces a conformational change (271), and this may promote the interaction between NORE1A and its binding partners.

NORE1A promotes the dephosphorylation of Rb at Serine 795 – The activity of Rb is primarily regulated by its phosphorylation status at several Ser/Thr residues, and Rb can be activated when Ser/Thr phosphatases promote its dephosphorylation (231). Since

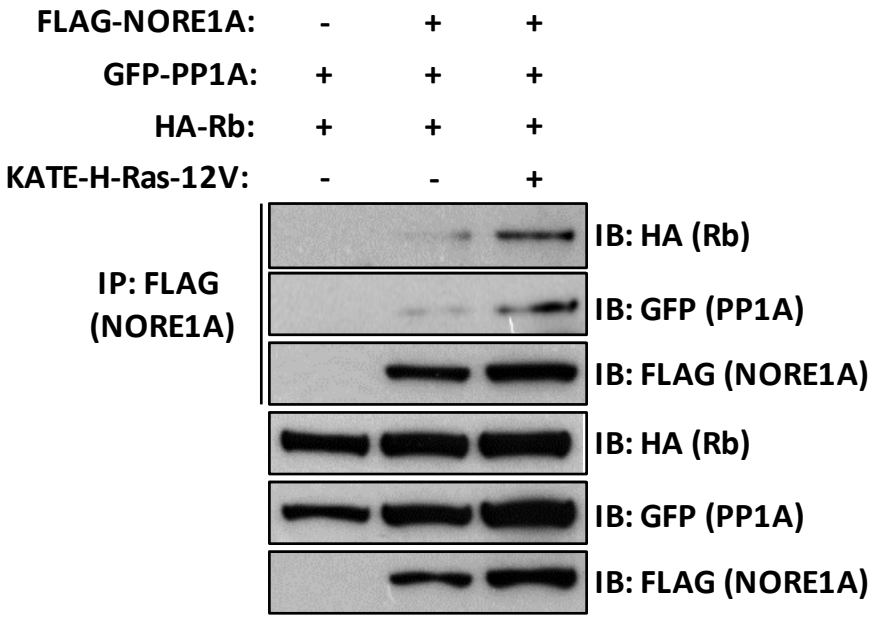


Figure 12. PP1A and Rb are found in a Ras regulated complex with NORE1A. HEK-293T cells were co-transfected with PP1A, Rb, NORE1A and activated H-Ras expression constructs for 24 hours. The cells were lysed and equal amounts of protein immunoprecipitated with anti-FLAG. The immunoprecipitates were analyzed by western blotting with anti-GFP, anti-HA, and anti-FLAG antibodies.

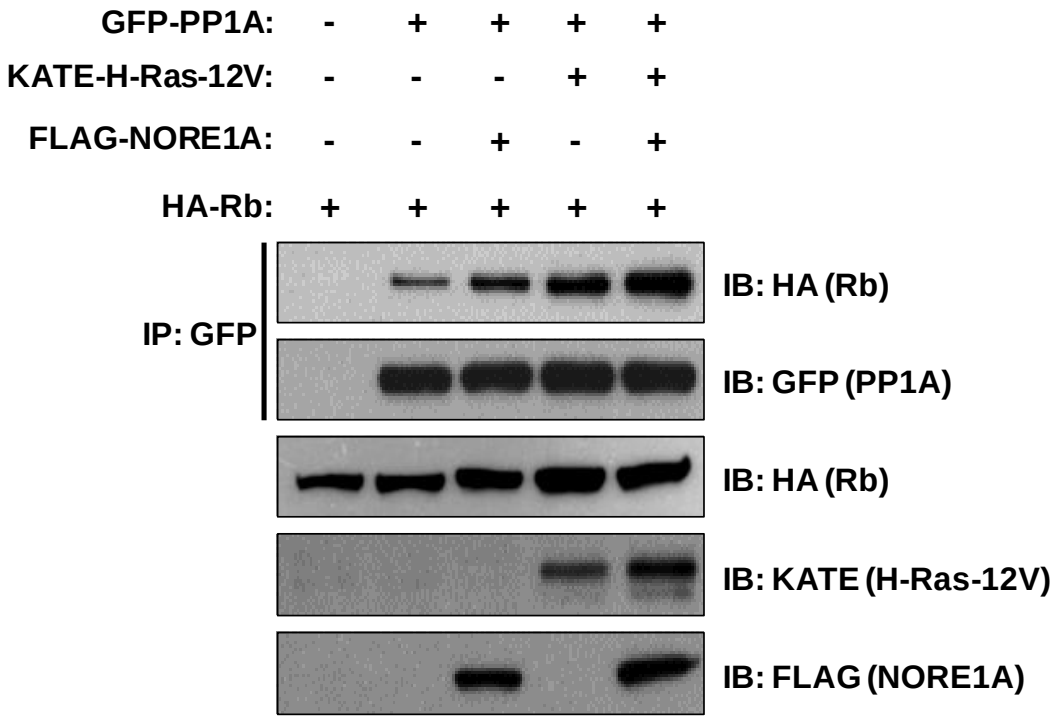


Figure 13. Ras regulates the interaction between PP1A and Rb via NORE1A. HEK-293T cells were co-transfected with expression constructs for PP1A, Rb, NORE1A, and activated H-Ras for 24 hours. Cells were lysed and immunoprecipitated for GFP-PP1A, and the immunoprecipitates analyzed by western blot analysis with anti-HA, GFP, and FLAG antibodies.

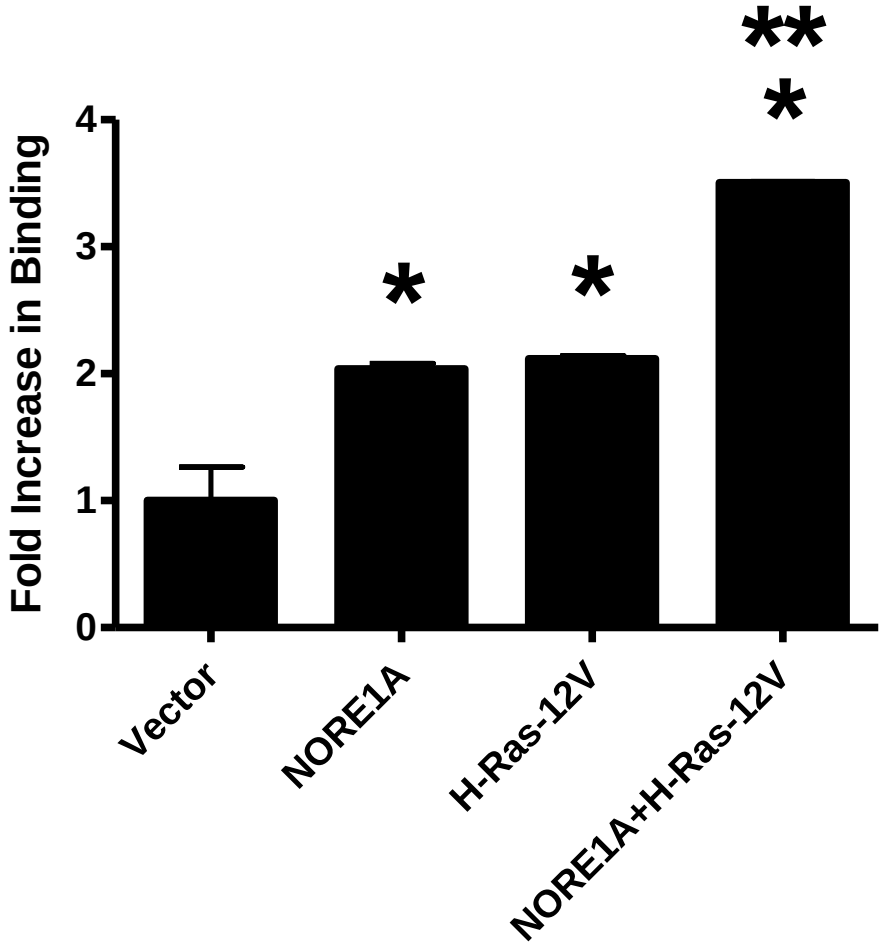


Figure 14. Quantification of the relative fold increase in Rb/PP1A interaction. Quantification of three independent experiments was performed, which is shown as a representative image in Figure 12. Briefly, the blots were quantified densitometrically to calculate the relative amount of Rb found in complex with PP1A. *, $P<0.05$ compared to cells transfected with empty vector, **, $P<0.05$ compared to cells transfected with either NORE1A or activated Ras alone.

NORE1A forms an endogenous complex with PP1A, a key mediator of Rb-dephosphorylation, and scaffolds it to Rb, we sought to determine if NORE1A could promote the dephosphorylation of Rb. By using a phospho-specific antibody to Ser-795, a residue known to be regulated in part by PP1A (272), we examined the effects of NORE1A on Rb phosphorylation. In transient transfections of A549 cells (a mutant K-Ras NORE1A negative, p53 positive lung tumor cell line (17)), we observed that NORE1A decreased the phosphorylation of endogenous Rb at Serine 795 (**Figure 15A**). In addition, we examined the phosphorylation status of endogenous Rb in NCI-H1299 lung cancer cells (mutant Ras positive, NORE1A negative, p53 negative (273)) stably expressing NORE1A at more physiological levels (194), and found similar results (**Figure 15B**). To confirm the link between NORE1A and Rb phosphorylation, we transiently knocked down NORE1A in HEK-293 cells using two previously validated shRNA constructs to NORE1A (20), and found that indeed loss of NORE1A enhanced the phosphorylation of Rb at Serine 795 (**Figure 16**). Thus, it seems that NORE1A may be a crucial mediator of Rb function by regulating its phosphorylation status.

Rb is a downstream effector of NORE1A induced senescence – We have recently shown that NORE1A is a critical Ras senescence effector that acts by forming a Ras regulated complex with p53 (20). However, although suppression of p53 strongly impaired the NORE1A senescence phenotype, it did not completely abolish it. This suggests that NORE1A may be able to promote senescence via additional mechanisms. Since the Rb pathway is one of the most powerful effector pathways of Ras-induced senescence, and we have now shown that NORE1A can regulate the dephosphorylation of Rb, we sought to determine if NORE1A can promote senescence through Rb in

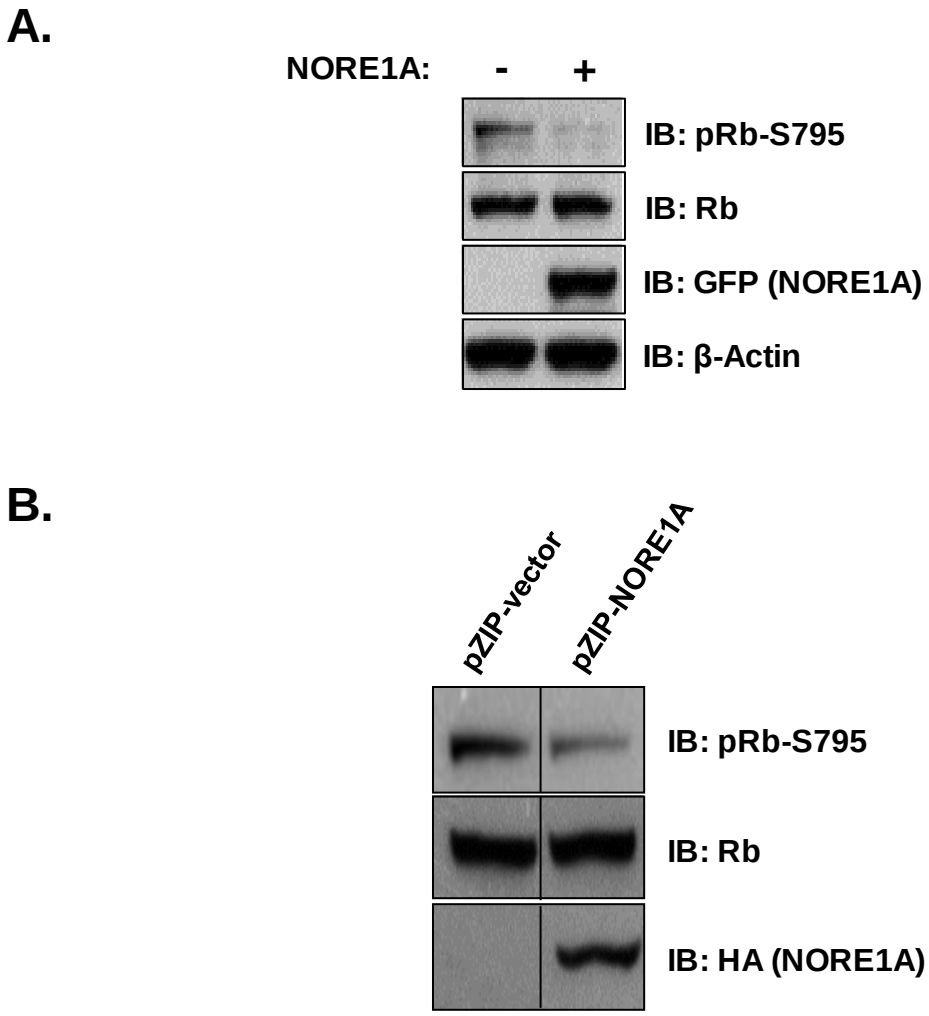


Figure 15. NORE1A promotes Rb dephosphorylation. A. A549 cells, which do not express NORE1A, were transiently transfected with GFP-NORE1A for 24 hours. Cells were lysed and immunoblotted for Rb phosphorylated at Serine-795 using a S795-specific antibody. **B.** NCI-H1299 cells stably expressing NORE1A or an empty vector were lysed an immunoblotted for phospho-RB at Serine 795. Shown are representative blots of two independent experiments.

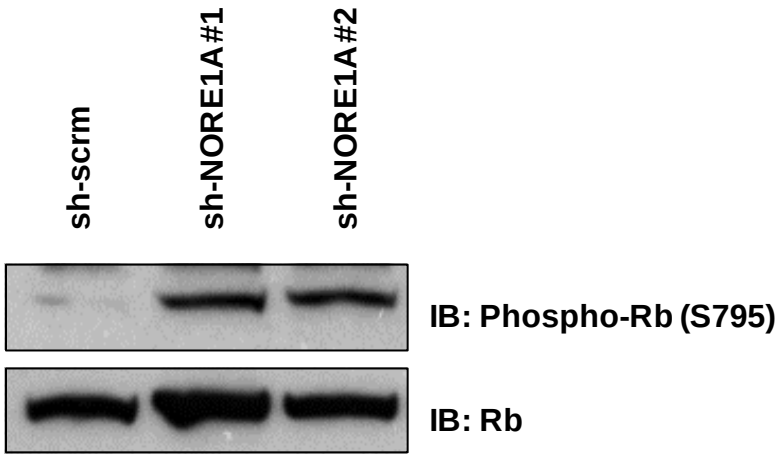


Figure 16. Loss of NORE1A enhances Rb phosphorylation. HEK-293 cells were transiently knocked down for NORE1A expression using two different shRNA constructs to NORE1A. The cells were lysed 48 hours following transfection and were immuno-blotted for phospho-Rb at Serine 795.

addition to p53 in order to fully promote its senescent phenotype. To address this question, we transfected NORE1A into wild type and $Rb^{-/-}$ MEFs and measured senescence by β-galactosidase activity. As we have previously shown, NORE1A induces senescence in wild-type MEFs, but in the absence of Rb, NORE1A was unable to induce senescence (**Figure 17A and B**). This suggests that NORE1A requires Rb in order to induce senescence in primary murine fibroblasts.

A459 cells are a human lung adenocarcinoma cell line that expresses wild type p53 and Rb, but do not express NORE1A (17). To confirm the role of Rb in NORE1A mediated senescence in human cells, we generated A549 cells that were knocked down for Rb using two different shRNAs to Rb (**Figure 18**). We then transiently transfected NORE1A into these cells, and assayed senescence by β-galactosidase activity. As expected, NORE1A was able to promote senescence in the cells stably transfected with the scramble control, but its ability to drive senescence was severely, although not completely, suppressed in the cells knocked down for Rb expression (**Figure 19A and B**). To substantiate that the elevated levels of β-galactosidase staining observed in these cells was senescence-related, we measured effects on IL-6 expression, an additional well-established marker of senescence (246). NORE1A induced a significant ($P<0.05$) increase in IL-6 promoter activity in the A549 cells, which was abrogated in the absence of Rb (**Figure 20**). Thus, in addition to p53, NORE1A also appears to act via Rb to fully promote senescence.

PP1A-mediated dephosphorylation of Rb is required for NORE1A Induced senescence – We have established that NORE1A interacts with PP1A, enhances the

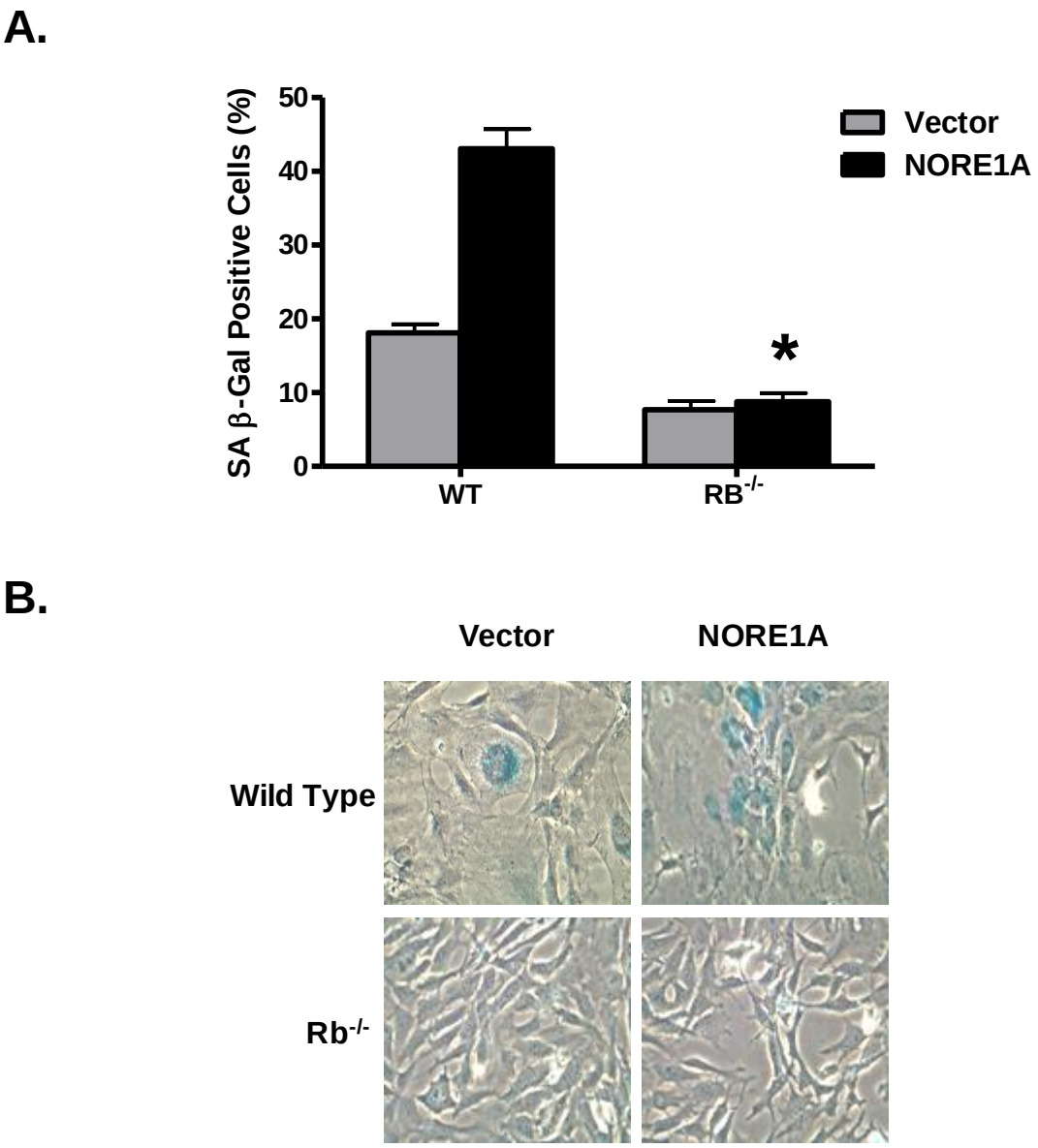


Figure 17. Loss of Rb significantly impairs NORE1A mediated senescence in MEFs. Wild-type and $Rb^{-/-}$ MEFs were transfected with 1 µg pcDNA-HA-Vector or NORE1A. Cells were incubated for 72 hours before assaying for β-galactosidase activity. *P≤0.05 compared with wild-type transfected MEFs.

