The discovery of a novel, Ras-mediated NORE1A/PMLIV tumor suppressor complex.

Jessica Mezzanotte Sharpe

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THE DISCOVERY OF A NOVEL, RAS-MEDIATED NORE1A/PMLIV TUMOR SUPPRESSOR COMPLEX

By:

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B.A., Vanderbilt University, 2010
M.S., University of Louisville, 2014

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THE DISCOVERY OF A NOVEL, RAS-MEDIATED NORE1A/PML IV TUMOR SUPPRESSOR COMPLEX

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A Dissertation Approved on

June 8, 2016

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DEDICATION

This dissertation is dedicated to my family for their endless love and support.
ACKNOWLEDGEMENTS

The common saying, “it takes a village to raise a child” is incredibly applicable to graduate students, although I would argue that it takes both a village and an entire university. I have to express my sincere thanks to everyone who has helped me along this journey, starting with my family. To my husband, Grayson Sharpe, thank you for your endless support, love, and encouragement. Thank you for sending me cat pictures when I was having a bad day, thank you for taking on all of the household maintenance while I was writing (and even before that), and thank you, most importantly, for being there always. To my mother, Madonna Mezzanotte, thank you for being my earliest cheerleader and for encouraging me to both pursue my dreams and to give everything 100%. To my father, John Mezzanotte, thank you for teaching me the value of hard work, for coaching me throughout my life, and for always being willing to listen when I have a problem. Thank you all for believing in me, even when I didn’t believe in myself. I also have to thank my “baby” brother, John Michael Mezzanotte. Thank you for always asking me what I’m working on and for listening, even when you have no idea what I’m talking about. To my grandparents, Mary and Jack Schafer and Rose Mezzanotte, thank you for your endless love, and for my grandfather, David Mezzanotte, who is no longer here, I miss you every day and love you so much. Thank you also to my in-laws and to my extended family for always encouraging me.

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ABSTRACT

THE DISCOVERY OF A NOVEL, RAS-MEDIATED NORE1A/PMLIV TUMOR SUPPRESSOR COMPLEX

Jessica Mezzanotte Sharpe

June 8, 2016

Ras is the most commonly activated oncogene in human cancer. Activated Ras drives cell growth and proliferation by activating multiple mitogenic signaling pathways. However, Ras also has the paradoxical ability to promote anti-growth, pro-apoptotic, and pro-senescent signaling. The signaling pathways of many of these biological effectors remain poorly defined. One group of proteins capable of promoting Ras-induced apoptosis and cell cycle arrest is the RASSF family of tumor suppressors. Novel Ras Effector 1A, or NORE1A, was the first member of this family discovered and is a bona fide tumor suppressor that is lost or inactivated in a number of different cancers. NORE1A promotes anti-growth properties of Ras and has recently been shown to act as a double barreled Ras senescence effector, driving Ras-induced senescence, or cell cycle arrest, by activating two major tumor suppressors, p53 and Rb. NORE1A interacts with proteins that promote post-translational modifications of p53 and Rb in small nuclear spots, and NORE1A alone also forms small dots in the nucleus. NORE1A’s localization in the nucleus has not been extensively studied, but the most famous protein that forms spots in the nucleus, referred to as nuclear bodies, is the Promyelocytic Leukemia protein, or PML. PML nuclear bodies are centers of protein regulation and post-translational modifications, and PML itself is a tumor suppressor that can be lost in certain cancers. PML is a critical component of Ras-induced senescence, and upon stimulation by activated Ras, PML nuclear bodies mediate the activation and post-
translational modifications of p53 and Rb, but the exact coordination of this process is unknown. Here, I show that NORE1A localizes to PML nuclear bodies and forms a novel, Ras-enhanced association with a specific isoform of PML, PMLIV. NORE1A promotes the recruitment of the senescence effectors p53 and Rb to PMLIV nuclear bodies. Moreover, the loss of PML expression prevents NORE1A from inducing senescence. Thus, NORE1A requires localization to PML, and specifically PMLIV, nuclear bodies to mediate its pro-senescent effects by promoting the localization of p53 and Rb to PMLIV, revealing a novel component of the PMLIV/Ras senescence signaling pathway.
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CHAPTER I

BACKGROUND AND INTRODUCTION

1.1 - Overview

Cancer is the second-leading cause of death in the United States, and approximately 40% of Americans will develop invasive cancer in their lifetime [1]. Although overall cancer mortality rates have declined since the 1990’s as a result of better screening techniques, which lead to earlier detection, and better treatment strategies, many cancers remain difficult or impossible to treat. As a result, over half a million people in the United States die from cancer each year, highlighting the urgent need for the development of better and novel therapeutic strategies and treatments [1]. In order for this to occur, a better understanding of tumor development and the intricate signaling processes that drive cancer progression is needed to develop the most effective targeting strategy for the disease.

The most commonly mutated genes in cancer are the Ras oncogenes; approximately thirty percent of human cancers contain activating point mutations of one of three Ras isoforms, H-, K-, or N-Ras [2]. Ras proteins, when activated, stimulate the signaling of a number of downstream pathways that promote cell growth, proliferation, and even invasion and metastasis. The three major pathways that Ras activates that mediate these functions are the MAP Kinase, the PI3K/AKT, and the RalGDS pathways [3, 4]. Functional studies have also shown that Ras drives tumor development and tumor maintenance through at least six different effector signaling pathways [5]. Surprisingly, however, studies dating back to the 1980’s showed that Ras could not transform cells alone [6, 7], and, in fact, the addition of activated Ras to some cells results in either apoptosis or a state of cell cycle arrest called senescence [8-11]. The mechanisms by
which Ras is able to induce cell death and inhibit tumorigenesis, however, remain relatively poorly characterized.

One group of proteins that is able to promote the pro-apoptotic and pro-senescent properties of Ras is the RASSF tumor suppressor family [12]. These proteins, which are often down regulated in cancer by epigenetic modifications, are hypothesized to act as molecular scaffolds by binding to Ras and mediating its interaction with other anti-growth signaling molecules [12]. The first member of this family discovered was Novel Ras Effector 1A, or NORE1A, which was later also named RASSF5. NORE1A can bind directly to Ras and is a bona fide tumor suppressor, as it often experiences epigenetic down regulation in tumors and experiences an inactivating translocation in a hereditary cancer syndrome [13-15]. NORE1A is also a potent mediator of Ras-induced senescence, as recent studies have shown that, under Ras stimulation, NORE1A can promote post-translational modifications of both the p53 and Rb tumor suppressors by binding to mediators of p53 acetylation and Rb phosphorylation [16, 17]. Ras thus activates senescence in part through its interaction with NORE1A by stimulating its interaction with and the activation of the p53 and Rb tumor suppressor pathways, both of which are major promoters of senescence [18].

Interestingly, another protein that is critical for Ras’s induction of senescence is the Promyelocytic Leukemia Protein, or PML [19, 20]. The PML gene can undergo alternative splicing to form seven PML protein isoforms that play various roles in the cell, and PML itself plays a major role in tumor suppression, as it is involved in mediating a number of important intracellular processes, including apoptosis and senescence [20, 21]. In fact, the loss of PML expression inhibits Ras-induced senescence, and activated Ras actually induces PML expression in the cell during the induction of senescence [19, 20]. PML forms specific structures in the cell nucleus called PML nuclear bodies (NBs), in which it scaffolds numerous proteins and mediates a number of important processes, including protein post-translational modifications [21]. Upon Ras stimulation, PML associates with both p53 and Rb in the NBs and mediates pro-senescent post-translational modifications of the two, thus promoting senescence [19, 20]. PML is therefore a critical mediator of Ras-induced senescence, and a specific PML isoform, PMLIV, is the only PML...
isoform capable of promoting senescence [20, 22]. The exact mechanism linking Ras to PMLIV-induced senescence, however, is unclear.

Using a number of different biological techniques, I discovered a novel interaction of NORE1A with PMLIV. I found that NORE1A and PMLIV co-localize in cells and associate with one another in a Ras-enhanced manner. Although NORE1A does not alter PML’s stability within the cell, it does promote PML acetylation, and NORE1A appears to promote the recruitment of p53 and Rb, two proteins that are critical mediators of Ras-induced senescence, to PMLIV. PML also appears to be a critical mediator of NORE1A-induced senescence, as cells lacking PML are resistant to senescence induced by NORE1A.

The interaction of these two proteins has numerous implications, especially since PML NBs mediate a number of important processes within the cell, and as a result, PML interacts with a wide range of proteins with diverse roles in tumor suppression [21]. PML NBs are centers of protein post-translational modifications, and NORE1A has recently been shown to play a role in protein acetylation—specifically, the acetylation of p53—phosphorylation, and ubiquitination [16, 17, 21, 23]. Therefore, Ras’s promotion of the interaction of NORE1A with PML, specifically the isoform PMLIV, could provide a novel mechanism by which Ras regulates protein function through both protein localization and post-translational modifications.

1.2 - The Ras Oncogene

Discovery

The origins of Ras research began with research on acutely transforming retroviruses. In the 1960’s, the Harvey murine sarcoma virus and the Kirsten murine sarcoma virus gave the first glimpse of the potent HRAS and KRAS oncogenes (For a timeline of milestones in Ras research, see Figure 1.) [3, 24, 25]. This began with research performed by Jennifer Harvey, who found that a sample of purified murine leukemia virus induced the formation of sarcomas in mice [24]. This virus, along with the Kirsten viral strain, was also able to transform cells in culture [3]. In the
1970’s, Ed Scolnick and colleagues began to determine the tumorigeneic potential of these viruses. They determined that the Harvey and Kirsten murine viral strains were actually recombinant viruses that contained sequences collected from the rat genome [26, 27]. Scholnick predicted that these viruses were actually inducing transformation through the normal cellular rat sequences contained in the recombinant viral genome, but there were not any methods available at the time to verify this theory [3, 26].

A major study in 1976 by Varmus, Bishop, Vogt and colleagues finally determined that Scholnick’s hypothesis was indeed correct: genes responsible for transformation were indeed normal genes in the genome [28]. This study examined the viral src oncogene and found that src was in fact a normal chicken gene that was transduced by the virus, converting it into an oncogene capable of forming sarcomas [28]. This provided the first evidence that proto-oncogenes exist in the genome that can be converted into activated oncogenes that promote transformation and tumorigenesis [3, 28].

Human Ras genes were initially named as variants of the src oncogene, although subsequent studies revealed that they were quite separate genes [3]. The term Ras was used because the genes were capable of promoting rat sarcoma formation. The two different variants of Ras were distinguished from one another by using the names of their discoverer: Jennifer Harvey (H) and Werner Kirsten (K) [3]. Thus, the Harvey and Kirsten viral Ras genes, or H-Ras and K-Ras, were named, but studies examining their horrifying oncogenic potential had only just begun [3].

*The Biochemical and Cellular Properties of Ras*

In the early 1970’s, Ed Scolnick and colleagues began a series of studies that would reveal much about the origin and biochemical properties of the H-Ras and K-Ras genes. First, they showed that both the Harvey and Kirsten Sarcoma Viruses were recombinant viruses that
Figure 1. Major Events in Ras Research. A timeline highlighting the major milestones in Ras research is shown.
contained mammalian genetic material, suggesting that the transforming properties of these viruses may have come from mammalian DNA itself [26, 27]. Again, this work provided the first insight into oncogenes: genes that are capable of promoting uncontrolled cell growth and proliferation that can contribute to tumorigenesis. Additionally, Scolnick found that these genes encode homologous 21 kDa proteins that are required for transformation of cells infected with virus, and these 21 kDa proteins bind GDP and GTP and localize to the cell membrane [29-32]. The human H-Ras and K-Ras genes were identified in 1982 [33] and were found to be homologous with the p21 viral genes in that they also bind GDP and GTP and associate with the cell membrane [34, 35].