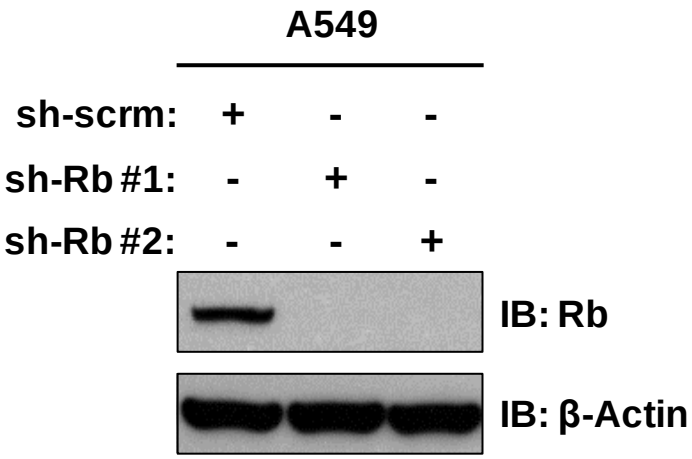
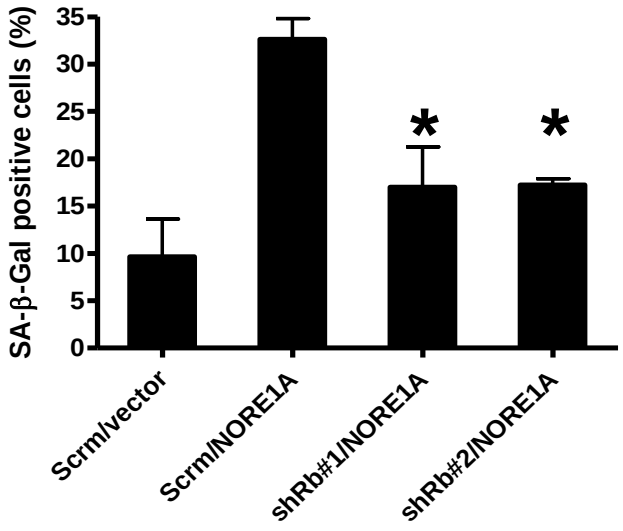


Figure 18. Generation of human lung cancer A549 +/- Rb matched pair cell system. To study the effects of NORE1A on Rb and senescence, we generated a matched pair cell system that would be wild-type or knocked down for Rb expression. The system was generated using two different lentiviral expression constructs containing shRNAs to Rb. Cells were validated by western blot analysis using an antibody to Rb.

A.



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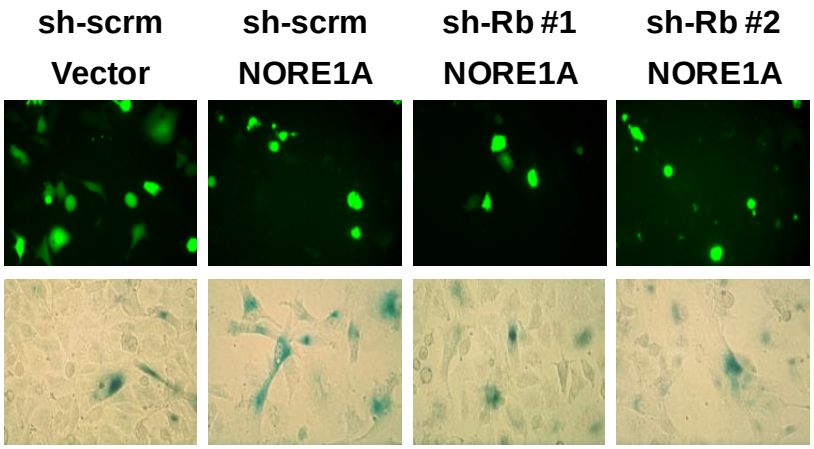


Figure 19. Rb is a downstream effector of NORE1A induced senescence in human cells. A. Stable scrambled or sh-Rb transduced A549 cells were transfected with 1 µg GFP-NORE1A. After 72 hours, the cells were assayed for β-galactosidase activity. *P<0.05 compared with scrambled control transfected cells. **B.** Representative images of NORE1A expression (upper panels) and β-galactosidase stained cells (lower panels).

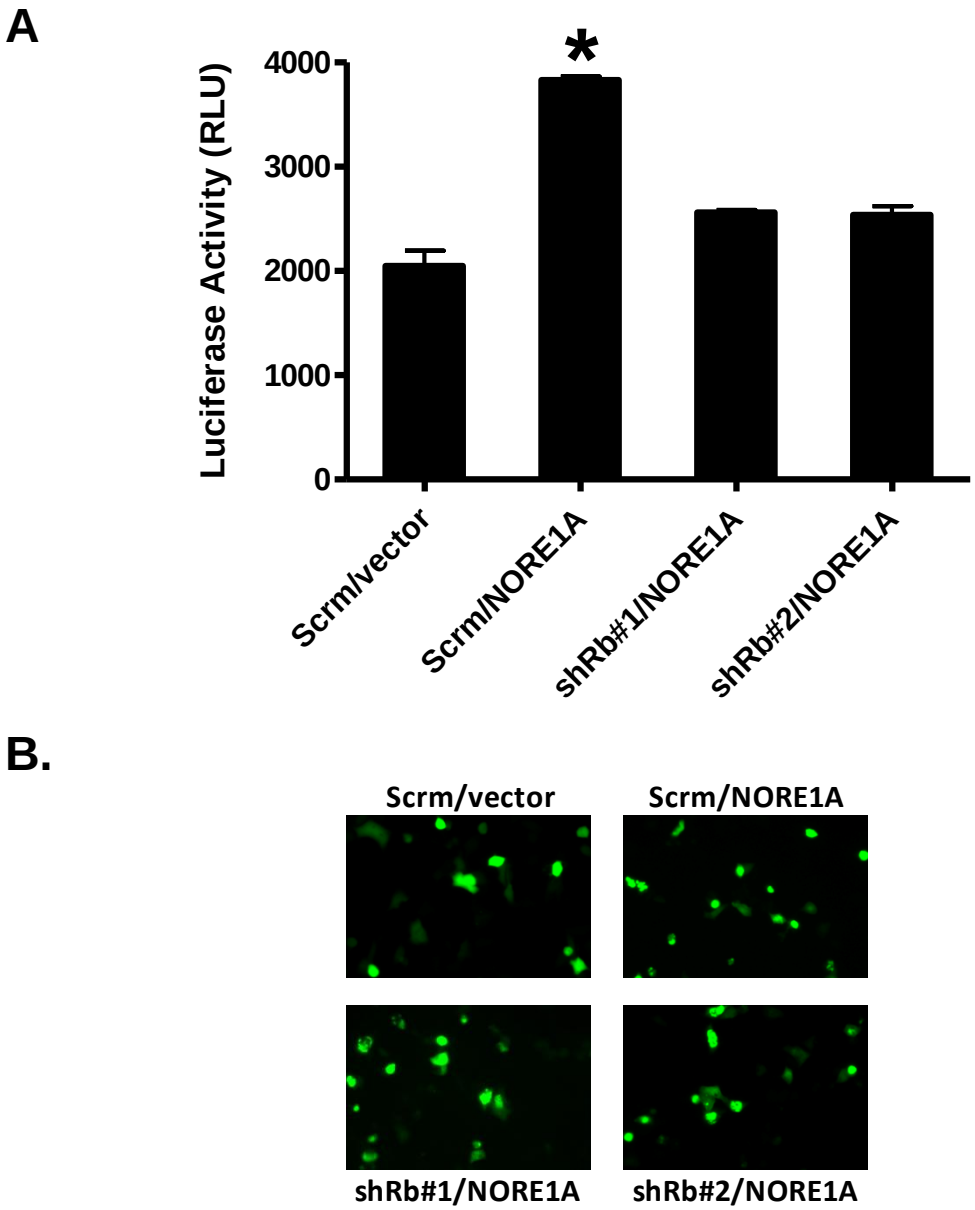


Figure 20. Rb is an effector of NORE1A induced IL-6 promoter activity. A. Control and A549 cells stably knocked down for Rb described previously were co-transfected with GFP-NORE1A and an IL-6 promoter luciferase reporter construct. Luciferase activity was measured 48 hours after transfection using a LightSwitch Luciferase Assay system. *, P<0.05. **B.** Representative images of NORE1A expression.

complex formation with Rb, promotes the dephosphorylation of Rb and requires Rb for senescence. To fully substantiate that the NORE1A mediated dephosphorylation of Rb by PP1A is required for NORE1A induced senescence, we examined the ability of NORE1A to promote the dephosphorylation of Rb and promote senescence in the absence of PP1A. In the absence of PP1A, both NORE1A mediated dephosphorylation (**Figure 21**) and senescence (**Figure 22**) were severely impaired, confirming that dephosphorylation of Rb via PP1A is required for NORE1A mediated senescence.

3.3 – Discussion:

Oncogenic Ras mutations are critical drivers of transformation via promotion of mitogenic signaling pathways (6). Paradoxically, Ras also regulates growth inhibitory pathways such as apoptosis and senescence (148,159,274). Ras-induced senescence is a major defense mechanism suppressing Ras driven transformation, and loss of functional senescence pathways is necessary for Ras to manifest its full transforming potential (246,275). While recent studies have confirmed the significance of Ras-induced senescence *in vivo,* the mechanisms by which Ras can promote a senescent phenotype both *in vitro* and *in vivo* remain poorly defined (246,276,277).

Two pathways that have been identified as key players in oncogene induced senescence involve the p53 and Rb tumor suppressors. Early studies in primary rodent cells suggested that loss of either the p53 or the Rb pathways was sufficient for Ras to bypass senescence and promote transformation (159). In contrast, the loss of one of these pathways typically only delays the onset of senescence in human cells. Recent studies

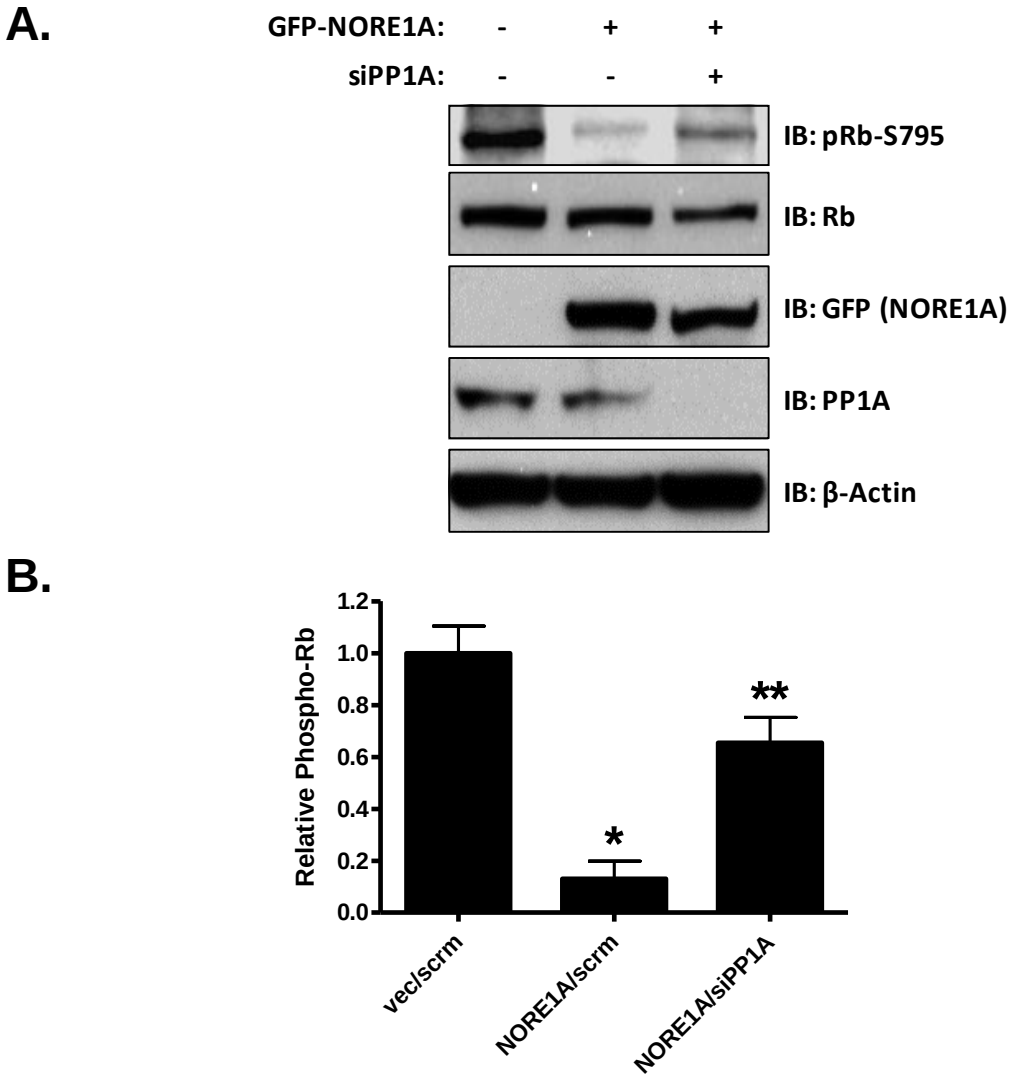
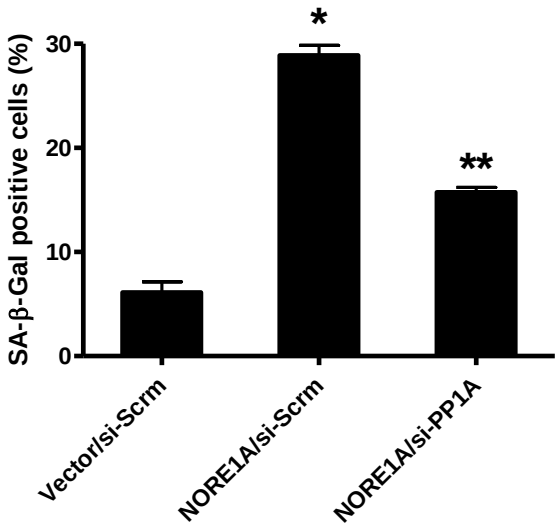


Figure 21. Loss of PP1A impairs NORE1A induced dephosphorylation of Rb. A. A549 cells were transiently knocked down for PP1A expression using a validated pool of PP1A siRNA. The cells were transfected with GFP-NORE1A and 24 hours later, lysed and immunoblotted for phosphor-Rb at Serine 795, Rb, PP1A, and NORE1A. **B.** Quantification of two independent experiments was performed. Blots were quantified densitometrically to calculate the relative amount of Rb phosphorylation at Ser-795. *, $P<0.05$ compared to vec/scrm transfected cells. **, $P<0.05$ compared to vec/scrm and NORE1A/scrm transfected cells.

A.



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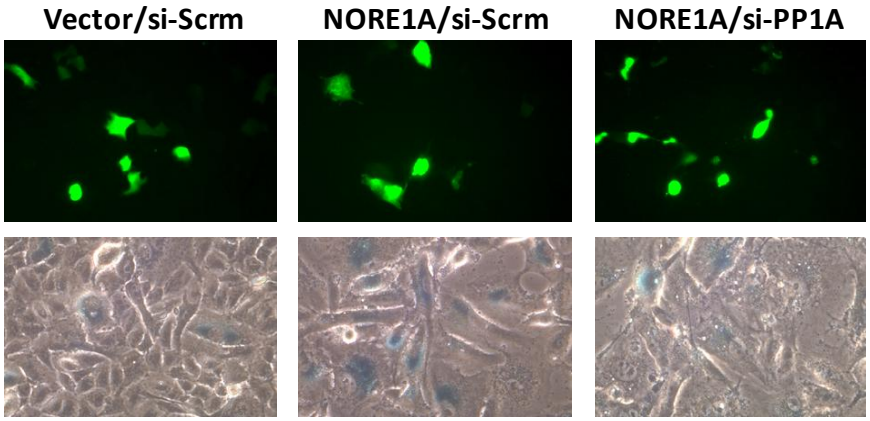

Figure 22. PP1A is required for NORE1A induced senescence. A. A549 cells were transiently knocked down for PP1A as previously described and transfected with GFP-NORE1A. 72 hours later, cells were assayed for β-galactosidase activity. *, $P<0.05$ compared with control cells transfected with vector, **, $P<0.05$ compared to si-Scrm cells transfected with NORE1A. **B.** Representative images of NORE1A expression and β-galactosidase staining.

have shown that the activation of both the p53 and the Rb pathways is essential for induction of senescence in a variety of human cell lines (159,164,253,278,279), and furthermore, cross-talk between the p53 and Rb pathways could allow for additional protection against oncogenic Ras bypassing senescence and promote tumorigenesis (250). Until now, the mechanisms by which Ras regulates the p53 and Rb pathways to drive senescence has been unclear.

NORE1A (RASSF5) is a member of the RASSF family of tumor suppressors (15). Like other members, it binds directly to Ras and serves as an effector to promote its growth inhibitory properties (12,19). The best characterized member of the RASSF family is RASSF1A, which shares considerable homology to NORE1A. RASSF1A binds and activates MST kinases, which then feed into the Hippo pathway to regulate the transcriptional co-activators YAP1, YAP2 and TAZ. However, although NORE1A also binds MST kinases (19), it does not seem to activate them (280). Moreover, deletion mutagenesis has shown that the interaction of NORE1A with MST kinases is not required for its ability to inhibit cellular growth (17). This suggests that NORE1A may be different to RASSF1A, and act via non-Hippo components.

By inducing physiological expression levels of NORE1A in cells, we found NORE1A plays a key role in p53-mediated cell-cycle arrest (191). This led us to determine that NORE1A is a potent senescence effector of Ras that precisely regulates the post-translational modification code of p53 (20). NORE1A forms a Ras-regulated complex with p53 and the kinase HIPK2. This scaffolding event is an essential component of Ras-induced senescence, and results in the enhanced acetylation of p53 at K320 and K382 residues. Acetylation of p53 at these residues activates pro-senescent

transcriptional programs (20,281). However, we noted that although suppression of p53 severely reduced NORE1A induced senescence, it did not completely abolish it (20). Thus, the role of NORE1A in Ras-induced senescence is likely more complex and may work through additional senescence pathways.

We hypothesized that NORE1A may also be able to promote senescence by regulating the Rb pathway, the other powerful senescence pathway in oncogene induced senescence. Indeed, we found that loss of Rb in both MEFs and human cells suppressed the powerful senescence phenotype promoted by NORE1A. Interestingly, the suppression of either Rb or p53 alone in A549 cells did not completely inhibit NORE1A induced senescence, consistent with the notion that human cells require the loss of both the p53 and Rb pathways in order to fully bypass senescence.

One of the main regulators of Rb activity is the PP1A phosphatase (282,283). PP1A has been implicated in Ras-induced senescence (22) and Ras has been shown to control the catalytic activity of PP1A (237). As PP1A specificity is often controlled by targeting proteins (25), we wondered if NORE1A might serve as a direct connection between Ras, PP1A and Rb. We found that NORE1A forms an endogenous, Ras regulated complex with the phosphatase PP1A. As PP1A has been detected in complex with NORE1A in a yeast two-hybrid system, the interaction is likely to be direct (23). Furthermore, we found that NORE1A appears to scaffold PP1A to Rb in a Ras dependent manner, as NORE1A could be co-precipitated with Rb and Ras/NORE1A enhanced the interaction between PP1A and Rb. The scaffolding of Rb to its phosphatase results in Rb dephosphorylation, a pro-senescent event (24). The exact mechanism by which Ras activates this NORE1A-PP1A-Rb axis is not entirely clear since Ras is found

predominantly on the cell membrane and the NORE1A/PP1A/Rb complex is located primarily in the nucleus. One possibility involves a Ras-induced conformational change of NORE1A (271) that enables it to interact with PP1A and/or Rb, and then shuttle them as a complex into the nucleus via various nuclear transport proteins (266). Another possible mechanism may involve additional Ras signaling pathways that act upon nuclear NORE1A to activate it, thereby inducing it to complex with PP1A and Rb. Interestingly, a pool of Ras has also been found located in the nucleus (284,285), raising the possibility that NORE1A/Ras stimulation of PP1A/Rb is entirely nuclear.

During these studies, we also noticed that NORE1A seems to modulate the stability of PP1A. Recent studies have shown that the ubiquitin ligase mdm2, a negative regulator of Rb that contributes to tumorigenesis in part by destabilizing Rb (286), can be found in an endogenous complex with PP1A (287), though the effects of mdm2 on PP1A stability have not been elucidated. Interestingly, NORE1A has been shown regulate the degradation of specific mdm2 targets (288). Thus, NORE1A could be regulating PP1A stability through its interaction with mdm2, potentially by antagonizing the ubiquitination/degradation properties of mdm2.

PP1A is not an Rb-specific phosphatase and can regulate the functions of a variety of proteins in the cell by modulating their phosphorylation status. The specificity of PP1A is dictated by targeting proteins (25). Our data suggests that NORE1A may serve as a Ras regulated PP1A-targeting protein, directing PP1A to a specific set of substrates, such as Rb. This may provide a novel mechanism whereby NORE1A mediates its tumor suppressor function through modulating specific protein phosphorylation.

Further studies will be necessary to identify any additional NORE1A targeted PP1A substrates.

Our data provide evidence that NORE1A provides a major link between Ras and Rb. Activated Ras signaling promotes the association of PP1A to Rb via NORE1A, resulting in the activation of Rb and senescence. In the absence of NORE1A, PP1A cannot effectively scaffold to Rb and activate it, resulting in senescence bypass and allowing the growth promoting effects of aberrant Ras signaling to predominate (**Figure 23**). Thus, NORE1A acts as a double-barreled Ras senescence effector that connects Ras to the two major senescence effectors in human cells, p53 (20) and Rb. This may explain why NORE1A is such a powerful senescence effector and why it is so frequently down-regulated during tumorigenesis (15), particularly in tumors with up-regulated Ras activity (256).