The biological properties of Ras proteins therefore closely resemble those of heterotrimeric G proteins, which modulate cellular signaling by shuttling between active, GTP-bound, and inactive, or GDP-bound, states [36]. G proteins, however, have an intrinsic GTP hydrolysis activity, which is commonly referred to as GTPase activity. This regulates the activity of the G protein by allowing it to hydrolyze its bound GTP to GDP, thus inducing a conformational change that shifts the G protein back into its inactive state [36]. In addition to binding GTP and GDP, Ras proteins also possess an intrinsic GTPase activity [37]. This allows the proteins to switch from an “on” conformation to an “off” conformation, and proteins are reactivated when GDP is exchanged for the more abundant GTP molecule (Figure 2) [37].

Landmark studies in the 1980’s also found that the human p21 homologue could induce transformation when over-expressed in cells, and the GTPase activity of these proteins was thought to be crucial factor in transformation [38, 39]. An oncogenic mutant form of p21 appeared have a decreased GTPase activity compared to the GTPase activity of the wild-type protein, although this was only inferred at the time and was later shown to be an incorrect assumption [38, 39]. All of these studies supported what is now common knowledge about the cellular and biochemical properties of Ras proteins: that Ras proteins are membrane-associated, GTP-binding proteins that promote the activation of cell growth and proliferation pathways when in their GTP-bound, or activated, state [3]. At the time, however, the nuances of Ras signaling—how Ras interacts with other proteins and drives different cellular processes—remained undetermined.
Figure 2. Model for Ras Activation. Growth factor signaling activates Ras, which is mainly situated on the inner leaflet of the plasma membrane. Ras exists in two forms: active, or GTP-bound Ras, and inactive, or GDP-bound Ras. Guanine nucleotide exchange factors, or GEFs, promote GTP binding, which promotes a change in the conformational state of Ras, allowing it to bind to and regulate numerous downstream effectors, like MAPK. Ras is inactivated by its intrinsic GTP hydrolysis, or GTPase activity, which is enhanced by GAP proteins.
The discovery of Ras as a major oncogene in human cancer occurred as a result of studies examining DNA with transforming properties in human cancer and mouse cell lines [40-44]. In 1982, these studies resulted in the surprising discovery that the transforming genes in these experiments were in fact the RAS genes previously identified in the Harvey and Kirsten sarcoma viruses [45-47]. These results lead to a deluge of papers describing human HRAS and KRAS activation in various solid tumors and hematological malignancies in the early 1980’s [44, 45, 47-51]. The most intriguing result at the time was that RAS signaling activation appeared to occur in cancer through a single missense mutation in codon 12, a mutation that has been subsequently identified in multiple cancer types, including bladder, lung, and colon tumors [48, 49, 51, 52]. Additionally, during this time, a third RAS gene was discovered from studies using homologous cloning to identify transforming genes [53]. This gene was named NRAS, as one of the cell lines expressing the activated form of this gene was derived from a neuroblastoma tumor [53, 54]. At this point, RAS mutations had been discovered in human tumor tissue, but two studies in 1984 confirmed that this was not simply a result of an accumulated mutation from passaging cells multiple times in culture, for RAS mutations were found in human cancer tissue but not in normal human tissue [50, 55]. This proved to be a key finding in the advancement of Ras research, and the finding of a single point mutation that activated a major oncogene ushered in a new era of research focus on cancer at a molecular level [3]. Essentially, the discovery of RAS mutations prompted studies that led to major findings in cancer, including oncogene activation as a molecular event that drives carcinogenesis [3].

Numerous studies following the revelation that RAS mutations occur in cancer showed that RAS activation is present in a number of different human tumor types and at a high frequency in certain cancers [3]. In particular, very high frequencies of RAS mutations are found in pancreatic, lung, and colon cancers [56-59]. As RAS mutational analysis expanded, more codon mutation sites were discovered, including mutations of codons 13 and 61. While there are other reported mutations that activate RAS in cancer, mutations at codons 12, 13, and 61
compose 97-99% of all activating mutations [3] (Figure 3). Additionally, the three RAS isoforms exhibit different frequencies of mutation. The RAS isoform that is the most likely to experience missense mutations is KRAS, at 85% [3]. HRAS and NRAS are mutated much less frequently, at three and twelve percent, respectively [3].

The Multi-hit Model of Tumorigenesis

Although mutationally activated RAS is able to transform immortalized mouse fibroblast cells, this observation lead to an oversimplified picture of the genetic mechanisms that promote cancer formation [3]. In 1983, this idea was challenged by studies reported by three independent groups showing that mutant H-Ras alone was not able to transform primary rodent fibroblasts [6, 7, 60]. Instead, Ras-induced transformation required either the concurrent activation of an oncogene, the inactivation of a tumor suppressor, or previous fibroblast immortalization to fully transform cells into cancer cells [6, 7, 60]. These results support the Knudson hypothesis that multiple mutations, or “hits,” are required in normal cells before they develop into cancer cells. Since early Ras research was performed in an already-immortalized cell line, it was proposed that those cells must have had enough “hits” to the point that the addition of activated Ras was able to transform the cells in a single step [7].

Today, Ras mutations are known to commonly occur in combination with other oncogenic events in cancer cells. This is especially true in pancreatic and colorectal cancers, in which KRAS mutations occur early and at a high frequency (90% and 50%, respectively) in cancer progression (Figure 4) [3]. Genome-wide sequencing of colon and pancreatic cancers has also revealed that KRAS is the most commonly mutated gene that contributes to cancer progression in these cancer types [3].