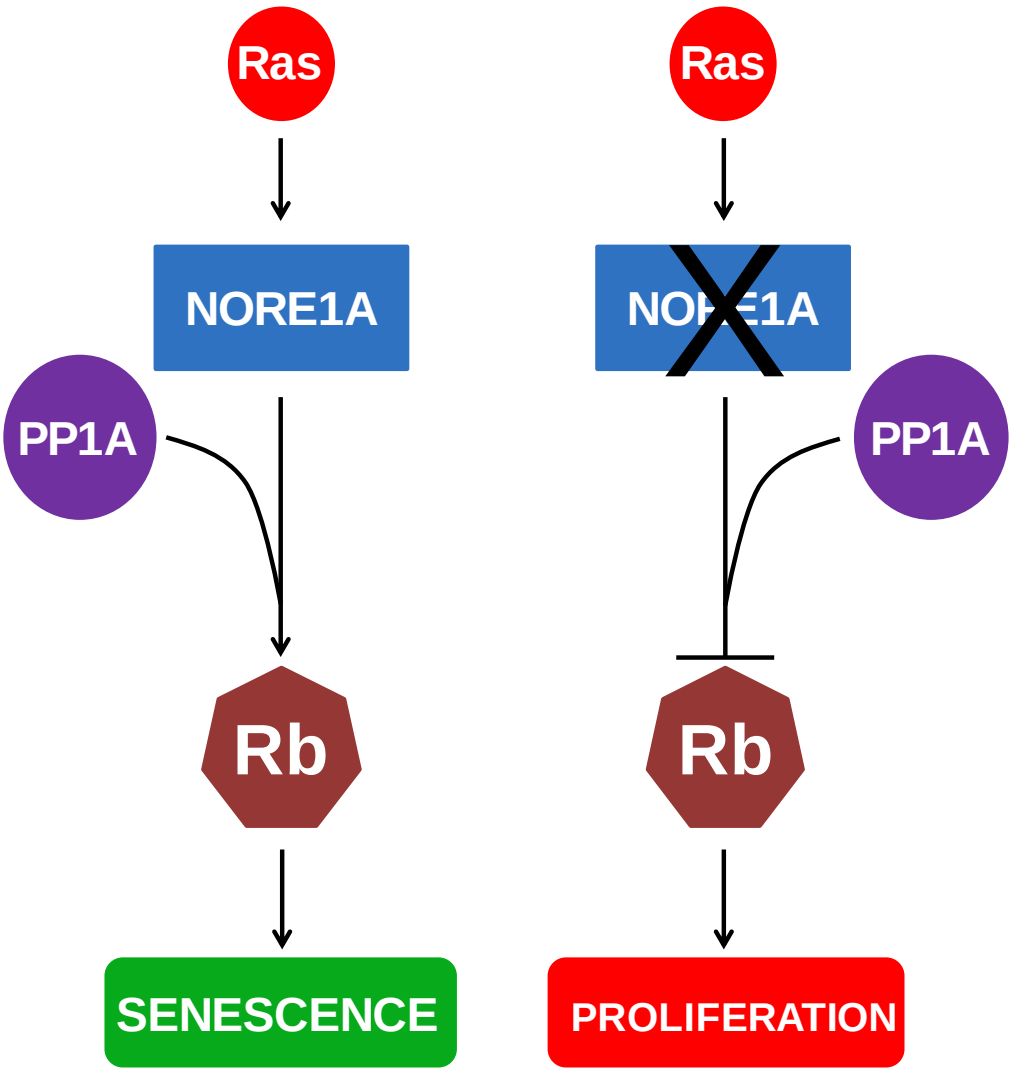

Figure 23. Schematic representation of the newly identified Ras/NORE1A/Rb pathway. Our data points to a novel Ras/NORE1A/Rb pathway whereby Ras promotes NORE1A mediated scaffolding of PP1A to Rb to promote its dephosphorylation. This results in Rb activation and subsequently activates the senescence machinery. Loss of NORE1A inhibits the proper dephosphorylation of Rb, in turn suppressing senescence induction, allowing Ras to bypass the senescence barrier allowing it to promote proliferation.

CHAPTER 4

NORE1A REGULATES ADDITIONAL RB POST-TRANSLATIONAL MODIFICATIONS AND PROMOTES ITS STABILITY

4.1 – Introduction:

While Ras oncoproteins have potent transforming properties, they can also activate important growth inhibitory pathways, including apoptosis and senescence (148,159,274,289). Work performed by Donninger and colleagues has recently identified NORE1A as a key component of Ras-driven senescence by regulating the pro-senescent post-translational modifications of p53 (20). The link between Ras/NORE1A and p53 appears to be primarily the kinase HIPK2, which has been shown to regulate apoptosis by directly phosphorylating p53 at Serine 46, in turn enhancing the affinity of p53 for pro-apoptotic gene promoters (290,291). In addition, HIPK2 can recruit acetyltransferases CBP/p300 and PCAF, which can acetylate p53 and modulate its transcriptional activity (291-293). Acetylation of p53 at Lysine 382, in combination with phosphorylation at Serine 46 shifts p53 towards apoptosis; however, without the phosphorylation of Serine 46, the acetylation of Lysine 382 drives p53 away from apoptosis and pushes it towards the induction of senescence (281,294). NORE1A forms an endogenous, Ras-regulated

complex with HIPK2, in turn promoting senescence not only by acetylating p53 at Lysine 382, but also by suppressing the phosphorylation of p53 at Serine 46 (20). It was also shown that NORE1A can promote the acetylation of p53 at Lysine 320 (20), which has been reported to enhance p53 association with the $p21^{CIP1}$ promoter, in turn enhancing its expression which can ultimately lead to senescence (295). Thus, NORE1A can regulate the post-translational signature of p53 in order to activate senescence.

Rb is the other critical tumor suppressor involved in Ras-induced senescence. In murine cells, abrogation of either p53 or Rb seems to be sufficient to suppress senescence; however, in human systems, both the p53 and Rb pathways must be inactivated in order for Ras to bypass senescence and drive transformation (253). As is the case with p53, Rb undergoes numerous post-translational modifications that regulate its tumor suppressive functions (296). Although the phosphorylation of Rb has been extensively studied, the significance of non-phosphorylation post-translational modifications of Rb, including the acetylation and SUMOylation, remains quite unclear.

It has been proposed that active Rb is both acetylated and hypophosphorylated. Rb has recently been shown to be acetylated at the C-terminus by p300, which hinders the phosphorylation of Rb by cyclin-dependent kinases (297), leading to the maintenance of Rb in its active, hypophosphorylated form. As a result, such cells are maintained in a growth arrested state. It is believed that some unknown cellular protein functions to bridge p300 and Rb into a multi-protein complex to facilitate Rb acetylation. In addition to p300, it has been recently shown that the p-300 Associated Factor (PCAF) can mediate Rb acetylation, as Rb interacts directly with the acetyltransferase domain of PCAF *in vitro* and can associate with PCAF in differentiated cells (298). PCAF acetylates Rb to

induce Rb mediated terminal cell-cycle exit and expression of late myogenic genes (298). Lastly, Tip60, a MYST-related HAT, catalyzes Rb acetylation in order to control Rb expression levels (299). Collectively, evidence points to a new level of Rb regulation, and stability, caused by differential acetylation of the Rb protein.

Protein acetylation is a reversible reaction. Interestingly, sirtuin 1 (SIRT1) not only deacetylates histones (300) but other non-histone proteins, such as p53 (301-303). A recent report by Wong and colleagues showed that Rb is a substrate for deacetylation by human SIRT1 (304). Their work showed that acetylated Rb increases in response to contact inhibition, and that over-expression of SIRT1 reduced the levels of Rb acetylation *in vivo*. They hypothesize that the active form of Rb is both acetylated and hypo-phosphorylated, and that SIRT1-mediated deacetylation is required to deactivate Rb (304). Again, evidence points to the role of Rb acetylation as an activating event the may be required to promote its tumor suppressive functions. We have now shown that NORE1A can regulate the pro-senescent function of Rb via dephosphorylation. But since NORE1A has been shown to regulate multiple post-translational modifications of p53 in order for senescence to fully manifest itself, we hypothesize that a similar mechanism might be occurring with Rb, whereby NORE1A regulates both the phosphorylation and the acetylation of Rb. The regulation of acetylation could involve the modulation of acetyltransferases, such as PCAF and p300, as well as deacetylases such as SIRT1.

In addition to acetylation, Rb can be regulated by SUMOylation. Similar to ubiquitination, SUMOylation involves the covalent attachment of a small peptide moiety called SUMO (Small Ubiquitin-like Modifier) to specific lysine residues on a target protein that can have profound effects on protein function, from influencing protein-

protein interactions to sub-cellular localization and protein stability (296). Though the significance of Rb SUMOylation remains somewhat undetermined, several studies have shown that hypophosphorylated Rb can be SUMOylated by SUMO-1 and by SUMO-2/3 (305,306). The site of Rb SUMOylation was mapped to Lysine 720, and SUMOylation enhanced the ability of Rb to repress E2F transcription (305). In addition, the conjugation of SUMO can in some instances block the sites of attachment for ubiquitin, and can thus protect proteins from degradation (307). Therefore, it is plausible that the SUMOylation of Rb can promote its stability, raising the possibility that Rb can be both qualitatively and quantitatively regulated. We hypothesize that the SUMOylation of Rb is critical for its activation. Perhaps a reason why this fairly ubiquitous modification has only been recently identified and studied lies in the reversibility of the process and the fact that, apart from a very few exceptions, the amount of any SUMO-modified protein within a cell only makes up a very small percentage of its total amount, thus making detection by various molecular and biochemical methods more challenging (308). Because preliminary evidence in our laboratory suggested that Ras/NORE1A can affect the SUMOylation levels of proteins, such as HIPK2 (data not shown), we hypothesized that NORE1A may also be regulating the SUMOylation of Rb to activate Rb function.

Protein inhibitors of activated STAT (PIAS) have been shown to function as E3 SUMO ligases, and data from various SUMO conjugates make them one of the largest families of SUMO-specific ligases (309). The expression levels of one of the family member, PIASy, was shown to be significantly elevated during senescence when compared to pre-senescent cells, as do levels of hyper-SUMOylated proteins (310). In addition, over-expression of PIASy induces premature senescence, and this effect

requires its E3 SUMO ligase activity. More importantly, Ras-induced senescence was significantly delayed in PIASy$^{-/-}$ MEFs, which was also reflected by their higher levels of hyperphosphorylated Rb (310). The study also showed that PIASy interacts with both p53 and Rb. The significance of these interactions was validated by the observation that the affinity for endogenous p53 and Rb for endogenous PIASy was significantly higher in bleomycin-induced senescent fibroblasts when compared to pre-senescent cells (310). Since NORE1A is a critical senescence effector of Ras, and preliminary evidence suggests that it can regulate the SUMOylation of proteins, it is plausible that NORE1A may be cooperating with PIASy to promote Ras-induced senescence.

Here we show that NORE1A, in addition to promoting Rb activation by de-phosphorylation, can also regulate other key post-translational modifications of Rb. Our evidence suggests that NORE1A enhances the overall acetylation as well as the SUMOylation of Rb, though the precise mechanisms are not fully defined. We show that NORE1A regulates Rb acetylation in a PCAF independent manner. Therefore, we hypothesize NORE1A mediated acetylation of Rb may be occurring via p300, as HIPK2, a binding partner of NORE1A, can recruit the acetyltransferase CBP/p300 into a complex to indirectly promote p53 acetylation (20,291). Thus, it is plausible that NORE1A can regulate Rb acetylation by a similar mechanism. We also show that NORE1A can enhance the SUMOylation of Rb. Though the mechanism by which NORE1A affects Rb SUMOylation remains unknown, we now show that NORE1A forms an exogenous complex with PIASy, one of the critical SUMO ligases involved in Ras-induced senescence. This finding does not confirm that PIASy is in fact the player involved in NORE1A enhanced Rb SUMOylation; however, it does bring up the intriguing

possibility that NORE1A could be regulating the SUMOylation of a number of proteins, as Rb is not the only target of PIASy. Lastly, we show that Ras and NORE1A cooperate to hyper-stabilize Rb, and that the loss of NORE1A leads to a decrease in overall Rb levels. Collectively, our results suggest that NORE1A regulates the post-translational modification code of Rb, just as is the case with p53, in order to drive its pro-senescent function, as well as stabilize the Rb protein, which highlights both a qualitative and quantitative means of NORE1A regulation of Rb.

4.2 – Results:

Ras/NORE1A enhance the acetylation of Rb – While the activity of Rb is currently thought to be primarily regulated by its phosphorylation status, Rb can also be regulated by acetylation (296-298,304). Since NORE1A can activate the pro-senescent function of p53 by altering its acetylation (20), we sought to determine if NORE1A could promote the acetylation of Rb. Overall levels of Rb acetylation can be studied by performing immunoprecipitations using Acetyl-Lysine conjugated beads followed by western blot analysis for the Rb protein. We observed that in transient transfections of HEK-293 cells, the over-expression of NORE1A and/or activated Ras promotes the overall acetylation of Rb ($P<0.05$), currently believed to be an activating event (**Figure 24**). The levels of Rb acetylation were elevated in a similar manner in the presence of NORE1A and activated Ras when compared to Ras/NORE1A individually, likely because the acetylation machinery has been stimulated to maximum capacity by either NORE1A or activated Ras over-expression and therefore no further increase is possible.

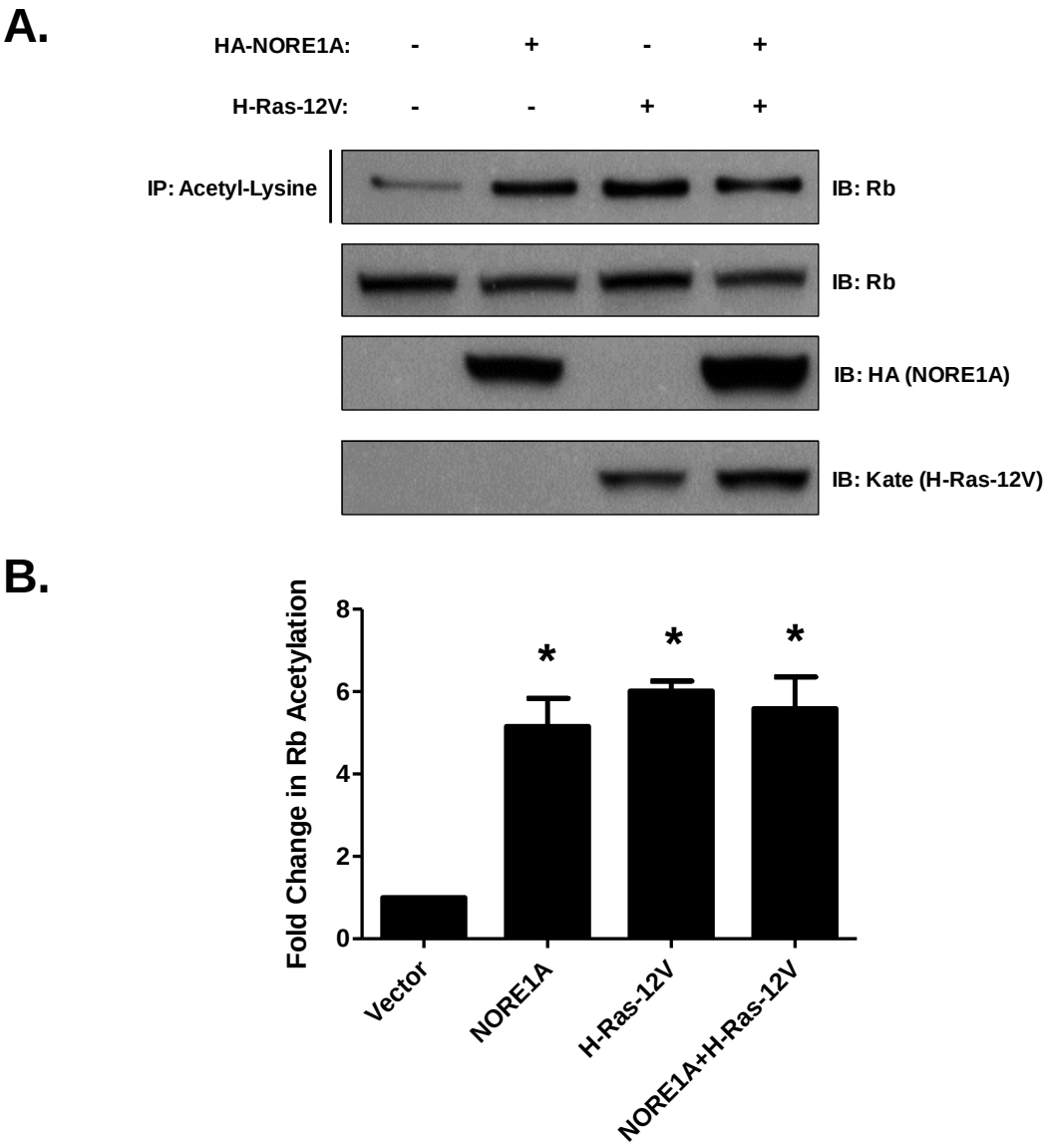

Figure 24. Ras/NORE1A enhance total Rb acetylation. A. HEK 293 cells were transfected with HA-NORE1A in the presence or absence of activated H-Ras for 24 hours. Cells were lysed and equal amounts of protein were immunoprecipitated using acetyl-lysine conjugated beads. The immunoprecipitates were analyzed by western blotting with anti-Rb, HA, and KATE antibodies. **B.** Blots were quantified densitometrically to calculate the fold change in Rb acetylation normalized to total levels of Rb. *, $P<0.05$ compared to vector transfected cells.

NORE1A forms an exogenous, Ras regulated complex with PCAF – While we have now shown that NORE1A can modulate the acetylation of Rb, it does not address the mechanism of action. Since NORE1A acts as a scaffolding molecule (20,194), we hypothesized that NORE1A could be interacting with one of the acetyltransferases known to regulate Rb acetylation, such as PCAF. We wanted to determine if NORE1A could be interacting with PCAF, one of the players involved in Rb acetylation (298). We co-transfected HEK-293T cells with NORE1A and PCAF in the presence or absence of activated Ras and found that NORE1A coimmunoprecipitated with over-expressed PCAF in a Ras dependent manner (**Figure 25**).

Ras/NORE1A modulate Rb acetylation in a PCAF-independent manner – Because we have shown that NORE1A can modulate the acetylation of Rb and interacts with PCAF, a known Rb acetyltransferase, we wanted to determine if the ability of NORE1A to enhance Rb acetylation was occurring via PCAF. We would anticipate that over-expressing PCAF would enhance the ability of NORE1A to promote Rb acetylation. To address this, we co-transfected HEK-293 cells with NORE1A in the presence or absence of activated Ras and/or PCAF. Our results suggest that Ras/NORE1A enhance the acetylation of Rb in a PCAF-independent fashion (**Figure 26**), as the addition of PCAF in this system did not affect Ras/NORE1A mediated acetylation of Rb.

Ras/NORE1A enhances Rb SUMOylation – In addition to its regulation by phosphorylation and acetylation, recent evidence has now shown that Rb can be modulated by SUMOylation at Lysine 720 (305), and that there may be a link between

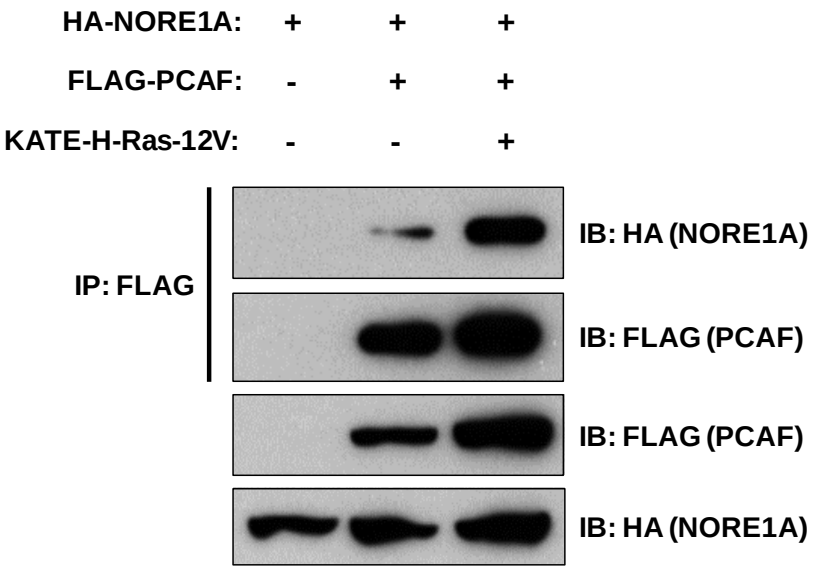

Figure 25. NORE1A forms an exogenous, Ras regulated complex with PCAF. HEK-293T cells were transfected with HA-NORE1A and FLAG-PCAF in the presence or absence of activated H-Ras for 24 hours. Cells were lysed and equal amounts of protein were immunoprecipitated for FLAG-PCAF, and the immunoprecipitates analyzed by western blot with anti-HA and anti-FLAG antibodies.

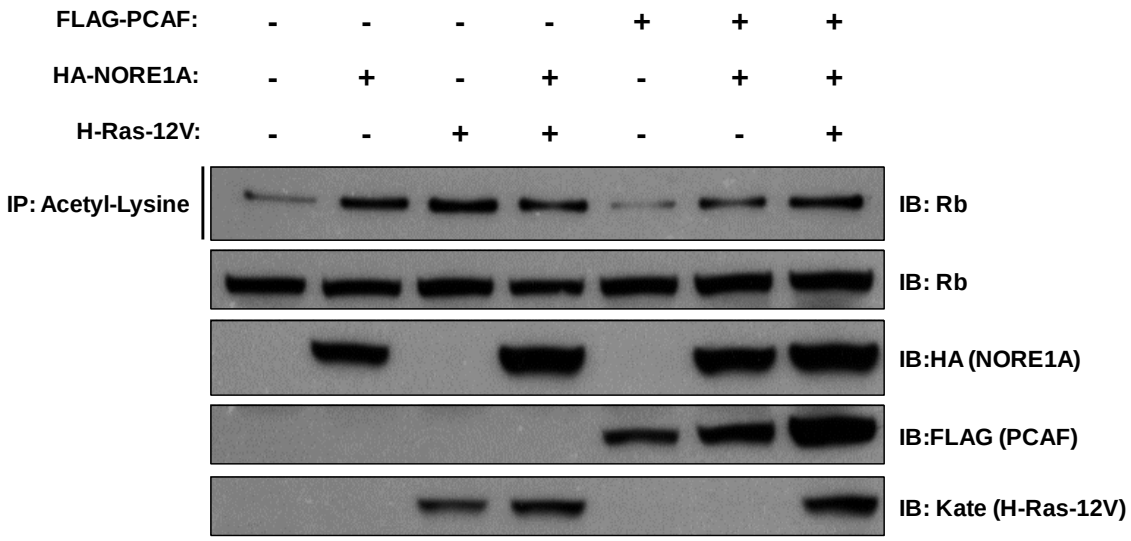

Figure 26. Ras/NORE1A enhance Rb acetylation in a PCAF-independent manner. HEK 293 cells were transfected with HA-NORE1A and FLAG-PCAF in the presence or absence of activated H-Ras for 24 hours. Cells were lysed and equal amounts of protein were immunoprecipitated using acetyl-lysine conjugated beads. The immunoprecipitates were analyzed by western blot using anti-Rb, HA, FLAG, and KATE antibodies. Results suggest that Ras/NORE1A do not cooperate with PCAF to promote Rb acetylation.

Rb SUMOylation and its pro-senescent function. Since preliminary evidence from the laboratory had shown that NORE1A may be able to regulate the SUMO modification of other proteins, we sought to determine if NORE1A could be regulating the SUMOylation of Rb. To address this, we transiently transfected HEK-293T cells with GFP-Rb and HA-SUMO-1 in the presence or absence of NORE1A and/or activated H-Ras. By immunoprecipitating with GFP-conjugated beads and western blotting for HA-SUMO-1, we can determine the levels of Rb-SUMOylation. Interestingly, our results suggest that Ras/NORE1A does enhance the SUMOylation of Rb. However, the levels of Rb SUMOylation were elevated in a similar manner in the presence of NORE1A and activated Ras when compared to Ras/NORE1A individually (**Figure 27**). This suggests that either the ability of NORE1A to enhance Rb SUMOylation might be Ras independent, or that the SUMOylation machinery has been stimulated to maximum capacity by NORE1A over-expression and therefore no further increase is possible.

NORE1A forms an exogenous, Ras-regulated complex with PIASy – In order to elucidate a mechanism of action for NORE1A mediated SUMOylation of Rb, we wanted to identify potential SUMO ligases that interact with NORE1A, in turn allowing NORE1A to promote Rb SUMOylation. The most intriguing possibility is the protein PIASy, an E3 SUMO ligase that has previously been implicated in Ras mediated senescence (310). To determine if NORE1A interacts with PIASy, we co-transfected HEK-293T cells with FLAG-PIASy and HA-NORE1A in the presence or absence of activated H-Ras. We found that NORE1A does form a complex with PIASy, albeit

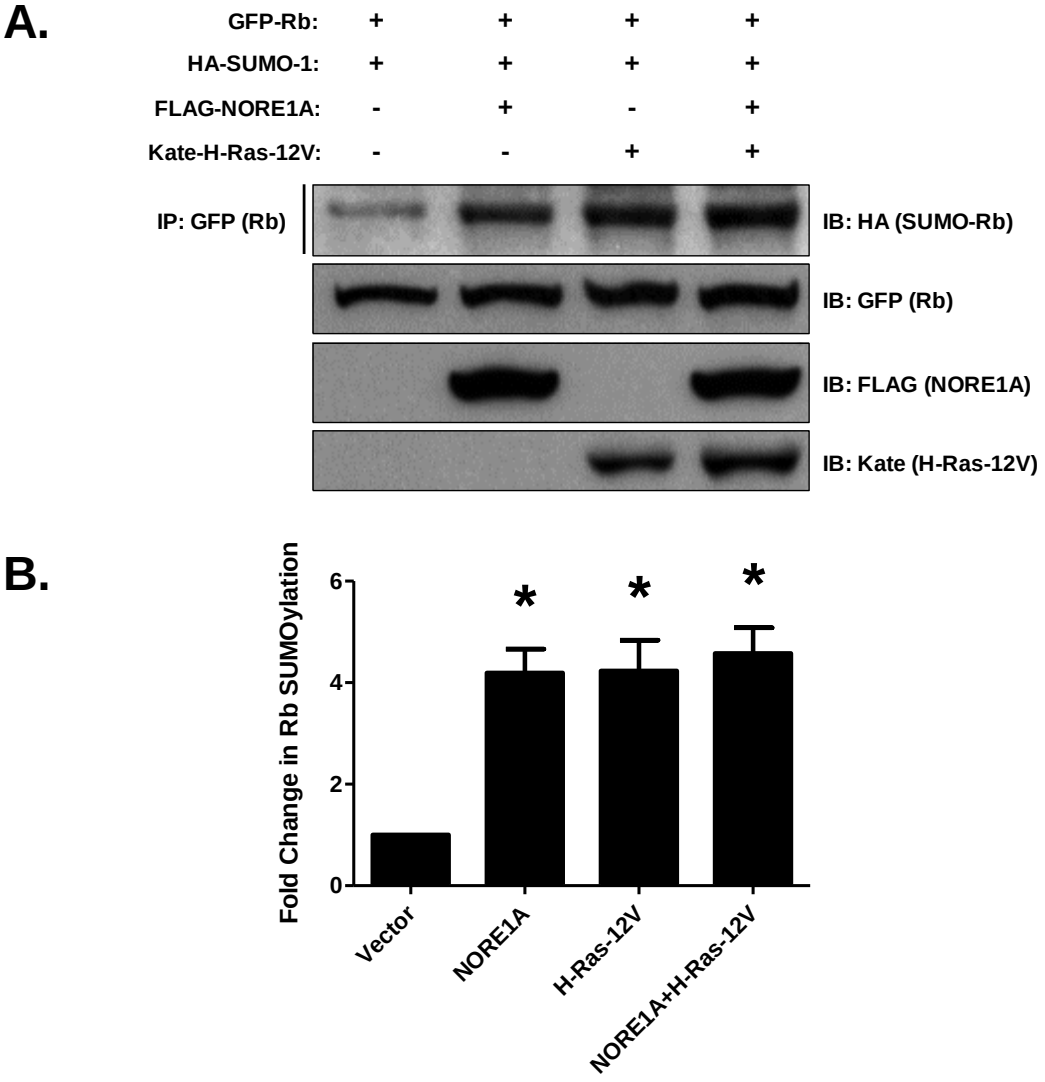

Figure 27. Ras/NORE1A enhance Rb SUMOylation. A. HEK-293T cells were transfected with GFP-Rb, HA-SUMO-1, and FLAG-NORE1A in the presence or absence of activated H-Ras for 24 hours. Cells were lysed and equal amounts of protein were immunoprecipitated using anti-GFP. The immunoprecipitates were analyzed by western blot using anti-HA, FLAG, GFP, and KATE antibodies. **B.** Blots were quantified densitometrically to calculate the fold change in Rb SUMOylation. *, $P<0.05$ compared to vector transfected cells.

a weak one; however, the results show that this interaction is enhanced in the presence of activated Ras (**Figure 28**), suggesting that this interaction is regulated by Ras.

NORE1A co-localizes with Pc2 in nuclear speckles – Because the significance of protein SUMOylation has only recently been discovered, far less is known about the proteins involved in the SUMOylation machinery compared to other post-translational modifications. To date, there are few proteins that are known to act as E3 SUMO ligases in addition to the known PIASy. Interestingly, the Polycomb Group protein Pc2 is a SUMO E3 ligase that has recently been identified, and is found in a subnuclear structure (nuclear foci) called a Polycomb body (PcG bodies) (311). While Polycomb-group proteins have been shown to remodel chromatin, little is known about their function. Work done by David Wotton and colleagues suggests that Pc2 has a much more limited repertoire than other SUMO E3s, but bring up the possibility that Pc2 represents a specialized polycomb protein (312). In addition, they demonstrated that although it appears to have a relatively weak E3 *in vitro*, Pc2 has robust SUMO E3 activity *in vivo* (312), suggesting that its activity may be modulated by adaptor proteins.

Interestingly, recent evidence showed that Pc2 binds to HIPK2, and that these proteins have an overlapping localization in distinct nuclear speckles. Furthermore, Pc2 serves as a SUMO E3 ligase for this kinase, and this SUMOylation was shown to enhance the ability of HIPK2 to mediate transcriptional repression (313). Because NORE1A was shown to regulate HIPK2 activity and is found in similar nuclear structures than the ones involving Pc2/HIPK2, we hypothesized that NORE1A might be found to have an overlapping localization with Pc2. To address this, we performed

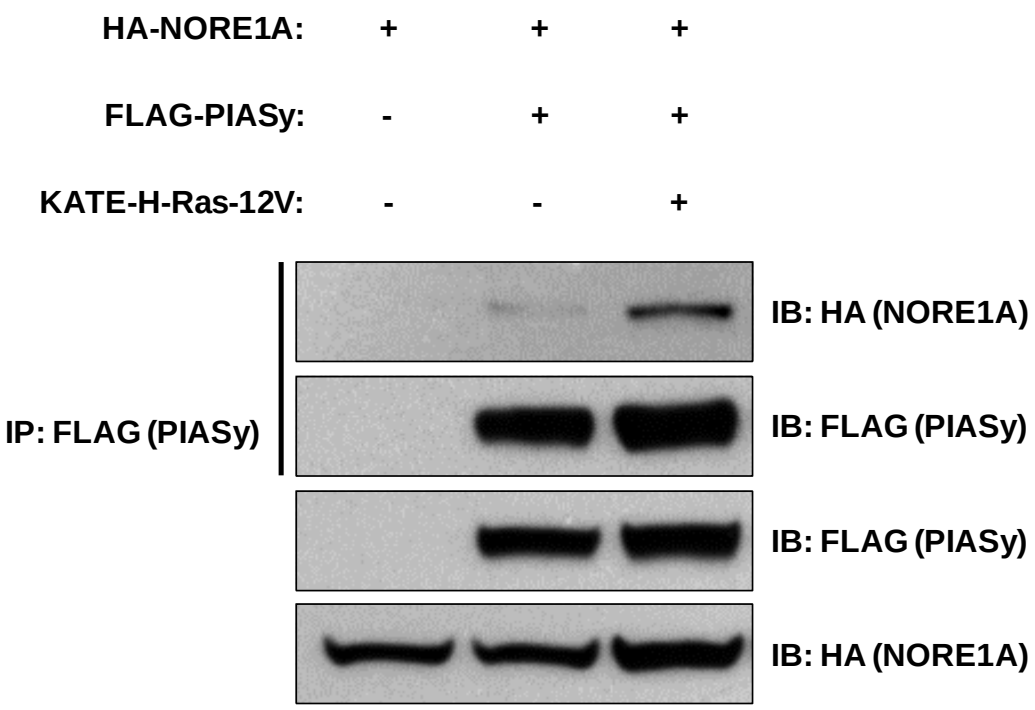

Figure 28. NORE1A forms an exogenous, Ras regulated complex with PIASy. HEK-293T cells were transfected with HA-NORE1A and FLAG-PIASy in the presence or absence of activated H-Ras for 24 hours. Cells were lysed and equal amounts of protein were immunoprecipitated using anti-FLAG. The immunoprecipitates were analyzed by western blot using anti-HA and anti-FLAG antibodies.

co-localization experiments using fluorescence microscopy. We transfected COS-7 cells with GFP-Pc2 and KATE-NORE1A in the presence or absence of activated Ras for 24hours. Indeed, we found that in a vast majority of cells that expressed both proteins, NORE1A and Pc2 strongly co-localized in distinct nuclear speckles (**Figure 29**), suggesting that NORE1A might be regulating the SUMOylation of proteins through its association with Pc2. However, co-localization does not confirm protein-protein interaction, and thus must be validated by coimmunoprecipitation. Furthermore, the presence of activated Ras in a number of cells caused co-localization of NORE1A and Pc2 along the microtubule network, raising the possibility that Ras may be regulating the sub-cellular localization of Pc2 via NORE1A, potentially regulating the SUMOylation of additional proteins in addition to nuclear proteins.

Ras and NORE1A cooperate to stabilize Rb – Post-translational modifications of proteins, such as acetylation and SUMOylation, have been shown to regulate protein stability (307,314). Since NORE1A forms an exogenous, Ras regulated complex with Rb, in turn modulating several key post-translational modifications of Rb, and because we found slightly elevated levels of Rb in the presence of Ras/NORE1A when performing experiments throughout this project, we hypothesized that Ras and NORE1A may be promoting the stability of Rb. To determine the effects of Ras and NORE1A on Rb stability, HEK-293 cells were transfected with Rb, NORE1A, and activated H-Ras expression constructs for 24 hours. The cells were then treated with cycloheximide and were lysed over a time course. The results show that after 8 hours following cycloheximide treatment, Rb expression is elevated in the presence of NORE1A or

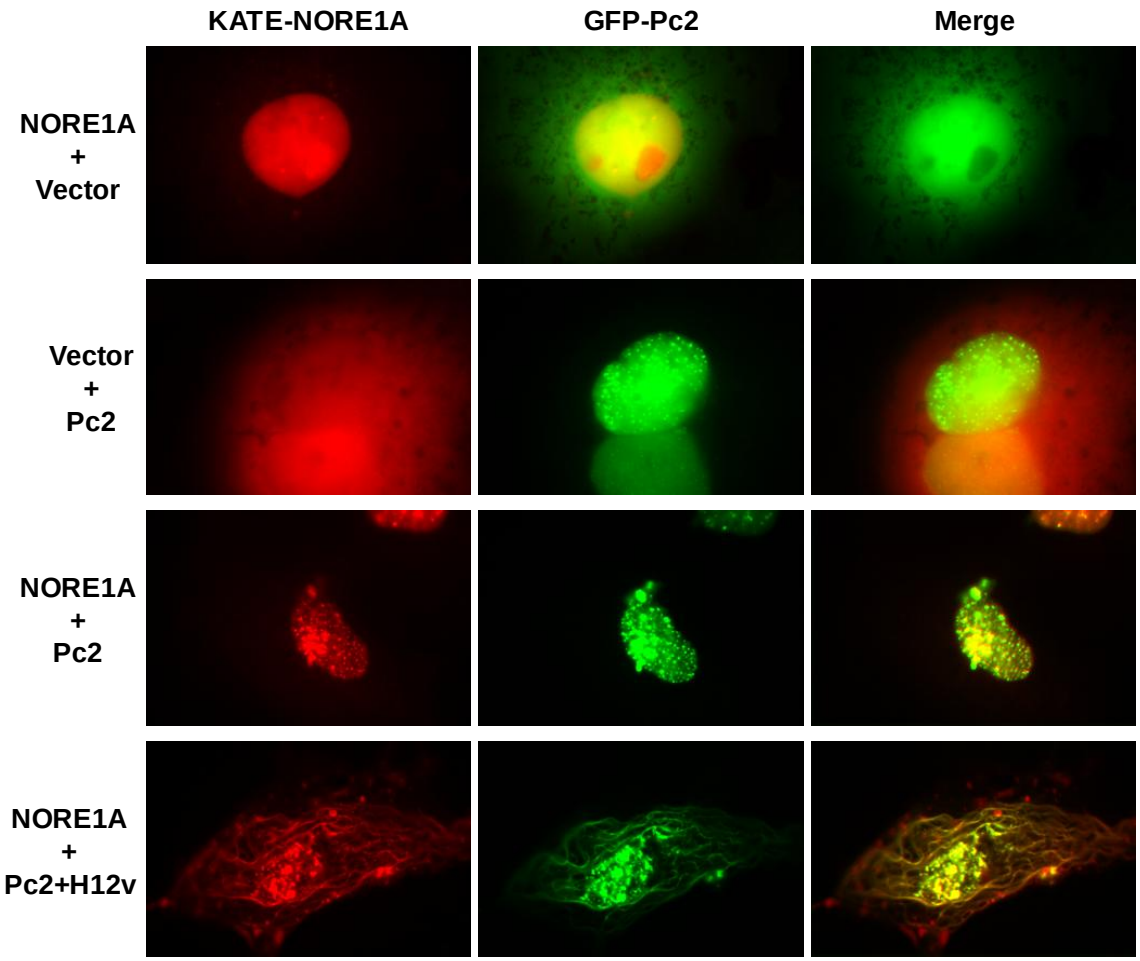

Figure 29. NORE1A co-localizes with Pc2 in the nucleus. COS-7 cells were transfected with GFP-Pc2 and RFP-NORE1A in the presence or absence of activated H-Ras for 24 hours. Fluorescence microscopy analysis revealed that in some cells, exogenously expressed NORE1A forms nuclear speckles with Pc2, and that activated Ras promotes additional co-localization onto the microtubule network of the cytoplasm.

activated Ras (**Figure 30**). Furthermore, there is a consistent elevation of Rb expression in the presence of both NORE1A and activated Ras, suggesting that the proteins are cooperating to promote Rb stability. Further studies using a set of lung epithelial HBEC-3KT cells, which we have engineered to be knocked down for NORE1A (20), showed that suppression of NORE1A acts to destabilize Rb levels (**Figure 31**). Collectively, these results suggest that Ras promotes Rb stability via NORE1A.

4.3 – Discussion:

It has been well established that Ras, in addition to its ability to promote transformation, can induce cellular senescence (159). It is widely believed that such a mechanism acts as a critical barrier against tumor development. There is now over-whelming evidence pointing to Ras being able to promote senescence through the critical p53 and Rb pathways, and that in human systems loss of both of these pathways is required in order for Ras to bypass senescence and promote transformation (169). However, the mechanisms by which Ras regulates p53 and Rb were not fully understood.

Interestingly, Ras was shown to promote the pro-senescent post-translational modifications of p53 (281), though how this was taking place remained unknown. It would seem logical that such tumor suppressive phenotypes would be occurring via senescence effectors of Ras. It was recently shown that NORE1A is a critical mediator of Ras-induced senescence. By forming a Ras regulated complex with HIPK2, NORE1A could scaffold HIPK2 to p53, in turn regulating the pro-senescent post-translational modifications of p53 (20). These findings provide two novel, and critical, lines of evidence. First, NORE1A is a critical mediator of Ras-induced senescence. Second,

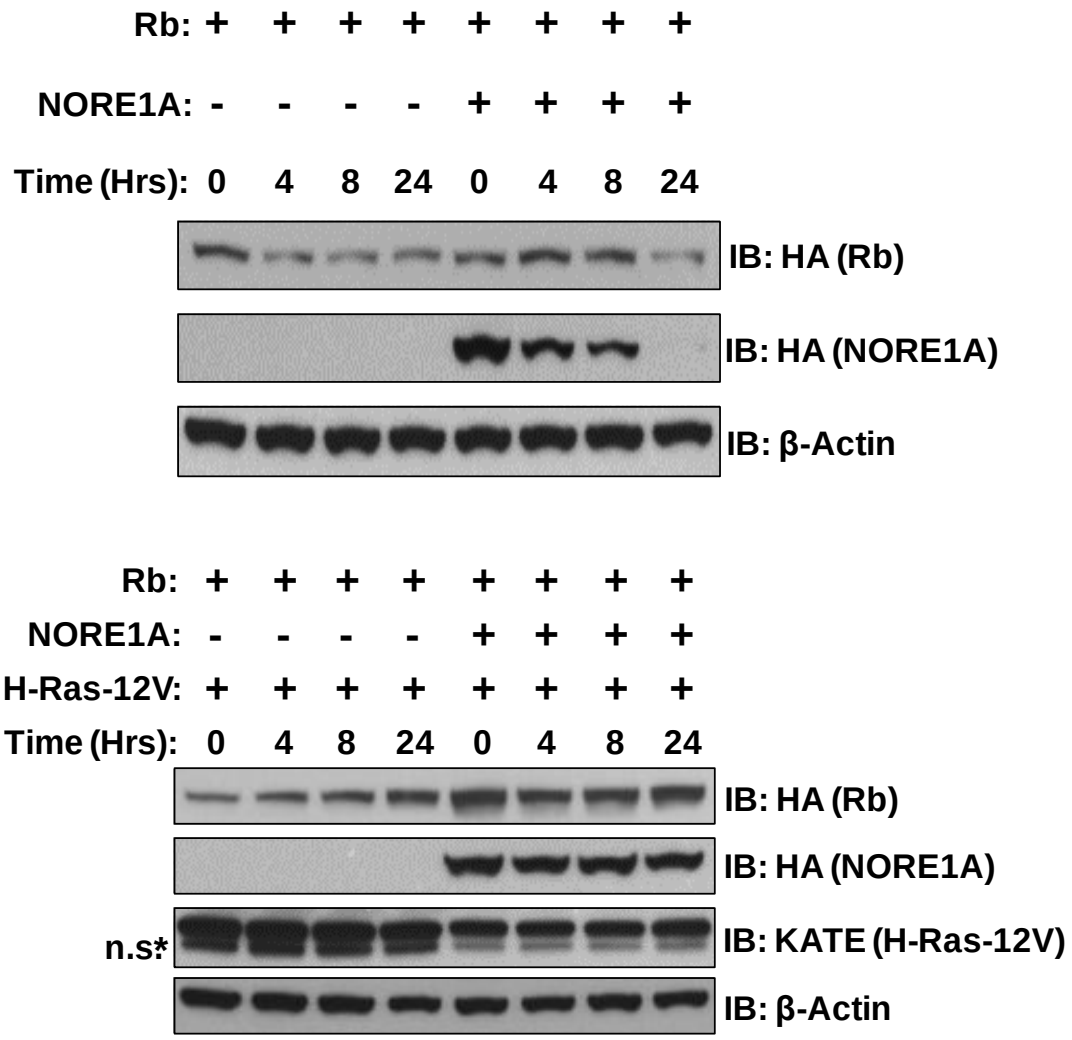

Figure 30. Ras/NORE1A cooperate to stabilize Rb. HEK 293 cells were transfected with HA-Rb and HA-NORE1A in the presence or absence of activated H-Ras for 24 hours. The cells were treated with cycloheximide (20 µg/mL) and lysed at the indicated times after addition of cycloheximide. Levels of Rb protein were measured by western blot analysis. Shown is a representative blot of two independent experiments.