Ras Mutants and GTPase Activity
Figure 3. **Common Mutations in H- and K-Ras.** Mutations in H- and K-Ras occur in multiple sites, but the most common mutations occur at codons 12, 13, and 61. Missense mutations found in cancer are indicated in the pink boxes, while those found in Ras-related developmental disorders are in green boxes. The numbers in parentheses indicate the number of cancer types and/or disorders in which the indicated mutation has been found.
Figure 4. The Progression of Colorectal and Pancreatic Cancers. Colorectal and pancreatic cancer both progress through a series of stages before progressing to full cancer. Those stages are indicated here (PanINs: precursor pancreatic lesions). Both cancers accumulate multiple mutations along the way, with mutations of Ras oncogenes indicated in green and mutations or the loss of tumor suppressor function indicated in red. In the case of pancreatic cancer, K-Ras mutations are early events that promote the formation of precursor lesions.
The study of Ras protein activation by a simple mutation at the genetic level caused researchers to ask the question: what does a single amino acid substitution do to the known biological function of Ras proteins? This led to a number of reports that investigated the different biological and biochemical properties of Ras mutants.

Ras proteins were first shown to bind guanine nucleotides, GTP and GDP, by Scolnick and colleagues in 1980 [29]. G proteins are well known as having an intrinsic GTPase activity that allows them to shuttle between active, GTP-bound, and inactive, GDP-bound, states [36]. Ras was hypothesized to act similar to G proteins, but this was not proven until 1984, when three different groups showed that normal and oncogenic Ras counterparts differed in their GTPase activity [39, 61, 62]. This lead to the theory that Ras-induced transformation was a result of the reduced GTPase activity of Ras mutants; however, this theory was later proved to be incorrect. Multiple studies investigating the GTPase activity of Ras mutants revealed that there is not a quantitative relationship between Ras’s GTPase activity and transforming potential, and in fact, the GTPase activity of mutant Ras is only slightly lower than that of wild-type Ras [63-66].

Ras GAPs and GEFs: Regulating the GTP/GDP Cycle of Ras

Ras cycles between active and inactive states based off its binding to GTP or GDP (Figure 2). The observation that Ras mutants have somewhat impaired GTPase activity provided insight into Ras activation and aberrant function in cancer, yet this impairment alone did not explain Ras-induced transformation in all cases [63]. In 1987, a landmark study by Trahey and McCormick revealed that a cytoplasmic protein was capable of stimulating GTP hydrolysis of wild-type Ras by over 200-fold, but it had no effect on Ras mutants [66]. This protein, termed a GTPase Activating Protein (GAP), explains why mutant Ras proteins are locked in the active conformation: Ras mutants can become resistant to inactivation by GAPs, leading to a constitutively GTP-bound, active Ras [3]. The first RasGAP, p120 RasGAP, was characterized in 1987 [66], and in 1990, a study examining tumor suppressor proteins involved in neurofibromatosis type I (NFI) revealed that neurofibromin (NF1) was also a RasGAP [67, 68].
More RasGAPs have been identified over time [69]. Additionally, mutations and/or epigenetic inactivation of RasGAPs can occur in numerous cancers, providing yet another mechanism by which Ras activation can occur in cancer [70-72].

This resistance to inactivation by GAPs is thus far more important than any small differences in intrinsic GTPase activity for Ras-induced transformation. An example of this occurs in a familial case of breast cancer [73]. In this case of early-onset breast cancer, loss of heterozygosity was observed in the NF1 gene in the absence of BRCA1/2 mutations, suggesting that the loss of this GAP protein is capable of promoting tumorigenesis through Ras activation in the absence of Ras structural mutation [73].

The discovery of RasGAPs started the race to determine what proteins were capable of activating Ras by promoting its binding to GTP. Termed Guanine Nucleotide Exchange factors, or GEFs, these proteins activate Ras by stimulating its exchange of GDP to GTP (Figure 2). The first RasGEF identified was a yeast protein found in S. cerevisiae, cell-division-cycle 25 (CDC25), although at the time of discovery, the mechanism of how CDC25 activated Ras was unknown [74, 75]. CDC25's ability to act as a RasGEF was identified in 1991 [76]. A CDC25 homologue, Son of Sevenless (SOS), found in Drosophila was the second RasGEF identified, and in 1992, the mammalian homologues of CDC25 and SOS were identified, serving as the basis for the Cdc25 family of RasGEFs [4, 77-80].

**Ras Structure**

To further understand Ras activation, studies examining the three-dimensional structure of Ras and Ras mutants were performed using crystallography in the late 1980’s and 1990 [81-83]. Ras contains a hydrophobic core consisting of six β-sheets and five α-helices that are connected by a series of ten loops, five of which play a role in regulating Ras’s interactions with nucleotides and GTP hydrolysis (Figure 5) [4].

Additional structural analysis revealed that Ras exists in two main structural conformations when bound to GDP and GTP, called switch I and switch II [82, 84]. These
Figure 5. The Three-Dimensional Structure of Ras. Ras consists of five α-helices and six β-sheets that are connected by ten loops. Inactive, or GDP-Ras is shown on the left, and active, GTP-Ras is on the right. The switch I and switch II regions are in red and green, respectively. A. Displays the crystallographic structures of these proteins, while B. shows the nucleotide rearrangements that occur upon GDP and GTP binding.
regions contain sequences that are critical for Ras's interactions with both its effectors and regulators [3, 4]. Additionally, switch I and switch II play crucial roles in the interaction of Ras with GAPs and GEFs. Mutations in these regions also provide an explanation for how Ras is activated by point mutations in cancer. For example, mutations of Gly12 and Gln61 confer insensitivity to RasGAPs, as they stabilize the GTP-bound state of Ras [3, 85].