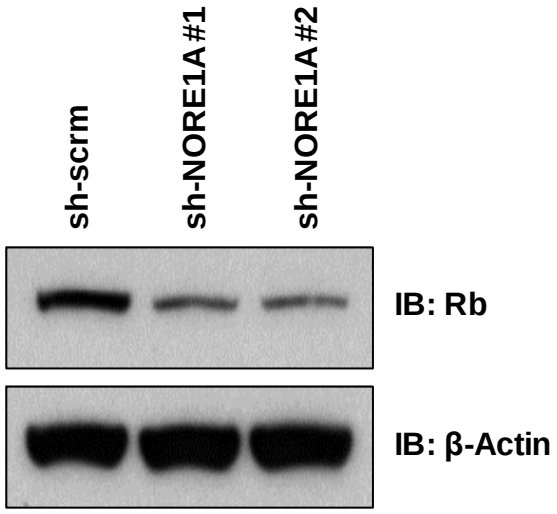

Figure 31. Loss of NORE1A destabilizes Rb. A matched pair of HBEC-3KT cells containing either the scrambled shRNA control or two different NORE1A shRNAs were generated as previously described (20). Cells were lysed and equal amount of whole cell lysates were subjected to western blot analysis using an anti-Rb antibody.

Ras regulates the post-translational modification signature of proteins, including tumor suppressors such as p53, via NORE1A.

The secondary pathway in Ras-induced senescence involves Rb, another tumor suppressor that, like p53, is primarily regulated by its post-translational modifications. We have shown that NORE1A can also promote Ras-induced senescence by regulating the Rb pathway. Ras can promote NORE1A mediated scaffolding of PP1A onto Rb in order to activate it via dephosphorylation. However, similar to p53, Rb is regulated by additional post-translational modifications. While the biological consequence of these modifications is not as clear as the phosphorylation events, the current evidence suggests that both the acetylation and SUMOylation of Rb as being activating events.

We have now shown that Ras and NORE1A enhance to overall acetylation of Rb. However, the mechanism of action remains unclear. Three proteins have been currently identified as being able to regulate Rb acetylation: PCAF, p300, and Tip60. Interestingly, we found that NORE1A forms an exogenous complex with PCAF; however, our over-expression studies suggest that the ability of Ras/NORE1A to promote Rb acetylation is occurring in a PCAF independent manner. A previous report showed that acetylation of Rb at the C-terminus by p300 maintains Rb in its active, hypophosphorylated form (297). Interestingly, the kinase HIPK2 has been shown to recruit CBP/p300 to its effector proteins, such as p53, to regulate their function (291). Because we have previously established that NORE1A forms an endogenous, Ras regulated complex with HIPK2, in turn promoting the pro-senescent acetylation of p53 (20), it is possible that NORE1A can regulate Rb acetylation by a similar mechanism in order to maintain Rb in its active state.

In addition to being regulated by phosphorylation and acetylation, Rb can also be modified by SUMOylation. SUMOylation of proteins is a relatively new concept, but one that has quickly shown to have drastic effects on protein function, such as protein stability and affecting protein-protein interactions. While the site for Rb SUMOylation has been mapped to Lysine 720 (305), the significance of this modification remains poorly understood. Interestingly, the SUMOylation machinery has been linked to senescence, as processed forms of SUMO-2/3 promotes senescence in a p53 and Rb dependent manner (306). Because the link between Rb, SUMOylation, and senescence is quite unclear, and that prior evidence from our laboratory showed that NORE1A could modulate the SUMOylation of other proteins, we believed that NORE1A might be regulating Rb SUMOylation. Indeed, over-expressing Ras/NORE1A did lead to an increase in the levels of Rb SUMOylation. What remains unknown is the mechanism by which NORE1A regulates this Rb modification. Currently, there are two intriguing possibilities that have arisen from our preliminary findings. First, we have shown that NORE1A forms an exogenous, Ras regulated complex with the E3 SUMO ligase PIASy, which is already established to play a role in Ras mediated senescence. Furthermore, we have shown that NORE1A strongly co-localizes with the newly identified E3 SUMO ligase Pc2, a protein known to regulate HIPK2 SUMOylation, and that activated Ras seems to affect some of this co-localization to the microtubule network in addition to nuclear speckles. While both of these potential mechanisms are quite interesting, there is a substantial amount of work that will need to be performed in order to confirm that one of these pathways is indeed the mechanism of action for NORE1A mediated Rb SUMOylation. Even with the possibility of NORE1A regulating Rb SUMOylation

independently of these potential pathways, the data strongly implicates NORE1A with the SUMOylation machinery, raising the intriguing possibility that NORE1A could be mediating the function of a variety of unknown target proteins via SUMOylation.

In addition to being regulated at a qualitative level by post-translational modifications, Rb can also be regulated from a quantitative perspective, as it has been shown to be degraded through a proteasome-dependent pathway (315,316). It was later found that the ubiquitin ligase mdm2 promotes Rb protein degradation via a proteasome-dependent pathway (286). We have now shown that Ras and NORE1A cooperate to stabilize Rb protein, and that the loss of NORE1A destabilizes Rb. NORE1A has been shown to associate with mdm2 to regulate the stability of other targets (288). Thus, it would be interesting to determine if NORE1A could be stabilizing Rb by antagonizing the effects of mdm2 on Rb. In addition, one of the physiological functions of protein SUMOylation is that it can regulate protein stability (307). Thus, the ability of Ras/NORE1A to modulate Rb stability might come for the ability to enhance Rb SUMOylation. It would be interesting to determine if generating a SUMO-deficient Rb mutant (via site-directed mutagenesis) would show a decrease in stability, even in the presence of Ras/NORE1A. In parallel, it would also be intriguing to determine what the outcome of such a mutant might have on NORE1A mediated senescence. However, it is quite possible that SUMOylated Rb may serve to regulate different Rb functions in addition to senescence, which may explain why Rb is such a critical tumor suppressor as well as the reason for its inactivation in such a high percentage in human cancers.

It has been well established that Ras can regulate critical signaling cascades by phosphorylation and dephosphorylation events (108), suggesting that this may be the

fundamental mechanism by which Ras modulates its effector proteins. However, we have recently described a novel mechanism by which Ras, through its effector NORE1A, can regulate the acetylation of p53 to induce senescence (20). This was the first description showing that Ras can regulate protein acetylation. Furthermore, we now show evidence suggesting that Ras can also regulate Rb acetylation via NORE1A. Therefore, it is reasonable to suggest that Ras mediated protein acetylation may be just as important, if not more important, than phosphorylation. Indeed, recent proteomics analysis has identified thousands of acetylated mammalian proteins, which has now given rise to the "*in vivo* acetylome" (317). More importantly, a large number of the acetylation sites identified were present on proteins involved in numerous, vital biological processes, such as chromatin remodeling, DNA replication, and nuclear transport, suggesting that these processes may be influenced by such a modification (317). Thus, from a clinical standpoint, a true understanding of how Ras regulates the "acetylome" will be critical in terms of evaluating the proper therapeutic approaches for cancer treatments. This is of particular interest with regards to targeting HDACs or SIRTs using pharmacological approaches. For example, SIRT1 is over expressed in some cancers harboring Ras mutations, and targeting SIRT1 has been shown to suppress transformation and sensitize such cancers to conventional therapy (318). Mechanistically, SIRT1 mediated deacetylation suppresses the functions of several tumor suppressors, including p53 (302). Additionally, HDAC inhibitors are another class of anti-cancer agents that are cytotoxic to cancer cells, and some studies have shown that HDAC inhibitors can activate the pro-apoptotic ability of oncogenic Ras (319). Ultimately, it will be of utmost importance to

determine the expression patterns of Ras effectors that play a role in the regulation of the "acetylome" in order to optimize therapeutic options targeting the acetylation machinery.

Lastly, our data highlights the first example by which Ras can promote the SUMOylation of proteins. Here, we show that by using its effector NORE1A, Ras can promote the SUMOylation of Rb, and may be doing so in part by promoting the interaction of NORE1A with SUMO ligases. While much less is known about protein SUMOylation compared to other post-translational modifications, the SUMOylation machinery has now been implicated in several important biological processes, such as the onset of cellular senescence (307). Collectively, these findings suggest a potential paradigm shift for Ras action. While it has been clearly shown that Ras regulates several key signaling pathways by modulation of protein phosphorylation, it now seems as if Ras can regulate additional post-translational modifications that may be just as critical in regulating protein function.

CHAPTER 5

CONCLUSION

Collectively, the work presented in this dissertation highlights a novel mechanism that explains an unanswered question: how does Ras regulate Rb? It has been known for over thirty years that while Ras can be powerfully transforming, it can also regulate growth inhibitory pathways. While it has now been clearly established that Ras can induce senescence, primarily by activating p53 and Rb, how Ras specifically regulates the p53 and Rb pathways remained unknown. Our work shows that Ras uses NORE1A as a senescence effector to regulate powerful tumor suppressors, p53 and Rb, that play crucial roles in Ras mediated senescence.

We found that NORE1A forms an endogenous, Ras regulated complex with the phosphatase PP1A. Not only does NORE1A stabilize PP1A, it scaffolds PP1A to Rb in a Ras dependent manner, in turn activating Rb via dephosphorylation. Furthermore, we show that NORE1A requires Rb in order to fully promote senescence. These findings, along with previous work performed in the laboratory, define NORE1A as a double barreled senescence effector of Ras by regulating pro-senescent post-translational modifications of p53 and Rb (**Figure 32**). Loss of NORE1A subverts these two critical pathways, which allows the Ras mediated growth promoting pathways to predominate, in

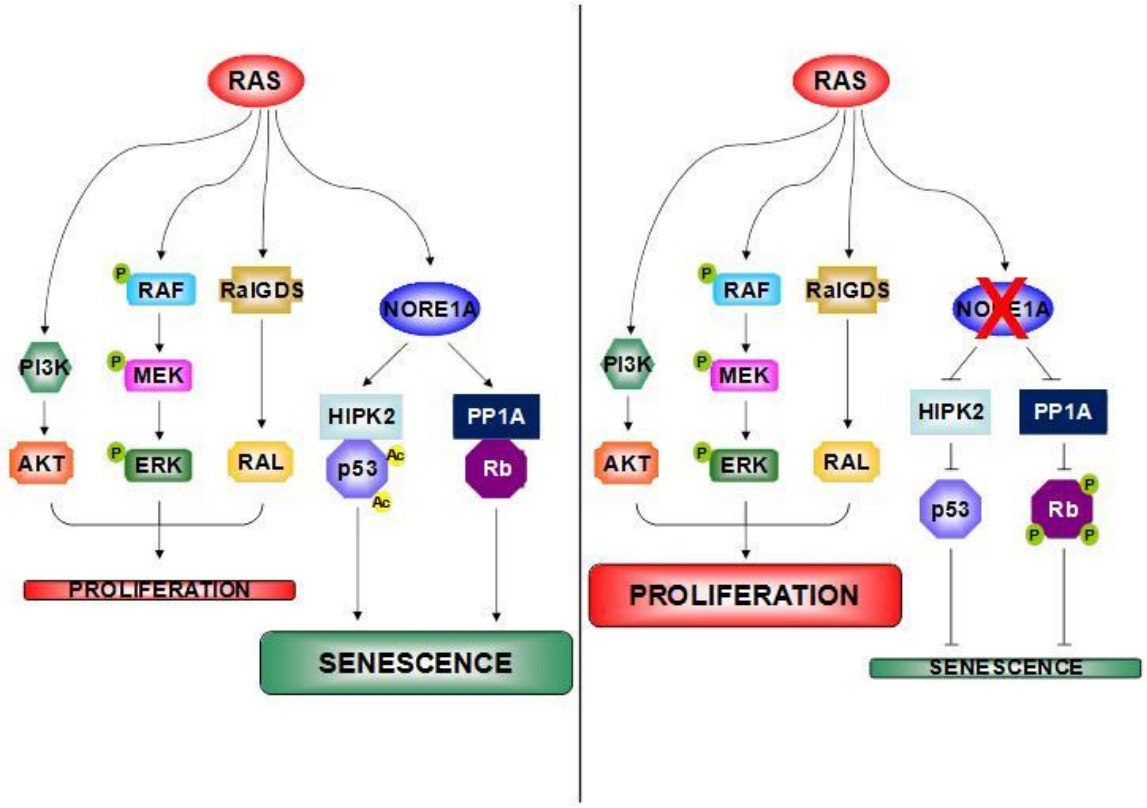

Figure 32. Molecular mechanisms whereby Ras promotes the induction of senescence via its effector NORE1A. In normal cells, Ras promotes the acetylation of p53 via the NORE1A/HIPK2 as well as the dephosphorylation of Rb via the NORE1A/PP1A axis. Together, these pathways serve as a protective barrier against unrestricted Ras mediated transformation. In the absence of NORE1A (right), the pro-senescent functions of p53 and Rb cannot be activated, resulting in the bypass of senescence induction and allows for the Ras mediated proliferative signals to dominate. Ac, acetylated lysine; P, phosphorylation.

turn driving Ras towards transformation. This validates previous findings showing that Ras driven tumors are frequently associated with reduced NORE1A expression.

Interestingly, PP1A is not an Rb-specific phosphatase, and can modulate the function of proteins by regulating their phosphorylation status. The specificity of PP1A for its substrates is dictated by other targeting proteins. Indeed, PP1A can associate with over two hundred regulatory proteins which can dephosphorylate hundreds of targets. Our data shows that NORE1A may serve as a PP1A targeting protein. However, our work has not confirmed whether the NORE1A/PP1A complex specifically targets Rb. Further experiments will be vital to determine any additional NORE1A targeted substrates.

In addition to regulating Rb phosphorylation, we found that NORE1A can regulate additional post-translational modifications of Rb, including acetylation and SUMOylation. Unlike phosphorylation, the significance of Rb acetylation/SUMOylation remains poorly understood; however, it is believed that these modifications are activating events. It will be interesting to determine if these modifications are necessary for Rb mediated senescence. There is some belief that Rb modifications, such as SUMOylation, may actually lead to a specific sub-set of Rb protein that perform distinct Rb functions in addition to the prototypical cell cycle arrest and senescence induction.

This work highlights novel mechanisms by which Ras can regulate protein acetylation as well as SUMOylation, suggesting the need for a new paradigm for Ras action. It has been well established that Ras can regulate the function of proteins by modulating their phosphorylation status. However, our evidence strongly suggests that in addition to such regulation, Ras can regulate multiple post-translational modifications by

using its effector proteins, such as NORE1A, to target specific substrates. Therefore, Ras must be thought of as a critical mediator of protein function by playing a pivotal role in regulating the post-translational modification code of downstream proteins, including the previously unidentified modifications of acetylation and SUMOylation.

Throughout the project, we found that Ras/NORE1A stabilize both PP1A and Rb. These findings were not unexpected for several reasons. First, we had previously shown that NORE1A can affect the stability of other proteins, such as HIPK2 (20). Second, it seems logical that if NORE1A is a senescence effector of Ras, it would stabilize the PP1A/Rb complex in order to activate the pro-senescent functions of Rb. While these findings are interesting, they do not provide a mechanism of action. Future studies are crucial to determine the mechanisms by which NORE1A promotes the stability of PP1A and Rb. One attractive possibility is via the ubiquitin ligase mdm2, for two reasons. First, mdm2 has been shown to promote tumorigenesis by destabilizing Rb. Second, mdm2 has been found in an endogenous complex with PP1A. Intriguingly, NORE1A has been implicated in regulating the degradation of specific mdm2 targets. Thus, NORE1A could be promoting the stability of both PP1A and Rb by antagonizing the degradation properties of mdm2.

Some standard therapies invoke a senescence response as part of their therapeutic effect. Indeed, the concept of pro-senescence therapy has emerged over the recent years as a novel therapeutic approach to treat cancers, and it is now believed that the inclusion of deliberate pro-senescence therapy may be critical as part of cancer treatment regimens (320). Such approaches include drugs that enhance p53 activity and function, as well as drugs with the ability to modulate Rb activity through targeting Cdks and CDKIs (320).

Our studies here indicate that the status of NORE1A in a tumor may play a role in dictating the sensitivity of tumor cells to any pro-senescence type therapy.

As loss of NORE1A expression primarily occurs via promoter methylation, it is a candidate for epigenetic therapy. It has already been shown that restoring NORE1A expression in several NORE1A-negative cancer cell lines using the DNA methyl-transferase inhibitor 5-Azacytidine restores NORE1A signaling pathways (191). Unfortunately, treatment with 5-Aza-C results in non-specific overall DNA methylation and this process may affect multiple regulatory pathways (321). This can likely reactivate expression of multiple silenced genes, including oncogenes and tumor suppressors in different cell types and in different cancers (322). However, the continued development of more specific epigenetic therapy agents, such as Nanaomycin A, which preferentially inhibits the key DNMT3B enzyme, may enhance the practicality of this approach. Recent evidence has shown that Nanaomycin A reduced global methylation levels while reactivating transcription of several RASSF family members (323,324), though its effects on NORE1A expression have not yet been studied. Thus, a novel approach in NORE1A negative cancers might be to combine epigenetic therapy with senescence therapy to induce tumor regression.

REFERENCES:

1. (2014) Cancer Facts & Figures 2014. American Cancer Society
2. (2005) The Burden of Chronic Diseases and Their Risk Factors: National and State Perspectives 2004. Centers for Disease Control and Prevention
3. Mariotto, A. B., Yabroff, K. R., Shao, Y., Feuer, E. J., and Brown, M. L. (2011) Projections of the cost of cancer care in the United States: 2010-2020. *J Natl Cancer Inst* **103**, 117-128
4. Croce, C. M. (2008) Oncogenes and cancer. *N Engl J Med* **358**, 502-511
5. Bishop, J. M. (1991) Molecular themes in oncogenesis. *Cell* **64**, 235-248
6. Pylayeva-Gupta, Y., Grabocka, E., and Bar-Sagi, D. (2011) RAS oncogenes: weaving a tumorigenic web. *Nature reviews. Cancer* **11**, 761-774
7. Adjei, A. A. (2001) Blocking oncogenic Ras signaling for cancer therapy. *J Natl Cancer Inst* **93**, 1062-1074
8. Campbell, P. M., and Der, C. J. (2004) Oncogenic Ras and its role in tumor cell invasion and metastasis. *Seminars in cancer biology* **14**, 105-114
9. Augsten, M., Bottcher, A., Spanbroek, R., Rubio, I., and Friedrich, K. (2014) Graded inhibition of oncogenic Ras-signaling by multivalent Ras-binding domains. *Cell Commun Signal* **12**, 1

10. Cox, A. D., Fesik, S. W., Kimmelman, A. C., Luo, J., and Der, C. J. (2014) Drugging the undruggable RAS: Mission possible? *Nat Rev Drug Discov* **13**, 828-851

11. Newbold, R. F., and Overell, R. W. (1983) Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. *Nature* **304**, 648-651

12. Vos, M. D., Martinez, A., Ellis, C. A., Vallecorsa, T., and Clark, G. J. (2003) The pro-apoptotic Ras effector Nore1 may serve as a Ras-regulated tumor suppressor in the lung. *The Journal of biological chemistry* **278**, 21938-21943

13. van der Weyden, L., and Adams, D. J. (2007) The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochimica et biophysica acta* **1776**, 58-85

14. Vavvas, D., Li, X., Avruch, J., and Zhang, X. F. (1998) Identification of Nore1 as a potential Ras effector. *The Journal of biological chemistry* **273**, 5439-5442

15. Donninger, H., Vos, M. D., and Clark, G. J. (2007) The RASSF1A tumor suppressor. *Journal of cell science* **120**, 3163-3172

16. Geli, J., Kogner, P., Lanner, F., Natalishvili, N., Juhlin, C., Kiss, N., Clark, G. J., Ekstrom, T. J., Farnebo, F., and Larsson, C. (2008) Assessment of NORE1A as a putative tumor suppressor in human neuroblastoma. *International journal of cancer. Journal international du cancer* **123**, 389-394

17. Aoyama, Y., Avruch, J., and Zhang, X. F. (2004) Nore1 inhibits tumor cell growth independent of Ras or the MST1/2 kinases. *Oncogene* **23**, 3426-3433

18. Chen, J., Lui, W. O., Vos, M. D., Clark, G. J., Takahashi, M., Schoumans, J., Khoo, S. K., Petillo, D., Lavery, T., Sugimura, J., Astuti, D., Zhang, C., Kagawa,

S., Maher, E. R., Larsson, C., Alberts, A. S., Kanayama, H. O., and Teh, B. T. (2003) The t(1;3) breakpoint-spanning genes LSAMP and NORE1 are involved in clear cell renal cell carcinomas. *Cancer cell* **4**, 405-413

19. Khokhlatchev, A., Rabizadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X. F., Seed, B., and Avruch, J. (2002) Identification of a novel Ras-regulated proapoptotic pathway. *Current biology : CB* **12**, 253-265
20. Donninger, H., Calvisi, D. F., Barnoud, T., Clark, J., Schmidt, M. L., Vos, M. D., and Clark, G. J. (2015) NORE1A is a Ras senescence effector that controls the apoptotic/senescent balance of p53 via HIPK2. *J Cell Biol* **208**, 777-789
21. Burkhart, D. L., and Sage, J. (2008) Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nature reviews. Cancer* **8**, 671-682
22. Castro, M. E., Ferrer, I., Cascon, A., Guijarro, M. V., Lleonart, M., Ramon y Cajal, S., Leal, J. F., Robledo, M., and Carnero, A. (2008) PPP1CA contributes to the senescence program induced by oncogenic Ras. *Carcinogenesis* **29**, 491-499
23. Schwarz, D. (2002) *Novel Ras Effector 1 (Nore1).* Ph.D. thesis, Ruhr-Universität Bochum
24. Futreal, P. A., and Barrett, J. C. (1991) Failure of senescent cells to phosphorylate the RB protein. *Oncogene* **6**, 1109-1113
25. Bollen, M., Peti, W., Ragusa, M. J., and Beullens, M. (2010) The extended PP1 toolkit: designed to create specificity. *Trends in biochemical sciences* **35**, 450-458
26. Fernandez-Medarde, A., and Santos, E. (2011) Ras in cancer and developmental diseases. *Genes Cancer* **2**, 344-358

27. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., and Thun, M. J. (2009) Cancer statistics, 2009. *CA Cancer J Clin* **59**, 225-249

28. Cox, A. D., and Der, C. J. (2010) Ras history: The saga continues. *Small GTPases* **1**, 2-27

29. Harvey, J. J. (1964) An Unidentified Virus Which Causes the Rapid Production of Tumours in Mice. *Nature* **204**, 1104-1105

30. Kirsten, W. H., and Mayer, L. A. (1967) Morphologic responses to a murine erythroblastosis virus. *J Natl Cancer Inst* **39**, 311-335

31. Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S., and Barbacid, M. (1982) T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. *Nature* **298**, 343-347

32. Der, C. J., Krontiris, T. G., and Cooper, G. M. (1982) Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 3637-3640

33. Parada, L. F., Tabin, C. J., Shih, C., and Weinberg, R. A. (1982) Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. *Nature* **297**, 474-478

34. O'Toole, C. M., Povey, S., Hepburn, P., and Franks, L. M. (1983) Identity of some human bladder cancer cell lines. *Nature* **301**, 429-430

35. Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M., and Wigler, M. (1982) Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. *Nature* **300**, 762-765

36. Reddy, E. P., Reynolds, R. K., Santos, E., and Barbacid, M. (1982) A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature* **300**, 149-152

37. Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R., and Chang, E. H. (1982) Mechanism of activation of a human oncogene. *Nature* **300**, 143-149

38. Roberts, P. J., and Der, C. J. (2007) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26**, 3291-3310

39. Fasano, O., Aldrich, T., Tamanoi, F., Taparowsky, E., Furth, M., and Wigler, M. (1984) Analysis of the transforming potential of the human H-ras gene by random mutagenesis. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 4008-4012

40. Walter, M., Clark, S. G., and Levinson, A. D. (1986) The oncogenic activation of human p21ras by a novel mechanism. *Science* **233**, 649-652

41. Sigal, I. S., Gibbs, J. B., D'Alonzo, J. S., Temeles, G. L., Wolanski, B. S., Socher, S. H., and Scolnick, E. M. (1986) Mutant ras-encoded proteins with altered nucleotide binding exert dominant biological effects. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 952-956

42. Seeburg, P. H., Colby, W. W., Capon, D. J., Goeddel, D. V., and Levinson, A. D. (1984) Biological properties of human c-Ha-ras1 genes mutated at codon 12. *Nature* **312**, 71-75

43. Bos, J. L., Toksoz, D., Marshall, C. J., Verlaan-de Vries, M., Veeneman, G. H., van der Eb, A. J., van Boom, J. H., Janssen, J. W., and Steenvoorden, A. C. (1985) Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukaemia. *Nature* **315**, 726-730

44. Der, C. J., Finkel, T., and Cooper, G. M. (1986) Biological and biochemical properties of human rasH genes mutated at codon 61. *Cell* **44**, 167-176

45. Barbacid, M. (1987) ras genes. *Annu Rev Biochem* **56**, 779-827

46. Hall, A., Marshall, C. J., Spurr, N. K., and Weiss, R. A. (1983) Identification of transforming gene in two human sarcoma cell lines as a new member of the ras gene family located on chromosome 1. *Nature* **303**, 396-400

47. Taparowsky, E., Shimizu, K., Goldfarb, M., and Wigler, M. (1983) Structure and activation of the human N-ras gene. *Cell* **34**, 581-586

48. Hirai, H., Okabe, T., Anraku, Y., Fujisawa, M., Urabe, A., and Takaku, F. (1985) Activation of the c-K-ras oncogene in a human pancreas carcinoma. *Biochem Biophys Res Commun* **127**, 168-174

49. Hand, P. H., Thor, A., Wunderlich, D., Muraro, R., Caruso, A., and Schlom, J. (1984) Monoclonal antibodies of predefined specificity detect activated ras gene expression in human mammary and colon carcinomas. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 5227-5231

50. Gambke, C., Hall, A., and Moroni, C. (1985) Activation of an N-ras gene in acute myeloblastic leukemia through somatic mutation in the first exon. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 879-882

51. Gambke, C., Signer, E., and Moroni, C. (1984) Activation of N-ras gene in bone marrow cells from a patient with acute myeloblastic leukaemia. *Nature* **307**, 476-478

52. Janssen, J. W., Steenvoorden, A. C., Collard, J. G., and Nusse, R. (1985) Oncogene activation in human myeloid leukemia. *Cancer research* **45**, 3262-3267

53. Sklar, M. D., and Kitchingman, G. R. (1985) Isolation of activated ras transforming genes from two patients with Hodgkin's disease. *Int J Radiat Oncol Biol Phys* **11**, 49-55

54. Padua, R. A., Barrass, N. C., and Currie, G. A. (1985) Activation of N-ras in a human melanoma cell line. *Molecular and cellular biology* **5**, 582-585

55. Fujita, J., Yoshida, O., Yuasa, Y., Rhim, J. S., Hatanaka, M., and Aaronson, S. A. (1984) Ha-ras oncogenes are activated by somatic alterations in human urinary tract tumours. *Nature* **309**, 464-466

56. Bos, J. L. (1989) ras oncogenes in human cancer: a review. *Cancer research* **49**, 4682-4689

57. Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**, 615-649

58. Gilman, A. G. (1984) G proteins and dual control of adenylate cyclase. *Cell* **36**, 577-579

59. Gibbs, J. B., Sigal, I. S., Poe, M., and Scolnick, E. M. (1984) Intrinsic GTPase activity distinguishes normal and oncogenic ras p21 molecules. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 5704-5708

60. McGrath, J. P., Capon, D. J., Goeddel, D. V., and Levinson, A. D. (1984) Comparative biochemical properties of normal and activated human ras p21 protein. *Nature* **310**, 644-649

61. Sweet, R. W., Yokoyama, S., Kamata, T., Feramisco, J. R., Rosenberg, M., and Gross, M. (1984) The product of ras is a GTPase and the T24 oncogenic mutant is deficient in this activity. *Nature* **311**, 273-275

62. Manne, V., Bekesi, E., and Kung, H. F. (1985) Ha-ras proteins exhibit GTPase activity: point mutations that activate Ha-ras gene products result in decreased GTPase activity. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 376-380

63. Trahey, M., Milley, R. J., Cole, G. E., Innis, M., Paterson, H., Marshall, C. J., Hall, A., and McCormick, F. (1987) Biochemical and biological properties of the human N-ras p21 protein. *Molecular and cellular biology* **7**, 541-544

64. Lacal, J. C., Srivastava, S. K., Anderson, P. S., and Aaronson, S. A. (1986) Ras p21 proteins with high or low GTPase activity can efficiently transform NIH/3T3 cells. *Cell* **44**, 609-617

65. Colby, W. W., Hayflick, J. S., Clark, S. G., and Levinson, A. D. (1986) Biochemical characterization of polypeptides encoded by mutated human Ha-ras1 genes. *Molecular and cellular biology* **6**, 730-734

66. McCormick, F. (1998) Going for the GAP. *Current biology : CB* **8**, R673-674

67. Trahey, M., and McCormick, F. (1987) A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* **238**, 542-545

68. Willumsen, B. M., Papageorge, A. G., Kung, H. F., Bekesi, E., Robins, T., Johnsen, M., Vass, W. C., and Lowy, D. R. (1986) Mutational analysis of a ras catalytic domain. *Molecular and cellular biology* **6**, 2646-2654

69. Willingham, M. C., Pastan, I., Shih, T. Y., and Scolnick, E. M. (1980) Localization of the src gene product of the Harvey strain of MSV to plasma membrane of transformed cells by electron microscopic immunocytochemistry. *Cell* **19**, 1005-1014

70. Willumsen, B. M., Norris, K., Papageorge, A. G., Hubbert, N. L., and Lowy, D. R. (1984) Harvey murine sarcoma virus p21 ras protein: biological and biochemical significance of the cysteine nearest the carboxy terminus. *The EMBO journal* **3**, 2581-2585

71. Srivastava, S. K., Lacal, J. C., Reynolds, S. H., and Aaronson, S. A. (1985) Antibody of predetermined specificity to a carboxy-terminal region of H-ras gene products inhibits their guanine nucleotide-binding function. *Molecular and cellular biology* **5**, 3316-3319

72. Cox, A. D., and Der, C. J. (1992) The ras/cholesterol connection: implications for ras oncogenicity. *Crit Rev Oncog* **3**, 365-400

73. Khosravi-Far, R., Cox, A. D., Kato, K., and Der, C. J. (1992) Protein prenylation: key to ras function and cancer intervention? *Cell Growth Differ* **3**, 461-469

74. Magee, T., and Newman, C. (1992) The role of lipid anchors for small G proteins in membrane trafficking. *Trends in cell biology* **2**, 318-323

75. Hancock, J. F. (2003) Ras proteins: different signals from different locations. *Nature reviews. Molecular cell biology* **4**, 373-384

76. Clarke, S. (1992) Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu Rev Biochem* **61**, 355-386

77. Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., and Brown, M. S. (1990) Inhibition of purified p21ras farnesyl:protein transferase by Cys-AAX tetrapeptides. *Cell* **62**, 81-88

78. Schaber, M. D., O'Hara, M. B., Garsky, V. M., Mosser, S. C., Bergstrom, J. D., Moores, S. L., Marshall, M. S., Friedman, P. A., Dixon, R. A., and Gibbs, J. B. (1990) Polyisoprenylation of Ras in vitro by a farnesyl-protein transferase. *The Journal of biological chemistry* **265**, 14701-14704

79. Schafer, W. R., Trueblood, C. E., Yang, C. C., Mayer, M. P., Rosenberg, S., Poulter, C. D., Kim, S. H., and Rine, J. (1990) Enzymatic coupling of cholesterol intermediates to a mating pheromone precursor and to the ras protein. *Science* **249**, 1133-1139

80. Hancock, J. F., Paterson, H., and Marshall, C. J. (1990) A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* **63**, 133-139

81. Rajalingam, K., Schreck, R., Rapp, U. R., and Albert, S. (2007) Ras oncogenes and their downstream targets. *Biochimica et biophysica acta* **1773**, 1177-1195

82. Kamata, T., and Feramisco, J. R. (1984) Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of ras oncogene proteins. *Nature* **310**, 147-150

83. Emanoil-Ravier, R., Pochart, F., Canivet, M., Garcette, M., Tobaly-Tapiero, J., and Peries, J. (1985) Interferon-mediated regulation of myc and Ki-ras oncogene expression in long-term-treated murine viral transformed cells. *J Interferon Res* **5**, 613-619

84. Hurley, J. B., Simon, M. I., Teplow, D. B., Robishaw, J. D., and Gilman, A. G. (1984) Homologies between signal transducing G proteins and ras gene products. *Science* **226**, 860-862

85. Samid, D., Chang, E. H., and Friedman, R. M. (1985) Development of transformed phenotype induced by a human ras oncogene is inhibited by interferon. *Biochem Biophys Res Commun* **126**, 509-516

86. Samid, D., Schaff, Z., Chang, E. H., and Friedman, R. M. (1985) Interferon-induced modulation of human ras oncogene expression. *Prog Clin Biol Res* **192**, 265-268

87. Rowell, C. A., Kowalczyk, J. J., Lewis, M. D., and Garcia, A. M. (1997) Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. *The Journal of biological chemistry* **272**, 14093-14097

88. Bar-Sagi, D., and Feramisco, J. R. (1986) Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. *Science* **233**, 1061-1068

89. Fukami, K., Matsuoka, K., Nakanishi, O., Yamakawa, A., Kawai, S., and Takenawa, T. (1988) Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 9057-9061

90. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205-214

91. Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260**, 1658-1661

92. Warne, P. H., Viciana, P. R., and Downward, J. (1993) Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature* **364**, 352-355

93. Zhang, X. F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* **364**, 308-313

94. Molina, J. R., and Adjei, A. A. (2006) The Ras/Raf/MAPK pathway. *J Thorac Oncol* **1**, 7-9

95. Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Zhu, H. J., Walker, F., Frenkel, M. J., Hoyne, P. A., Jorissen, R. N., Nice, E. C., Burgess, A. W., and Ward, C. W. (2002) Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell* **110**, 763-773

96. Zhang, X., Gureasko, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* **125**, 1137-1149

97. Hofman, E. G., Bader, A. N., Voortman, J., van den Heuvel, D. J., Sigismund, S., Verkleij, A. J., Gerritsen, H. C., and van Bergen en Henegouwen, P. M. (2010)

Ligand-induced EGF receptor oligomerization is kinase-dependent and enhances internalization. *The Journal of biological chemistry* **285**, 39481-39489

98. Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211-225

99. Marais, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *The EMBO journal* **14**, 3136-3145

100. Liebmann, C. (2001) Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. *Cell Signal* **13**, 777-785

101. McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. M., and Franklin, R. A. (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochimica et biophysica acta* **1773**, 1263-1284

102. Garnett, M. J., and Marais, R. (2004) Guilty as charged: B-RAF is a human oncogene. *Cancer cell* **6**, 313-319

103. Chang, F., and McCubrey, J. A. (2001) P21(Cip1) induced by Raf is associated with increased Cdk4 activity in hematopoietic cells. *Oncogene* **20**, 4354-4364

104. Malumbres, M., Perez De Castro, I., Hernandez, M. I., Jimenez, M., Corral, T., and Pellicer, A. (2000) Cellular response to oncogenic ras involves induction of the Cdk4 and Cdk6 inhibitor p15(INK4b). *Molecular and cellular biology* **20**, 2915-2925

105. Vivanco, I., and Sawyers, C. L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nature reviews. Cancer* **2**, 489-501

106. Castellano, E., and Downward, J. (2010) Role of RAS in the regulation of PI 3-kinase. *Curr Top Microbiol Immunol* **346**, 143-169

107. Yuan, T. L., and Cantley, L. C. (2008) PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**, 5497-5510

108. Castellano, E., and Downward, J. (2011) RAS Interaction with PI3K: More Than Just Another Effector Pathway. *Genes Cancer* **2**, 261-274

109. Cantley, L. C. (2002) The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657

110. Engelman, J. A., Luo, J., and Cantley, L. C. (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* **7**, 606-619

111. Duronio, V. (2008) The life of a cell: apoptosis regulation by the PI3K/PKB pathway. *The Biochemical journal* **415**, 333-344

112. Rodriguez-Viciana, P., Warne, P. H., Vanhaesebroeck, B., Waterfield, M. D., and Downward, J. (1996) Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. *The EMBO journal* **15**, 2442-2451

113. Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S. M., Riggins, G. J., Willson, J. K., Markowitz, S., Kinzler, K. W., Vogelstein, B., and Velculescu, V. E. (2004) High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304**, 554

114. Lim, K. H., and Counter, C. M. (2005) Reduction in the requirement of oncogenic Ras signaling to activation of PI3K/AKT pathway during tumor maintenance. *Cancer cell* **8**, 381-392

115. Etienne-Manneville, S., and Hall, A. (2002) Rho GTPases in cell biology. *Nature* **420**, 629-635

116. Chiarugi, P., and Giannoni, E. (2008) Anoikis: a necessary death program for anchorage-dependent cells. *Biochem Pharmacol* **76**, 1352-1364

117. Zhan, M., Zhao, H., and Han, Z. C. (2004) Signalling mechanisms of anoikis. *Histol Histopathol* **19**, 973-983

118. Simpson, C. D., Anyiwe, K., and Schimmer, A. D. (2008) Anoikis resistance and tumor metastasis. *Cancer Lett* **272**, 177-185

119. D'Adamo, D. R., Novick, S., Kahn, J. M., Leonardi, P., and Pellicer, A. (1997) rsc: a novel oncogene with structural and functional homology with the gene family of exchange factors for Ral. *Oncogene* **14**, 1295-1305

120. Feig, L. A. (2003) Ral-GTPases: approaching their 15 minutes of fame. *Trends in cell biology* **13**, 419-425

121. Kikuchi, A., Demo, S. D., Ye, Z. H., Chen, Y. W., and Williams, L. T. (1994) ralGDS family members interact with the effector loop of ras p21. *Molecular and cellular biology* **14**, 7483-7491

122. Gentry, L. R., Martin, T. D., Reiner, D. J., and Der, C. J. (2014) Ral small GTPase signaling and oncogenesis: More than just 15minutes of fame. *Biochimica et biophysica acta* **1843**, 2976-2988

123. Rodriguez-Viciana, P., and McCormick, F. (2005) RalGDS comes of age. *Cancer cell* **7**, 205-206

124. Gonzalez-Garcia, A., Pritchard, C. A., Paterson, H. F., Mavria, G., Stamp, G., and Marshall, C. J. (2005) RalGDS is required for tumor formation in a model of skin carcinogenesis. *Cancer cell* **7**, 219-226

125. Hamad, N. M., Elconin, J. H., Karnoub, A. E., Bai, W., Rich, J. N., Abraham, R. T., Der, C. J., and Counter, C. M. (2002) Distinct requirements for Ras oncogenesis in human versus mouse cells. *Genes & development* **16**, 2045-2057

126. Illenberger, D., Schwald, F., and Gierschik, P. (1997) Characterization and purification from bovine neutrophils of a soluble guanine-nucleotide-binding protein that mediates isozyme-specific stimulation of phospholipase C beta2. *Eur J Biochem* **246**, 71-77

127. Illenberger, D., Schwald, F., Pimmer, D., Binder, W., Maier, G., Dietrich, A., and Gierschik, P. (1998) Stimulation of phospholipase C-beta2 by the Rho GTPases Cdc42Hs and Rac1. *The EMBO journal* **17**, 6241-6249

128. Harden, T. K., and Sondek, J. (2006) Regulation of phospholipase C isozymes by ras superfamily GTPases. *Annu Rev Pharmacol Toxicol* **46**, 355-379

129. Bai, Y., Edamatsu, H., Maeda, S., Saito, H., Suzuki, N., Satoh, T., and Kataoka, T. (2004) Crucial role of phospholipase Cepsilon in chemical carcinogen-induced skin tumor development. *Cancer research* **64**, 8808-8810

130. Ikuta, S., Edamatsu, H., Li, M., Hu, L., and Kataoka, T. (2008) Crucial role of phospholipase C epsilon in skin inflammation induced by tumor-promoting phorbol ester. *Cancer research* **68**, 64-72

131. Jin, T. G., Satoh, T., Liao, Y., Song, C., Gao, X., Kariya, K., Hu, C. D., and Kataoka, T. (2001) Role of the CDC25 homology domain of phospholipase Cepsilon in amplification of Rap1-dependent signaling. *The Journal of biological chemistry* **276**, 30301-30307

132. Kelley, G. G., Reks, S. E., Ondrako, J. M., and Smrcka, A. V. (2001) Phospholipase C(epsilon): a novel Ras effector. *The EMBO journal* **20**, 743-754

133. Habets, G. G., Scholtes, E. H., Zuydgeest, D., van der Kammen, R. A., Stam, J. C., Berns, A., and Collard, J. G. (1994) Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. *Cell* **77**, 537-549