*Ras Post-translational Modifications*

Although Ras was indeed regulated by its GTP/GDP cycle, studies began to show that the regulation of Ras signaling was a much more complicated process. Ras strongly localizes to the cell membrane, an observation that was first reported in the early 1980's [86]. Soon after this discovery, the importance of lipid modifications of the C-terminus of Ras was demonstrated for the viral H-Ras protein, which localized to the plasma membrane in response to C-terminal lipid modifications [87]. In 1984, Willumsen *et al.* showed that the C-terminus of Ras undergoes farnesylation, a post-translational modification that involves the attachment of a hydrophobic farnesyl group to the protein, and can bind membrane lipids, localizing the protein to the inner leaflet of the plasma membrane (*Figure 2*) [88]. Moreover, mutational analysis revealed that C-terminal amino acids are required for Ras to maintain its transforming ability and its membrane binding, suggesting that Ras activity is not controlled simply by its GTP/GDP association but is also influenced by its ability to bind the plasma membrane [88]. The same year, Willumsen *et al.* also found that a specific residue, Cys186, is required for Ras to associate with membranes, bind lipids, and exert transforming activity [89]. They also showed that Ras can incorporate the fatty acid 3H-palmitate, leading to the hypothesis that this cysteine residue was the site of Ras palmitoylation, a modification that would stabilize Ras membrane binding [89].

It took several more years and numerous studies to finally understand the biochemical processing of Ras. In 1989, studies determined that all Ras proteins share a C-terminal CAAX motif (C = cysteine, A= aliphatic amino acid, X= terminal amino acid) and that Cys186 must be farnesylated before further modifications, like palmitoylation, can occur [90-92]. Briefly, Ras
modifications consist of an initial cytoplasmic addition of a farnesyl pyrophosphate to a Ras cysteine by a farnesyltransferase [93, 94]. Then, three amino acids at the C-terminus are cleaved by Ras-converting enzyme 1 (RCE1), and isoprenylcysteine carboxyl methyltransferase 1 (ICMT1) methylates the final cysteine residue [91]. Finally, Ras’s insertion into the membrane is stabilized by the palmitoylation of cysteine residues upstream of the C-terminus [91].

While the CAAX sequence is required for Ras to be targeted to membranes, studies of different Ras variants led to the discovery of a second signaling domain that could also recruit Ras to the membrane and allow it to function properly [91]. This sequence is present in K-Ras4B and is polybasic, consisting of a strand of lysine residues upstream of the C-terminus that allow the protein to attach to the membrane [91]. Other Ras variants, however, including H-Ras, N-Ras, and K-Ras4A, must be palmitoylated on their C-terminal cysteine before their insertion into the membrane is fully complete [91]. The enzymes that catalyze these reactions have been an intense focus of drug development efforts, since preventing Ras from undergoing this post-translational processing and anchorage into the membrane could prevent its activation in cancer [4].

Ras Activates a Number of Different Effector Proteins

Throughout the 1980’s, little was known about the details of Ras’s function in cellular signaling. The first study exploring this occurred in 1986 and showed that injecting Ras into rat-embryo fibroblasts led to the activation of phospholipase A₂, an enzyme capable of hydrolyzing phospholipids into fatty acids [95]. Other studies reported numerous other changes associated with Ras expression, but at the time, it was unclear if these effects were simply a result of the forced over-expression of Ras or if they were actually a result of Ras signaling [4]. In 1988, a study used antibodies to inhibit a specific downstream effector of Ras and showed that this disrupted Ras signaling, showing that Ras indeed signals to downstream effectors that are critical mediators of its biological effects [96]. This led to a plethora of studies exploring different “Ras
effectors,” which is the term given to proteins that mediate Ras downstream signaling effects (Figure 6).

In 1993, two groups identified the first mammalian Ras effector, the RAF1 Ser/Thr kinase [97, 98]. Extensive research on RAF1 showed that upon stimulation with mitogenic factors such as insulin, RAF1 activated the mitogen-activated protein kinase (MAP kinase) signaling cascade [99]. There are three Raf isoforms (A-Raf, B-Raf, and c-Raf), and all can directly activate the first component of the MAPK pathway, the MEK1/2 kinases (MAPK/extracellular signal-related kinases) [99]. MEK1/2 then activate the ERK1/2 kinases (extracellular signal-related kinases), which then activate the E26-transcription (ETS) factors, which activate the transcription of genes involved in cell growth and proliferation [99]. Under normal conditions, this pathway exists under tight regulation, but the constitutive activation of Ras or other factors can lead to MAPK pathway hyperactivation and oncogenic transformation. Both of the studies in 1993 showed via yeast two-hybrid screens that Raf interacts directly with Ras, and GST pull-down studies revealed that Raf preferentially binds with GTP-bound Ras, suggesting that Ras is the membrane protein that transmits signals from mitogenic receptors to Raf, thus activating the MAPK pathway and making Raf the first bona fide Ras effector protein [97, 98].

A year after the discovery of the Ras-Raf interaction, two more Ras effectors were identified: phosphoinositide 3-kinase (PI3K) and RalGDS (ral guanine nucleotide dissociation stimulator) [100-103]. PI3K activates the Ser/Thr kinase AKT and can regulate the actin cytoskeleton upon stimulation by growth factors like PDGF and insulin [104-106]. Rodriguez-Viciana et al. revealed that, just as Ras must activate the MAPK pathway via Raf to induce transformation, Ras also requires PI3K activation to transform NIH 3T3 cells [102]. These studies provided the basis for mechanistic studies that concluded that Ras’s anti-apoptotic effects were attributed to the pro-survival activities of PI3K signaling, which includes the activation of AKT/Protein Kinase B (PKB) and the nuclear factor-kB transcription factor [107-109]. This pathway highlights yet another mechanism by which activated Ras signaling can mediate cell survival and the evasion of apoptosis in cancer.