134. Huang, J., Ye, X., Guan, J., Chen, B., Li, Q., Zheng, X., Liu, L., Wang, S., Ding, Y., Ding, Y., and Chen, L. (2013) Tiam1 is associated with hepatocellular carcinoma metastasis. *International journal of cancer. Journal international du cancer* **132**, 90-100

135. Ding, Y., Chen, B., Wang, S., Zhao, L., Chen, J., Ding, Y., Chen, L., and Luo, R. (2009) Over-expression of Tiam1 in hepatocellular carcinomas predicts poor prognosis of HCC patients. *International journal of cancer. Journal international du cancer* **124**, 653-658

136. Michiels, F., Habets, G. G., Stam, J. C., van der Kammen, R. A., and Collard, J. G. (1995) A role for Rac in Tiam1-induced membrane ruffling and invasion. *Nature* **375**, 338-340

137. Minard, M. E., Kim, L. S., Price, J. E., and Gallick, G. E. (2004) The role of the guanine nucleotide exchange factor Tiam1 in cellular migration, invasion, adhesion and tumor progression. *Breast Cancer Res Treat* **84**, 21-32

138. Liu, H., Shi, G., Liu, X., Wu, H., Fan, Q., and Wang, X. (2011) Over-expression of Tiam1 predicts poor prognosis in patients with esophageal squamous cell carcinoma. *Oncol Rep* **25**, 841-848

139. Wang, S., Li, S., Tang, Q., Yang, S., Wang, S., Liu, J., Yang, M., and Yang, X. (2015) Over-expression of Tiam1 promotes the progression of laryngeal squamous cell carcinoma. *Oncol Rep* **33**, 1807-1814

140. Lambert, J. M., Lambert, Q. T., Reuther, G. W., Malliri, A., Siderovski, D. P., Sondek, J., Collard, J. G., and Der, C. J. (2002) Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism. *Nat Cell Biol* **4**, 621-625

141. Colicelli, J., Nicolette, C., Birchmeier, C., Rodgers, L., Riggs, M., and Wigler, M. (1991) Expression of three mammalian cDNAs that interfere with RAS function in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 2913-2917

142. Han, L., and Colicelli, J. (1995) A human protein selected for interference with Ras function interacts directly with Ras and competes with Raf1. *Molecular and cellular biology* **15**, 1318-1323

143. Han, L., Wong, D., Dhaka, A., Afar, D., White, M., Xie, W., Herschman, H., Witte, O., and Colicelli, J. (1997) Protein binding and signaling properties of RIN1 suggest a unique effector function. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 4954-4959

144. Wang, Y., Waldron, R. T., Dhaka, A., Patel, A., Riley, M. M., Rozengurt, E., and Colicelli, J. (2002) The RAS effector RIN1 directly competes with RAF and is regulated by 14-3-3 proteins. *Molecular and cellular biology* **22**, 916-926

145. Tall, G. G., Barbieri, M. A., Stahl, P. D., and Horazdovsky, B. F. (2001) Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. *Dev Cell* **1**, 73-82

146. Milstein, M., Mooser, C. K., Hu, H., Fejzo, M., Slamon, D., Goodglick, L., Dry, S., and Colicelli, J. (2007) RIN1 is a breast tumor suppressor gene. *Cancer research* **67**, 11510-11516

147. Hueber, A. O., and Evan, G. I. (1998) Traps to catch unwary oncogenes. *Trends Genet* **14**, 364-367

148. Cox, A. D., and Der, C. J. (2003) The dark side of Ras: regulation of apoptosis. *Oncogene* **22**, 8999-9006

149. Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997) Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544-548

150. Chen, C. Y., and Faller, D. V. (1995) Direction of p21ras-generated signals towards cell growth or apoptosis is determined by protein kinase C and Bcl-2. *Oncogene* **11**, 1487-1498

151. Chen, C. Y., Liou, J., Forman, L. W., and Faller, D. V. (1998) Differential regulation of discrete apoptotic pathways by Ras. *The Journal of biological chemistry* **273**, 16700-16709

152. di Jeso, B., Ulianich, L., Racioppi, L., D'Armiento, F., Feliciello, A., Pacifico, F., Consiglio, E., and Formisano, S. (1995) Serum withdrawal induces apoptotic cell death in Ki-ras transformed but not in normal differentiated thyroid cells. *Biochem Biophys Res Commun* **214**, 819-824

153. Parrizas, M., Blakesley, V. A., Beitner-Johnson, D., and Le Roith, D. (1997) The proto-oncogene Crk-II enhances apoptosis by a Ras-dependent, Raf-1/MAP kinase-independent pathway. *Biochem Biophys Res Commun* **234**, 616-620

154. Hayflick, L. (1965) The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Experimental cell research* **37**, 614-636

155. Campisi, J., and d'Adda di Fagagna, F. (2007) Cellular senescence: when bad things happen to good cells. *Nature reviews. Molecular cell biology* **8**, 729-740

156. Hayflick, L., and Moorhead, P. S. (1961) The serial cultivation of human diploid cell strains. *Experimental cell research* **25**, 585-621

157. Campisi, J. (1996) Replicative senescence: an old lives' tale? *Cell* **84**, 497-500

158. Dimauro, T., and David, G. (2010) Ras-induced senescence and its physiological relevance in cancer. *Curr Cancer Drug Targets* **10**, 869-876

159. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593-602

160. Dimri, G. P., Itahana, K., Acosta, M., and Campisi, J. (2000) Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor. *Molecular and cellular biology* **20**, 273-285

161. Zhu, J., Woods, D., McMahon, M., and Bishop, J. M. (1998) Senescence of human fibroblasts induced by oncogenic Raf. *Genes & development* **12**, 2997-3007

162. Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V., Kolettas, E., Niforou, K., Zoumpourlis, V. C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C. L., Dyrskjot, L., Orntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T. D., Bartek, J., and Gorgoulis, V. G. (2006) Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* **444**, 633-637

163. Bianchi-Smiraglia, A., and Nikiforov, M. A. (2012) Controversial aspects of oncogene-induced senescence. *Cell Cycle* **11**, 4147-4151

164. Kuilman, T., Michaloglou, C., Mooi, W. J., and Peeper, D. S. (2010) The essence of senescence. *Genes & development* **24**, 2463-2479

165. Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R. A. (1996) Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85**, 27-37

166. Tanaka, N., Ishihara, M., Kitagawa, M., Harada, H., Kimura, T., Matsuyama, T., Lamphier, M. S., Aizawa, S., Mak, T. W., and Taniguchi, T. (1994) Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell* **77**, 829-839

167. Wei, W., Hemmer, R. M., and Sedivy, J. M. (2001) Role of p14(ARF) in replicative and induced senescence of human fibroblasts. *Molecular and cellular biology* **21**, 6748-6757

168. Wei, W., Herbig, U., Wei, S., Dutriaux, A., and Sedivy, J. M. (2003) Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts. *EMBO reports* **4**, 1061-1066

169. Courtois-Cox, S., Jones, S. L., and Cichowski, K. (2008) Many roads lead to oncogene-induced senescence. *Oncogene* **27**, 2801-2809

170. Vos, M. D., Ellis, C. A., Bell, A., Birrer, M. J., and Clark, G. J. (2000) Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. *The Journal of biological chemistry* **275**, 35669-35672

171. Gordon, M., and Baksh, S. (2011) RASSF1A: Not a prototypical Ras effector. *Small GTPases* **2**, 148-157

172. Richter, A. M., Pfeifer, G. P., and Dammann, R. H. (2009) The RASSF proteins in cancer; from epigenetic silencing to functional characterization. *Biochimica et biophysica acta* **1796**, 114-128

173. Fujita, H., Fukuhara, S., Sakurai, A., Yamagishi, A., Kamioka, Y., Nakaoka, Y., Masuda, M., and Mochizuki, N. (2005) Local activation of Rap1 contributes to directional vascular endothelial cell migration accompanied by extension of microtubules on which RAPL, a Rap1-associating molecule, localizes. *The Journal of biological chemistry* **280**, 5022-5031

174. Eckfeld, K., Hesson, L., Vos, M. D., Bieche, I., Latif, F., and Clark, G. J. (2004) RASSF4/AD037 is a potential ras effector/tumor suppressor of the RASSF family. *Cancer research* **64**, 8688-8693

175. Matallanas, D., Romano, D., Al-Mulla, F., O'Neill, E., Al-Ali, W., Crespo, P., Doyle, B., Nixon, C., Sansom, O., Drosten, M., Barbacid, M., and Kolch, W. (2011) Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wild-type K-Ras. *Molecular cell* **44**, 893-906

176. Ponting, C. P., and Benjamin, D. R. (1996) A novel family of Ras-binding domains. *Trends in biochemical sciences* **21**, 422-425

177. Volodko, N., Gordon, M., Salla, M., Ghazaleh, H. A., and Baksh, S. (2014) RASSF tumor suppressor gene family: biological functions and regulation. *FEBS Lett* **588**, 2671-2684

178. Avruch, J., Xavier, R., Bardeesy, N., Zhang, X. F., Praskova, M., Zhou, D., and Xia, F. (2009) Rassf family of tumor suppressor polypeptides. *The Journal of biological chemistry* **284**, 11001-11005

179. Lerman, M. I., and Minna, J. D. (2000) The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. *Cancer research* **60**, 6116-6133

180. Dammann, R., Li, C., Yoon, J. H., Chin, P. L., Bates, S., and Pfeifer, G. P. (2000) Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* **25**, 315-319

181. Tommasi, S., Dammann, R., Zhang, Z., Wang, Y., Liu, L., Tsark, W. M., Wilczynski, S. P., Li, J., You, M., and Pfeifer, G. P. (2005) Tumor susceptibility of Rassf1a knockout mice. *Cancer research* **65**, 92-98

182. Dammann, R., Schagdarsurengin, U., Seidel, C., Strunnikova, M., Rastetter, M., Baier, K., and Pfeifer, G. P. (2005) The tumor suppressor RASSF1A in human carcinogenesis: an update. *Histol Histopathol* **20**, 645-663

183. Pan, Z. G., Kashuba, V. I., Liu, X. Q., Shao, J. Y., Zhang, R. H., Jiang, J. H., Guo, C., Zabarovsky, E., Ernberg, I., and Zeng, Y. X. (2005) High frequency somatic mutations in RASSF1A in nasopharyngeal carcinoma. *Cancer biology & therapy* **4**, 1116-1122

184. Shivakumar, L., Minna, J., Sakamaki, T., Pestell, R., and White, M. A. (2002) The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Molecular and cellular biology* **22**, 4309-4318

185. Whang, Y. M., Kim, Y. H., Kim, J. S., and Yoo, Y. D. (2005) RASSF1A suppresses the c-Jun-NH2-kinase pathway and inhibits cell cycle progression. *Cancer research* **65**, 3682-3690

186. Xie, R., Nguyen, S., McKeehan, K., Wang, F., McKeehan, W. L., and Liu, L. (2011) Microtubule-associated protein 1S (MAP1S) bridges autophagic components with microtubules and mitochondria to affect autophagosomal biogenesis and degradation. *The Journal of biological chemistry* **286**, 10367-10377

187. Donninger, H., Clark, J., Rinaldo, F., Nelson, N., Barnoud, T., Schmidt, M. L., Hobbing, K. R., Vos, M. D., Sils, B., and Clark, G. J. (2015) The RASSF1A

tumor suppressor regulates XPA-mediated DNA repair. *Molecular and cellular biology* **35**, 277-287

188. Agathanggelou, A., Cooper, W. N., and Latif, F. (2005) Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer research* **65**, 3497-3508

189. Steiner, G., Cairns, P., Polascik, T. J., Marshall, F. F., Epstein, J. I., Sidransky, D., and Schoenberg, M. (1996) High-density mapping of chromosomal arm 1q in renal collecting duct carcinoma: region of minimal deletion at 1q32.1-32.2. *Cancer research* **56**, 5044-5046

190. Wohlgemuth, S., Kiel, C., Kramer, A., Serrano, L., Wittinghofer, F., and Herrmann, C. (2005) Recognizing and defining true Ras binding domains I: biochemical analysis. *J Mol Biol* **348**, 741-758

191. Calvisi, D. F., Donninger, H., Vos, M. D., Birrer, M. J., Gordon, L., Leaner, V., and Clark, G. J. (2009) NORE1A tumor suppressor candidate modulates p21CIP1 via p53. *Cancer research* **69**, 4629-4637

192. Avruch, J., Praskova, M., Ortiz-Vega, S., Liu, M., and Zhang, X. F. (2006) Nore1 and RASSF1 regulation of cell proliferation and of the MST1/2 kinases. *Methods Enzymol* **407**, 290-310

193. Yu, F. X., and Guan, K. L. (2013) The Hippo pathway: regulators and regulations. *Genes & development* **27**, 355-371

194. Schmidt, M. L., Donninger, H., and Clark, G. J. (2014) Ras regulates SCF(beta-TrCP) protein activity and specificity via its effector protein NORE1A. *The Journal of biological chemistry* **289**, 31102-31110

195. Saifo, M. S., Rempinski, D. R., Jr., Rustum, Y. M., and Azrak, R. G. (2010) Targeting the oncogenic protein beta-catenin to enhance chemotherapy outcome against solid human cancers. *Mol Cancer* **9**, 310

196. Campisi, J. (2000) Cancer, aging and cellular senescence. *In Vivo* **14**, 183-188

197. Gartel, A. L., Serfas, M. S., and Tyner, A. L. (1996) p21--negative regulator of the cell cycle. *Proc Soc Exp Biol Med* **213**, 138-149

198. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**, 817-825

199. Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323**, 643-646

200. Viatour, P., and Sage, J. (2011) Newly identified aspects of tumor suppression by RB. *Dis Model Mech* **4**, 581-585

201. Mastrangelo, D., Hadjistilianou, T., De Francesco, S., and Lore, C. (2009) Retinoblastoma and the genetic theory of cancer: an old paradigm trying to survive to the evidence. *J Cancer Epidemiol* **2009**, 301973

202. Knudson, A. G., Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proceedings of the National Academy of Sciences of the United States of America* **68**, 820-823

203. Dunn, J. M., Phillips, R. A., Becker, A. J., and Gallie, B. L. (1988) Identification of germline and somatic mutations affecting the retinoblastoma gene. *Science* **241**, 1797-1800

204. Harbour, J. W., Lai, S. L., Whang-Peng, J., Gazdar, A. F., Minna, J. D., and Kaye, F. J. (1988) Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* **241**, 353-357

205. Lohmann, D. R. (1999) RB1 gene mutations in retinoblastoma. *Hum Mutat* **14**, 283-288

206. Harbour, J. W., and Dean, D. C. (2000) The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes & development* **14**, 2393-2409

207. Sherr, C. J. (1996) Cancer cell cycles. *Science* **274**, 1672-1677

208. Weinberg, R. A. (1992) The retinoblastoma gene and gene product. *Cancer Surv* **12**, 43-57

209. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., and Livingston, D. M. (1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**, 275-283

210. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988) Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334**, 124-129

211. Dyson, N., Howley, P. M., Munger, K., and Harlow, E. (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**, 934-937

212. Sherr, C. J., and McCormick, F. (2002) The RB and p53 pathways in cancer. *Cancer cell* **2**, 103-112

213. Gabellini, C., Del Bufalo, D., and Zupi, G. (2006) Involvement of RB gene family in tumor angiogenesis. *Oncogene* **25**, 5326-5332

214. Sanseverino, F., Santopietro, R., Torricelli, M., D'Andrilli, G., Russo, G., Cevenini, G., Bovicelli, A., Leoncini, L., Scambia, G., Petraglia, F., Claudio, P. P., and Giordano, A. (2006) pRb2/p130 and VEGF expression in endometrial carcinoma in relation to angiogenesis and histopathologic tumor grade. *Cancer biology & therapy* **5**, 84-88

215. Chicas, A., Wang, X., Zhang, C., McCurrach, M., Zhao, Z., Mert, O., Dickins, R. A., Narita, M., Zhang, M., and Lowe, S. W. (2010) Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer cell* **17**, 376-387

216. Lundberg, A. S., and Weinberg, R. A. (1999) Control of the cell cycle and apoptosis. *European journal of cancer* **35**, 1886-1894

217. Weinberg, R. A. (1995) The retinoblastoma protein and cell cycle control. *Cell* **81**, 323-330

218. Claudio, P. P., De Luca, A., Howard, C. M., Baldi, A., Firpo, E. J., Koff, A., Paggi, M. G., and Giordano, A. (1996) Functional analysis of pRb2/p130 interaction with cyclins. *Cancer research* **56**, 2003-2008

219. Claudio, P. P., Tonini, T., and Giordano, A. (2002) The retinoblastoma family: twins or distant cousins? *Genome Biol* **3**, reviews3012

220. Adams, P. D., and Kaelin, W. G., Jr. (1995) Transcriptional control by E2F. *Seminars in cancer biology* **6**, 99-108

221. Qian, Y., Luckey, C., Horton, L., Esser, M., and Templeton, D. J. (1992) Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. *Molecular and cellular biology* **12**, 5363-5372

222. Giacinti, C., and Giordano, A. (2006) RB and cell cycle progression. *Oncogene* **25**, 5220-5227

223. Shao, Z., and Robbins, P. D. (1995) Differential regulation of E2F and Sp1-mediated transcription by G1 cyclins. *Oncogene* **10**, 221-228

224. Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998) Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391**, 601-605

225. Zhang, H. S., Gavin, M., Dahiya, A., Postigo, A. A., Ma, D., Luo, R. X., Harbour, J. W., and Dean, D. C. (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* **101**, 79-89

226. Sage, J., Mulligan, G. J., Attardi, L. D., Miller, A., Chen, S., Williams, B., Theodorou, E., and Jacks, T. (2000) Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes & development* **14**, 3037-3050

227. Peeper, D. S., Dannenberg, J. H., Douma, S., te Riele, H., and Bernards, R. (2001) Escape from premature senescence is not sufficient for oncogenic transformation by Ras. *Nat Cell Biol* **3**, 198-203

228. Rangarajan, A., and Weinberg, R. A. (2003) Opinion: Comparative biology of mouse versus human cells: modelling human cancer in mice. *Nature reviews. Cancer* **3**, 952-959

229. Sherr, C. J., and Roberts, J. M. (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes & development* **9**, 1149-1163

230. Wei, W., Jobling, W. A., Chen, W., Hahn, W. C., and Sedivy, J. M. (2003) Abolition of cyclin-dependent kinase inhibitor p16Ink4a and p21Cip1/Waf1 functions permits Ras-induced anchorage-independent growth in telomerase-immortalized human fibroblasts. *Molecular and cellular biology* **23**, 2859-2870

231. Kolupaeva, V., and Janssens, V. (2013) PP1 and PP2A phosphatases--cooperating partners in modulating retinoblastoma protein activation. *The FEBS journal* **280**, 627-643

232. Alberts, A. S., Thorburn, A. M., Shenolikar, S., Mumby, M. C., and Feramisco, J. R. (1993) Regulation of cell cycle progression and nuclear affinity of the retinoblastoma protein by protein phosphatases. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 388-392

233. Ludlow, J. W., Glendening, C. L., Livingston, D. M., and DeCarprio, J. A. (1993) Specific enzymatic dephosphorylation of the retinoblastoma protein. *Molecular and cellular biology* **13**, 367-372

234. Yan, Y., and Mumby, M. C. (1999) Distinct roles for PP1 and PP2A in phosphorylation of the retinoblastoma protein. PP2a regulates the activities of G(1) cyclin-dependent kinases. *The Journal of biological chemistry* **274**, 31917-31924

235. Rubin, E., Mittnacht, S., Villa-Moruzzi, E., and Ludlow, J. W. (2001) Site-specific and temporally-regulated retinoblastoma protein dephosphorylation by protein phosphatase type 1. *Oncogene* **20**, 3776-3785

236. Wang, R. H., Liu, C. W., Avramis, V. I., and Berndt, N. (2001) Protein phosphatase 1alpha-mediated stimulation of apoptosis is associated with dephosphorylation of the retinoblastoma protein. *Oncogene* **20**, 6111-6122

237. Ayllon, V., Martinez, A. C., Garcia, A., Cayla, X., and Rebollo, A. (2000) Protein phosphatase 1alpha is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. *The EMBO journal* **19**, 2237-2246

238. Rajesh, D., Schell, K., and Verma, A. K. (1999) Ras mutation, irrespective of cell type and p53 status, determines a cell's destiny to undergo apoptosis by okadaic acid, an inhibitor of protein phosphatase 1 and 2A. *Mol Pharmacol* **56**, 515-525

239. Ellis, C. A., Vos, M. D., Howell, H., Vallecorsa, T., Fults, D. W., and Clark, G. J. (2002) Rig is a novel Ras-related protein and potential neural tumor suppressor. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 9876-9881

240. Michaud, K., Solomon, D. A., Oermann, E., Kim, J. S., Zhong, W. Z., Prados, M. D., Ozawa, T., James, C. D., and Waldman, T. (2010) Pharmacologic inhibition of cyclin-dependent kinases 4 and 6 arrests the growth of glioblastoma multiforme intracranial xenografts. *Cancer research* **70**, 3228-3238

241. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**, 59-74

242. Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175-182

243. Ferraz da Costa, D. C., Casanova, F. A., Quarti, J., Malheiros, M. S., Sanches, D., Dos Santos, P. S., Fialho, E., and Silva, J. L. (2012) Transient transfection of a wild-type p53 gene triggers resveratrol-induced apoptosis in cancer cells. *PloS one* **7**, e48746