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**Figure 6. The Ras Interactome.** Different proteins capable of interacting with Ras are indicated, and a number of described Ras effectors are shown. Ras can interact with a wide range of proteins and is capable of promoting a number of different processes within the cell.
The third Ras Effector identified in 1994 was RalGDS, which was found to bind Ras directly in a yeast two-hybrid study [101]. Initially dismissed as having a limited involvement in Ras-mediated transformation in rodent cells, studies in human cells quickly revealed that the RalGDS pathway plays an important role in Ras-mediated transformation and is indeed the only Ras Effector pathway that can promote Ras transformation of human kidney epithelial cells by itself; the PI3K and Raf pathways could not transform cells alone [110]. The two Ral isoforms, RalA and RalB, are members of the Ras superfamily of small GTPases and share a significant sequence homology with Ras, and RalGDS specifically catalyzes nucleotide exchange on these, but not other, small GTPases [111, 112]. Ras activates RalGDS, which in turn activates Ral to drive transformation, constituting the third major pathway by which Ras is thought to drive cellular transformation, including through the binding and activation of Raf and PI3K [4]. Ras is capable, however, of binding to and activating numerous other downstream effectors that are involved in the modulation of a number of different cellular processes, including actin organization, autophagy, transcription, endocytosis, cytokineses, and the production of second messengers [111]. While Raf, PI3K, and RalGDS are the three most well characterized Ras effectors, several additional Ras effectors with diverse functions have been identified.

Additional Ras Effectors

In 2001, phospholipase C-ε (PLCε) was shown to be a direct effector of Ras [113]. PLCε regulates both intracellular calcium signaling and the activation of Protein Kinase C (PKC), and PLCε’s activation allows it to cleave phosphatidylinositol-4,5-bisphosphate into inositol-4,5-trisphosphate and diacylglycerol (DAG), which promote calcium release and PKC activation, respectively [4]. Additionally, PLCε contains a Ras GEF domain called CDC25. This isolated domain and the full PLCε protein are both capable of promoting the formation of GTP-Ras and of activating the Ras-MAPK pathway [114].

In 2002, another Ras effector was identified, T-cell invasion and metastasis-1 (TIAM1) [115]. A Rac-specific GEF, TIAM1 is capable of binding directly to GTP-bound Ras and
cooperates with Ras to promote the formation of Rac-GTP [115]. Rac, like Ras, is a small GTPase protein that plays a role in regulating cell growth, motility, and invasion. Additionally, studies from another group found that knockout of \textit{TiAM1} in mice provided resistance to Ras-induced skin tumors following DMBA treatment, showing that the loss or inhibition of yet another Ras effector has deleterious effects on downstream Ras signaling [116].

There are other Ras effectors that are considered less well characterized than those described above. These include ALL-1 fused gene on chromosome 6 (AF-6), Ras interaction/interference protein-1 (RIN1), and the Ras association domain-containing family (RASSF) of proteins. AF-6, often found in human leukemia as part of a chimeric fusion protein with ALL-1, was identified as a potential Ras-binding protein in 1996 and was shown to contain both actin and microtubule binding motifs, linking Ras to control of the cytoskeleton [117, 118]. The other proteins, RIN1 and the RASSF proteins, actually interact with Ras to promote tumor suppressor functions, highlighting a surprising aspect of Ras signaling, which is the ability of Ras to mediate tumor suppressor functions, including apoptosis and senescence [8] (Figure 7).

The first example of a Ras effector capable of blocking Ras-induced transformation, RIN1, was discovered in yeast in 1995 [119]. RIN1 binds to GTP-bound Ras with such a high affinity that it competes with Raf for Ras binding, providing a potential mechanism for how RIN1 blocks Ras-induced transformation [119]. More recent models propose that RIN1 actually promotes the endocytosis of Ras-activating growth receptors, like EGFR [120]. In 2007, a study was published showing that RIN1 protein levels were reduced in breast cancer both through transcriptional repression and epigenetic inactivation, providing evidence that the loss of this protein may be required for Ras-induced transformation [121]. Another protein family of negative Ras effectors, the RASSF proteins, also has family members that exhibit a loss of expression in various cancers and have been shown to mediate pro-apoptotic and pro-senescent functions of Ras.
Figure 7. Ras Mediates Both Pro-Apoptotic and Anti-Apoptotic Pathways. Activated Ras can interact with and stimulate a number of different pathways within the cell, and depending on the context, Ras can promote two different cell fates: growth and survival, or apoptosis.
The RASSF Family of Proteins

The RASSF family of proteins consists of ten family members that all contain a common Ras Association, or RA domain, hence the name RASSF: Ras-Association Domain Family. This family consists of two types of RASSF proteins: those that have their RA domains towards the C-terminus, the C-terminal RASSF proteins, and those that have their RA domains towards the N-terminus, the N-terminal RASSF proteins [12, 122]. The C-terminal RASSF proteins consist of RASSF1 to RASSF6 and have been widely implicated as having powerful roles in tumor suppression, while the N-terminal family members, RASSF7 to RASSF10, have not been as widely studied [14, 16, 17, 23, 122-125].

A general feature of RASSF proteins is that they do not have any apparent enzymatic activity; instead, they seem to act as scaffolding molecules, binding to Ras through their RA domains and facilitating its interactions with various proteins that promote cell growth arrest, apoptosis, and/or senescence [126]. In addition to the RA domain, the C-terminal RASSF proteins also contain a number of different protein-interaction domains, including the Salvador/RASSF/Hippo, or SARAH, domain, which has been hypothesized to allow them to directly bind the MST kinases, connecting them to the Hippo signaling pathway [124, 127]. Two family members, RASSF1A and NORE1A, also contain a cysteine-rich C1 domain, a central zinc finger domain, and an ATM domain that can be phosphorylated by the ataxia telangiectasia (ATM) kinase [122, 128]. The structural domains of one of these RASSF proteins, NORE1A, are shown in Figure 8. It is important to note that, although the RASSF proteins contain similar structural domains, numerous studies have shown that each family member has unique functions in tumor suppression and different tissue expression profiles [14, 16-18, 23, 123-126, 129-131].

RASSF proteins also exhibit a high rate of epigenetic inactivation in cancer via hypermethylation of CpG islands in the promoter region of RASSF genes [122]. The loss of these negative Ras effectors shifts the balance of Ras signaling towards uncontrolled cell growth and
proliferation, thus facilitating Ras-induced transformation. One of these RASSF proteins in particular, RASSF5, or NORE1A, is often down-regulated in cancer and appears to act as a potent tumor suppressor, although much remains to be discovered about its mechanism of action [14, 16-18, 23, 124, 130, 132].

The NORE1A Tumor Suppressor (RASSF5)

RASSF5, or NORE1A, is the founding member of the RASSF family of proteins. First discovered in 1998, it was named Novel Ras Effector 1, as NORE1A was the first member of the RASSF family to be cloned [14, 15]. Two main isoforms, NORE1A and NORE1B (also known as RAPL), are produced from the RASSF5 gene locus. NORE1A is broadly expressed in tissue, whereas the smaller splice product, NORE1B, is mostly restricted to the lymphoid compartment. NORE1A was originally identified as a Ras binding protein in 1998, while NORE1B was shown to bind Rap in a similar screen [15, 132, 133]. NORE1A binds to Ras via its Ras Association, or RA, domain in a GTP-dependent manner (Figure 8) [134]. Similar to its close homolog RASSF1A, NORE1A and Ras form an endogenous complex [10, 14, 135]. While RASSF1A, however, binds K-Ras, NORE1A binds preferentially to H-Ras [14, 135]. NORE1A, like other RASSF proteins, does not have any inherent enzymatic activity; instead, it acts as a scaffolding molecule, facilitating the interactions of Ras with various proteins [14].

NORE1A has approximately 50% sequence homology with the well-characterized tumor suppressor RASSF1A [136], and evidence exists suggesting that NORE1A is also a potent tumor suppressor. Additionally, NORE1A is often down regulated in tumors by epigenetic inactivation via promoter hypermethylation and can undergo loss of heterozygosity in a rare form of kidney cancer [14, 137]. At a protein level, NORE1A can also be down regulated in tumor cells by calpains and by ubiquitination [138, 139]. In liver cancer, more malignant primary tumor samples have an inverse correlation with NORE1A expression, whereas normal liver tissue expresses NORE1A [140]. Moreover, a hereditary cancer syndrome exists that involves an inactivating
Figure 8. Functional Domains of NORE1A (RASSF5). NORE1A consists of an N-terminal proline-rich region, a cysteine-rich zinc finger domain (ZnF), a central domain, a Ras Association (RA) domain that mediates Ras binding, and the Salvador/RASSF/HIPPO (SARAH) domain, which is hypothesized to allow NORE1A to bind the MST1 and MST2 kinases, thus connecting it to the Hippo signaling pathway.
translocation of the *NORE1A* gene [13]. Therefore, there is strong evidence that NORE1A is a tumor suppressor.

In 2002, evidence was presented that Ras uses NORE1A as a pro-apoptotic effector [124]. NORE1A binds the MST kinases and has the potential to modulate the pro-apoptotic Hippo pathway through its SARAH domain [141]. However, it is unclear if NORE1A can stimulate the canonical Hippo kinase cascade, and deletion mutagenesis studies have shown that the canonical Hippo pathway is not essential for the growth suppressing functions of NORE1A [141, 142]. These early studies on the relationship between Ras and NORE1A only began to highlight the complex nature of NORE1A’s role as a Ras effector and tumor suppressor.

*NORE1A and Ras-induced Senescence*

Although initial studies focused on NORE1A’s ability to induce apoptosis, more physiological studies implicated that NORE1A also plays a role in cell cycle arrest and senescence. Indeed, NORE1A was found to be a potent senescence effector of Ras [16-18]. Senescence is a state of permanent cell cycle arrest that was first observed in the 1960’s, in which researchers found that cells had a finite number of divisions in culture before entering a state of cell growth arrest, even though the cells were metabolically active and viable [143-145]. Senescent cells also undergo numerous changes, including changes in morphology, the activation of β-galactosidase activity, and the formation of senescence-associated heterochromatin foci (SAHF) [145, 146]. This type of senescence, now termed replicative, or cellular, senescence, is just one type of senescence and is often implicated in aging studies.

Senescence can also be caused prematurely by different factors, and one type of senescence, oncogene-induced senescence (OIS), can be caused by activated Ras expression. This was first observed in studies performed in the 1990’s, in which mutant H-Ras that was introduced into primary cells lead to a surprising arrest in cell growth [9]. Serrano *et al.* noticed that these cells looked phenotypically similar to cells that had undergone cellular senescence,
thus prompting the conclusion that activated Ras itself was able to induce a specific form of senescence, later termed OIS [9].

OIS involves the activation of a number of different signaling pathways, with two of the most crucial being the p53 and Rb pathways. OIS also presents a key barrier that must be overcome in the process of tumorigenesis [9, 145, 147]. Indeed, the presence of cellular senescence predicts a better treatment outcome in high-grade colorectal cancer, a finding that corroborated mouse studies from Serrano’s group showing that most premalignant lesions expressing mutant KRAS failed to progress to malignancy and were instead senescent [148, 149]. Additionally, work done in 2007 by two independent groups showed that the re-expression of p53 in tumors can result in widespread senescence and even tumor regression, suggesting that reactivating this process in tumor cells could provide another therapeutic strategy compared to traditional agents aimed at promoting tumor cell apoptosis [150, 151].

NORE1A was first implicated as having a role in Ras-induced senescence in 2009, when Calvisi et al. showed that NORE1A is capable of activating p21\(^\text{CIP1}\), a cyclin-dependent kinase involved in G\(_1\) cell cycle arrest and senescence [123]. They showed that NORE1A over-expression stimulates p21\(^\text{CIP1}\) expression, while loss of NORE1A expression reduces p21\(^\text{CIP1}\) expression in liver and hepatocellular carcinoma cell lines [123]. Moreover, they showed that the loss of NORE1A expression in hepatocellular carcinoma primary tumors correlated with a loss of p21\(^\text{CIP1}\) expression and with a poor prognosis in those tumors [123]. Perhaps most importantly, Calvisi et al. also showed NORE1A was acting to induce p21\(^\text{CIP1}\) expression via p53, an ability that was lost in the presence of mutant p53 or p53 siRNA [123]. However, NORE1A’s ability to act as a mediator of Ras-induced senescence and the mechanism by which it interacts with p53 was not discovered until 2015.