244. Ramirez, R. D., Sheridan, S., Girard, L., Sato, M., Kim, Y., Pollack, J., Peyton, M., Zou, Y., Kurie, J. M., Dimaio, J. M., Milchgrub, S., Smith, A. L., Souza, R. F., Gilbey, L., Zhang, X., Gandia, K., Vaughan, M. B., Wright, W. E., Gazdar, A. F., Shay, J. W., and Minna, J. D. (2004) Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. *Cancer research* **64**, 9027-9034

245. Desbois-Mouthon, C., Baron, A., Blivet-Van Eggelpoel, M. J., Fartoux, L., Venot, C., Bladt, F., Housset, C., and Rosmorduc, O. (2009) Insulin-like growth factor-1 receptor inhibition induces a resistance mechanism via the epidermal growth factor receptor/HER3/AKT signaling pathway: rational basis for cotargeting insulin-like growth factor-1 receptor and epidermal growth factor receptor in hepatocellular carcinoma. *Clin Cancer Res* **15**, 5445-5456

246. Collado, M., and Serrano, M. (2010) Senescence in tumours: evidence from mice and humans. *Nature reviews. Cancer* **10**, 51-57

247. DeNicola, G. M., and Tuveson, D. A. (2009) RAS in cellular transformation and senescence. *European journal of cancer* **45 Suppl 1**, 211-216

248. Malumbres, M., and Barbacid, M. (2003) RAS oncogenes: the first 30 years. *Nature reviews. Cancer* **3**, 459-465

249. Ferbeyre, G., de Stanchina, E., Lin, A. W., Querido, E., McCurrach, M. E., Hannon, G. J., and Lowe, S. W. (2002) Oncogenic ras and p53 cooperate to induce cellular senescence. *Molecular and cellular biology* **22**, 3497-3508

250. Ben-Porath, I., and Weinberg, R. A. (2005) The signals and pathways activating cellular senescence. *The international journal of biochemistry & cell biology* **37**, 961-976

251. Gao, J., Huang, H. Y., Pak, J., Cheng, J., Zhang, Z. T., Shapiro, E., Pellicer, A., Sun, T. T., and Wu, X. R. (2004) p53 deficiency provokes urothelial proliferation and synergizes with activated Ha-ras in promoting urothelial tumorigenesis. *Oncogene* **23**, 687-696

252. Carriere, C., Gore, A. J., Norris, A. M., Gunn, J. R., Young, A. L., Longnecker, D. S., and Korc, M. (2011) Deletion of Rb accelerates pancreatic carcinogenesis by oncogenic Kras and impairs senescence in premalignant lesions. *Gastroenterology* **141**, 1091-1101

253. Smogorzewska, A., and de Lange, T. (2002) Different telomere damage signaling pathways in human and mouse cells. *The EMBO journal* **21**, 4338-4348

254. Yamamoto, T., Taya, S., and Kaibuchi, K. (1999) Ras-induced transformation and signaling pathway. *Journal of biochemistry* **126**, 799-803

255. Park, J., Kang, S. I., Lee, S. Y., Zhang, X. F., Kim, M. S., Beers, L. F., Lim, D. S., Avruch, J., Kim, H. S., and Lee, S. B. (2010) Tumor suppressor ras association

domain family 5 (RASSF5/NORE1) mediates death receptor ligand-induced apoptosis. *The Journal of biological chemistry* **285**, 35029-35038

256. Calvisi, D. F., Pinna, F., Pellegrino, R., Sanna, V., Sini, M., Daino, L., Simile, M. M., De Miglio, M. R., Frau, M., Tomasi, M. L., Seddaiu, M. A., Muroni, M. R., Feo, F., and Pascale, R. M. (2008) Ras-driven proliferation and apoptosis signaling during rat liver carcinogenesis is under genetic control. *International journal of cancer. Journal international du cancer* **123**, 2057-2064

257. Benavente, C. A., and Dyer, M. A. (2015) Genetics and epigenetics of human retinoblastoma. *Annual review of pathology* **10**, 547-562

258. Doorbar, J. (2006) Molecular biology of human papillomavirus infection and cervical cancer. *Clinical science* **110**, 525-541

259. Meuwissen, R., Linn, S. C., Linnoila, R. I., Zevenhoven, J., Mooi, W. J., and Berns, A. (2003) Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model. *Cancer cell* **4**, 181-189

260. Dick, F. A., and Rubin, S. M. (2013) Molecular mechanisms underlying RB protein function. *Nature reviews. Molecular cell biology* **14**, 297-306

261. Zheng, L., and Lee, W. H. (2001) The retinoblastoma gene: a prototypic and multifunctional tumor suppressor. *Experimental cell research* **264**, 2-18

262. Dannenberg, J. H., and te Riele, H. P. (2006) The retinoblastoma gene family in cell cycle regulation and suppression of tumorigenesis. *Results and problems in cell differentiation* **42**, 183-225

263. Bollen, M., Gerlich, D. W., and Lesage, B. (2009) Mitotic phosphatases: from entry guards to exit guides. *Trends in cell biology* **19**, 531-541

264. Wurzenberger, C., and Gerlich, D. W. (2011) Phosphatases: providing safe passage through mitotic exit. *Nature reviews. Molecular cell biology* **12**, 469-482

265. Barr, F. A., Elliott, P. R., and Gruneberg, U. (2011) Protein phosphatases and the regulation of mitosis. *Journal of cell science* **124**, 2323-2334

266. Kumari, G., Singhal, P. K., Rao, M. R., and Mahalingam, S. (2007) Nuclear transport of Ras-associated tumor suppressor proteins: different transport receptor binding specificities for arginine-rich nuclear targeting signals. *J Mol Biol* **367**, 1294-1311

267. Trinkle-Mulcahy, L., Sleeman, J. E., and Lamond, A. I. (2001) Dynamic targeting of protein phosphatase 1 within the nuclei of living mammalian cells. *Journal of cell science* **114**, 4219-4228

268. Shcherbo, D., Merzlyak, E. M., Chepurnykh, T. V., Fradkov, A. F., Ermakova, G. V., Solovieva, E. A., Lukyanov, K. A., Bogdanova, E. A., Zaraisky, A. G., Lukyanov, S., and Chudakov, D. M. (2007) Bright far-red fluorescent protein for whole-body imaging. *Nat Methods* **4**, 741-746

269. Shcherbo, D., Murphy, C. S., Ermakova, G. V., Solovieva, E. A., Chepurnykh, T. V., Shcheglov, A. S., Verkhusha, V. V., Pletnev, V. Z., Hazelwood, K. L., Roche, P. M., Lukyanov, S., Zaraisky, A. G., Davidson, M. W., and Chudakov, D. M. (2009) Far-red fluorescent tags for protein imaging in living tissues. *The Biochemical journal* **418**, 567-574

270. Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., and Elledge, S. J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & development* **7**, 555-569

271. Klumpers, F., Gotz, U., Kurtz, T., Herrmann, C., and Gronewold, T. M. A. (2014) Conformational changes at protein-protein interaction followed online with an SAW biosensor. *Sensors and Actuators B: Chemical* **203**, 904-908

272. De Leon, G., Sherry, T. C., and Krucher, N. A. (2008) Reduced expression of PNUTS leads to activation of Rb-phosphatase and caspase-mediated apoptosis. *Cancer biology & therapy* **7**, 833-841

273. Shinmura, K., Tao, H., Nagura, K., Goto, M., Matsuura, S., Mochizuki, T., Suzuki, K., Tanahashi, M., Niwa, H., Ogawa, H., and Sugimura, H. (2011) Suppression of hydroxyurea-induced centrosome amplification by NORE1A and down-regulation of NORE1A mRNA expression in non-small cell lung carcinoma. *Lung Cancer* **71**, 19-27

274. Lowe, S. W., Cepero, E., and Evan, G. (2004) Intrinsic tumour suppression. *Nature* **432**, 307-315

275. Overmeyer, J. H., and Maltese, W. A. (2011) Death pathways triggered by activated Ras in cancer cells. *Frontiers in bioscience* **16**, 1693-1713

276. Morton, J. P., Timpson, P., Karim, S. A., Ridgway, R. A., Athineos, D., Doyle, B., Jamieson, N. B., Oien, K. A., Lowy, A. M., Brunton, V. G., Frame, M. C., Evans, T. R., and Sansom, O. J. (2010) Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 246-251

277. Chen, X., Mitsutake, N., LaPerle, K., Akeno, N., Zanzonico, P., Longo, V. A., Mitsutake, S., Kimura, E. T., Geiger, H., Santos, E., Wendel, H. G., Franco, A., Knauf, J. A., and Fagin, J. A. (2009) Endogenous expression of Hras(G12V)

induces developmental defects and neoplasms with copy number imbalances of the oncogene. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 7979-7984

278. Shay, J. W., Pereira-Smith, O. M., and Wright, W. E. (1991) A role for both RB and p53 in the regulation of human cellular senescence. *Experimental cell research* **196**, 33-39

279. Chen, Q. M., Bartholomew, J. C., Campisi, J., Acosta, M., Reagan, J. D., and Ames, B. N. (1998) Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *The Biochemical journal* **332 (Pt 1)**, 43-50

280. Praskova, M., Khoklatchev, A., Ortiz-Vega, S., and Avruch, J. (2004) Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. *The Biochemical journal* **381**, 453-462

281. Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., and Pelicci, P. G. (2000) PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. *Nature* **406**, 207-210

282. Tamrakar, S., Mittnacht, S., and Ludlow, J. W. (1999) Binding of select forms of pRB to protein phosphatase type 1 independent of catalytic activity. *Oncogene* **18**, 7803-7809

283. Tamrakar, S., and Ludlow, J. W. (2000) The carboxyl-terminal region of the retinoblastoma protein binds non-competitively to protein phosphatase type

1alpha and inhibits catalytic activity. *The Journal of biological chemistry* **275**, 27784-27789

284. Contente, S., Yeh, T. J., and Friedman, R. M. (2011) H-ras localizes to cell nuclei and varies with the cell cycle. *Genes Cancer* **2**, 166-172

285. Wurzer, G., Mosgoeller, W., Chabicovsky, M., Cerni, C., and Wesierska-Gadek, J. (2001) Nuclear Ras: unexpected subcellular distribution of oncogenic forms. *J Cell Biochem Suppl* **Suppl 36**, 1-11

286. Sdek, P., Ying, H., Chang, D. L., Qiu, W., Zheng, H., Touitou, R., Allday, M. J., and Xiao, Z. X. (2005) MDM2 promotes proteasome-dependent ubiquitin-independent degradation of retinoblastoma protein. *Molecular cell* **20**, 699-708

287. Lee, S. J., Lim, C. J., Min, J. K., Lee, J. K., Kim, Y. M., Lee, J. Y., Won, M. H., and Kwon, Y. G. (2007) Protein phosphatase 1 nuclear targeting subunit is a hypoxia inducible gene: its role in post-translational modification of p53 and MDM2. *Cell death and differentiation* **14**, 1106-1116

288. Lee, D., Park, S. J., Sung, K. S., Park, J., Lee, S. B., Park, S. Y., Lee, H. J., Ahn, J. W., Choi, S. J., Lee, S. G., Kim, S. H., Kim, D. H., Kim, J., Kim, Y., and Choi, C. Y. (2012) Mdm2 associates with Ras effector NORE1 to induce the degradation of oncoprotein HIPK1. *EMBO reports* **13**, 163-169

289. Feig, L. A., and Buchsbaum, R. J. (2002) Cell signaling: life or death decisions of ras proteins. *Current biology : CB* **12**, R259-261

290. D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., Piaggio, G., Fanciulli, M.,

Appella, E., and Soddu, S. (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nat Cell Biol* **4**, 11-19

291. Hofmann, T. G., Moller, A., Sirma, H., Zentgraf, H., Taya, Y., Droge, W., Will, H., and Schmitz, M. L. (2002) Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. *Nat Cell Biol* **4**, 1-10

292. Di Stefano, V., Mattiussi, M., Sacchi, A., and D'Orazi, G. (2005) HIPK2 inhibits both MDM2 gene and protein by, respectively, p53-dependent and independent regulations. *FEBS Lett* **579**, 5473-5480

293. Di Stefano, V., Soddu, S., Sacchi, A., and D'Orazi, G. (2005) HIPK2 contributes to PCAF-mediated p53 acetylation and selective transactivation of p21Waf1 after nonapoptotic DNA damage. *Oncogene* **24**, 5431-5442

294. Puca, R., Nardinocchi, L., Givol, D., and D'Orazi, G. (2010) Regulation of p53 activity by HIPK2: molecular mechanisms and therapeutical implications in human cancer cells. *Oncogene* **29**, 4378-4387

295. Taira, N., and Yoshida, K. (2012) Post-translational modifications of p53 tumor suppressor: determinants of its functional targets. *Histol Histopathol* **27**, 437-443

296. Macdonald, J. I., and Dick, F. A. (2012) Posttranslational modifications of the retinoblastoma tumor suppressor protein as determinants of function. *Genes Cancer* **3**, 619-633

297. Chan, H. M., Krstic-Demonacos, M., Smith, L., Demonacos, C., and La Thangue, N. B. (2001) Acetylation control of the retinoblastoma tumour-suppressor protein. *Nat Cell Biol* **3**, 667-674

298. Nguyen, D. X., Baglia, L. A., Huang, S. M., Baker, C. M., and McCance, D. J. (2004) Acetylation regulates the differentiation-specific functions of the retinoblastoma protein. *The EMBO journal* **23**, 1609-1618

299. Leduc, C., Claverie, P., Eymin, B., Col, E., Khochbin, S., Brambilla, E., and Gazzeri, S. (2006) p14ARF promotes RB accumulation through inhibition of its Tip60-dependent acetylation. *Oncogene* **25**, 4147-4154

300. Vaquero, A., Scher, M., Lee, D., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2004) Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Molecular cell* **16**, 93-105

301. Luo, J., Nikolaev, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001) Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* **107**, 137-148

302. Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001) hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* **107**, 149-159

303. Langley, E., Pearson, M., Faretta, M., Bauer, U. M., Frye, R. A., Minucci, S., Pelicci, P. G., and Kouzarides, T. (2002) Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. *The EMBO journal* **21**, 2383-2396

304. Wong, S., and Weber, J. D. (2007) Deacetylation of the retinoblastoma tumour suppressor protein by SIRT1. *The Biochemical journal* **407**, 451-460

305. Ledl, A., Schmidt, D., and Muller, S. (2005) Viral oncoproteins E1A and E7 and cellular LxCxE proteins repress SUMO modification of the retinoblastoma tumor suppressor. *Oncogene* **24**, 3810-3818

306. Li, T., Santockyte, R., Shen, R. F., Tekle, E., Wang, G., Yang, D. C., and Chock, P. B. (2006) Expression of SUMO-2/3 induced senescence through p53- and pRB-mediated pathways. *The Journal of biological chemistry* **281**, 36221-36227

307. Andreou, A. M., and Tavernarakis, N. (2010) Roles for SUMO modification during senescence. *Adv Exp Med Biol* **694**, 160-171

308. Johnson, E. S. (2004) Protein modification by SUMO. *Annu Rev Biochem* **73**, 355-382

309. Schmidt, D., and Muller, S. (2003) PIAS/SUMO: new partners in transcriptional regulation. *Cell Mol Life Sci* **60**, 2561-2574

310. Bischof, O., Schwamborn, K., Martin, N., Werner, A., Sustmann, C., Grosschedl, R., and Dejean, A. (2006) The E3 SUMO ligase PIASy is a regulator of cellular senescence and apoptosis. *Molecular cell* **22**, 783-794

311. Kagey, M. H., Melhuish, T. A., and Wotton, D. (2003) The polycomb protein Pc2 is a SUMO E3. *Cell* **113**, 127-137

312. Wotton, D., and Merrill, J. C. (2007) Pc2 and SUMOylation. *Biochem Soc Trans* **35**, 1401-1404

313. Roscic, A., Moller, A., Calzado, M. A., Renner, F., Wimmer, V. C., Gresko, E., Ludi, K. S., and Schmitz, M. L. (2006) Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Molecular cell* **24**, 77-89

314. Singh, B. N., Zhang, G., Hwa, Y. L., Li, J., Dowdy, S. C., and Jiang, S. W. (2010) Nonhistone protein acetylation as cancer therapy targets. *Expert Rev Anticancer Ther* **10**, 935-954

315. Boyer, S. N., Wazer, D. E., and Band, V. (1996) E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer research* **56**, 4620-4624

316. Wang, J., Sampath, A., Raychaudhuri, P., and Bagchi, S. (2001) Both Rb and E7 are regulated by the ubiquitin proteasome pathway in HPV-containing cervical tumor cells. *Oncogene* **20**, 4740-4749

317. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* **325**, 834-840

318. Sasca, D., Hahnel, P. S., Szybinski, J., Khawaja, K., Kriege, O., Pante, S. V., Bullinger, L., Strand, S., Strand, D., Theobald, M., and Kindler, T. (2014) SIRT1 prevents genotoxic stress-induced p53 activation in acute myeloid leukemia. *Blood* **124**, 121-133

319. Choudhary, S., and Wang, H. C. (2007) Proapoptotic ability of oncogenic H-Ras to facilitate apoptosis induced by histone deacetylase inhibitors in human cancer cells. *Mol Cancer Ther* **6**, 1099-1111

320. Nardella, C., Clohessy, J. G., Alimonti, A., and Pandolfi, P. P. (2011) Pro-senescence therapy for cancer treatment. *Nature reviews. Cancer* **11**, 503-511

321. Komashko, V. M., and Farnham, P. J. (2010) 5-azacytidine treatment reorganizes genomic histone modification patterns. *Epigenetics* **5**, 229-240

322. Hatzimichael, E., and Crook, T. (2013) Cancer epigenetics: new therapies and new challenges. *J Drug Deliv* **2013**, 529312

323. Kuck, D., Caulfield, T., Lyko, F., and Medina-Franco, J. L. (2010) Nanaomycin A selectively inhibits DNMT3B and reactivates silenced tumor suppressor genes in human cancer cells. *Mol Cancer Ther* **9**, 3015-3023

324. Mezzanotte, J. J., Hill, V., Schmidt, M. L., Shinawi, T., Tommasi, S., Krex, D., Schackert, G., Pfeifer, G. P., Latif, F., and Clark, G. J. (2014) RASSF6 exhibits promoter hypermethylation in metastatic melanoma and inhibits invasion in melanoma cells. *Epigenetics* **9**, 1496-1503

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Donninger, H., Calvisi, D.F., **Barnoud, T.**, Clark, J., Schmidt, M.L., Vos, M.D., and Clark, G.J., *NORE1A is a Ras senescence effector that controls the apoptotic/senescent balance of p53 via HIPK2*. J Cell Biol, 2015. **208**(6): p. 777-89.

Donninger, H., Clark, J., Rinaldo, F., Nelson, N., **Barnoud, T.**, Schmidt, M.L., Hobbing, K.R., Vos, M.D., Sils, B., and Clark, G.J., *The RASSF1A tumor suppressor regulates XPA-mediated DNA repair*. Mol Cell Biol, 2015. **35**(1): p. 277-87.

Donninger, H., **Barnoud, T.**, Nelson, N., Kassler, S., Clark, J., Cummins, T.D., Powell, D.W., Nyante, S., Milikan, R.C., and Clark, G.J. *RASSF1A and the rs2073498 cancer associated SNP*. Front in Oncol, 2011. **1**(54): p.1-7.

POSTER

PRESENTATIONS: **Barnoud, T.**, Donninger, H., and Clark, G.J. NORE1A links Ras to Rb Mediated Senescence. **Ras Initiative Symposium.** Frederick, MD, USA, 2015.

Henderson, S., **Barnoud, T.**, Trent, J.O., Clark, J.G., and Donninger, H. Novel Small Molecule Inhibitors of IL-6 block the transformed phenotype. **James Graham Brown Cancer Center, 14th Annual Retreat.** Louisville, Kentucky, USA, 2014.

Barnoud, T., Donninger, H., and Clark, G.J. Ras Regulates Rb Mediated Senescence via NORE1A. **James Graham Brown Cancer Center, 14th Annual Retreat.** Louisville, Kentucky, USA, 2014.

Barnoud, T., Donninger, H., and Clark, G.J. NORE1A is a Ras Senescence Node. **James Graham Brown Cancer Center, 12th Annual Retreat.** Louisville, Kentucky, USA, 2013.

Barnoud, T., Donninger, H., Clark, J., Gordon, L., and Clark, G.J. Analysis of a Novel Interaction Between the Tumor Suppressor RASSF1A and ACF7. **James Graham Brown Cancer Center, 11th Annual Retreat** and **Bi-Annual Biochemistry and Molecular Biology Retreat.** Louisville, KY, USA, 2012.

Donninger, H., Clark, J., **Barnoud, T.**, Gordon, L., and Clark, G.J. NORE1A Regulates p53 Activity through HIPK2. **James Graham Brown Cancer Center, 11th Annual Retreat.** Louisville, KY, USA, 2012.

Donninger, H., Nelson, N., **Barnoud, T.**, Pogue, J., Kassler, S., Cummins, T.C., Powell, D., and Clark, G.J. Functional Characterization of the RASSF1A A133S Tumor-Associated SNP. **James Graham Brown Cancer Center, 10th Annual Retreat.** Louisville, KY, USA, 2011.

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