In 2015, Donninger et al. found that NORE1A over-expression induces senescence at levels comparable to those induced by activated Ras, and suppression of NORE1A both impairs senescence and enhances Ras-mediated transformation [17]. This correlates with Calvisi’s finding that NORE1A expression in primary human tumors correlates with the expression of markers of senescence [123]. NORE1A can actually mediate Ras-induced senescence via two
major pathways (Figure 9). The first is that NORE1A can form a Ras-regulated complex with p53 and scaffolds p53 to the kinase HIPK2, providing the mechanistic basis for the initial findings of Calvisi in 2009 [17, 123]. HIPK2 can mediate post-translational modifications of p53 at various residues to mediate different functions. Phosphorylation at residue Ser46 by HIPK2 promotes apoptosis, but HIPK2 can also recruit acetyltransferases to acetylate p53 at specific lysine residues to promote pro-senescent effects (Reviewed in [152]). NORE1A promotes the scaffolding of HIPK2 to p53, resulting in the suppression of Ser46 phosphorylation of p53 and the enhancement of p53 acetylation at lysine residues 382 and 320, which drive p53-dependent senescence [17]. Without the presence of p53, however, NORE1A still retained some ability to induce senescence, suggesting that NORE1A regulates senescence through a multifaceted process [17].

The second major pathway by which NORE1A mediates Ras-induced senescence is via its interaction with the Rb tumor suppressor (Figure 9) [16]. Rb regulates a number of important processes in the cell, including cell cycle regulation and Ras-induced senescence, and it is frequently inactivated in human cancers [9, 153, 154]. Rb is regulated by phosphorylation: inhibitory phosphorylation by cyclin-dependent kinases inactivates the protein, while a phosphatase, PP1A, dephosphorylates and activates Rb [155-157]. Barnoud et al. showed that NORE1A forms a Ras-regulated endogenous complex with PP1A, scaffolding the phosphatase to Rb and promoting Rb dephosphorylation [16]. The loss of Rb also reduced NORE1A’s ability to induce senescence, showing that NORE1A mediates Ras-induced senescence through two major pro-senescent signaling pathways [16-18]. The loss of NORE1A expression in human tumors would therefore prevent Ras from promoting senescence through both p53 and Rb, allowing Ras to promote growth-promoting signaling pathways instead [18].

NORE1A can also affect the stability of proteins in the cell through its work as a scaffolding molecule. NORE1A can associate with MDM2, an E3 ubiquitin ligase that is a negative regulator of p53 and Rb, but NORE1A uses this association to induce the ubiquitination and degradation of the oncoprotein HIPK1 by scaffolding the HIPK1 to the MDM2 ubiquitin ligase,

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Figure 9. NORE1A Mediates Ras-Induced Senescence Via Two Major Pathways. Ras stimulates the interaction of NORE1A with HIPK2 and PP1A, mediating p53 acetylation and Rb dephosphorylation, respectively. Through these two mechanisms, NORE1A mediates Ras-induced senescence, acting as a double barreled Ras senescence effector.
highlighting the functionality of NORE1A as a scaffolding molecule [158]. Work by Schmidt et al. shows that the NORE1A/MDM2 interaction is Ras-regulated, and NORE1A promotes MDM2 degradation by the SCF-β-TrCP ubiquitin ligase complex [130]. Thus, NORE1A not only promotes senescence by promoting pro-senescent post-translational modifications of p53, it also stabilizes p53 in the cell by suppressing the main negative regulator of p53, MDM2 [17, 130].

Another major component of Ras/NORE1A signaling that was identified in 2015 is NORE1A’s regulation of the β-catenin protein [23]. β-catenin is an adherens junction protein and a transcription co-factor that serves as the terminal executor of the Wnt signaling pathway [159]. A multi-protein complex phosphorylates β-catenin under normal conditions, allowing it to bind the SCFβ-TrCP ubiquitin ligase complex [160]. Without Wnt stimulation, this process results in β-catenin’s degradation. When Wnt is active, unphosphorylated β-catenin translocates to the nucleus, where it activates the transcription of genes that promote survival and cell growth [159]. β-catenin can therefore act as an oncogene in cancer when it is either dysregulated or mutated, and β-TrCP, the E3 component of the SCFβ-TrCP complex, is the substrate recognition component and can act as a tumor suppressor because it influences β-catenin degradation [161]. Schmidt et al. found that NORE1A is able to prevent β-catenin activation by binding directly to β-TrCP in an interaction that is enhanced by activated Ras, allowing Ras to specifically stimulate SCFβ-TrCP-mediated degradation of β-catenin [23]. This reveals another critical barrier that NORE1A forms against tumorigenesis, for the loss of NORE1A expression in tumor cells can disrupt Ras’s negative regulation of β-catenin [23].

In vivo studies performed in 2010 to further investigate the role of NORE1A as a tumor suppressor found that NORE1A knockout mice appear overtly normal, yet mouse embryonic fibroblasts (MEFs) from the animals can be transformed by activated Ras alone [162]. Normal MEFs require the addition of multiple oncogenic events to overcome oncogene-induced senescence in order to transform, including the mutation of p53 or the activation of additional oncogenes [6], so loss of NORE1A expression greatly increases the susceptibility to transformation [162]. A study by Park et al. also provided further evidence that NORE1A is a tumor suppressor by showing that NORE1A helps mediate TNF-α-mediated, or death receptor
mediated, apoptosis, probably through its interaction with MST1 [162]. Few studies have examined the results of NORE1A inactivation and Ras activation in human tumors, but one study of hepatocellular carcinoma tumors with activated Ras signaling showed that NORE1A promoter methylation was specifically present in a subset of tumors with poor patient survival, indicating that the impact of the loss of NORE1A expression in human tumors can be great [140]. In order to further examine the impact of the loss of NORE1A expression in tumor cells with activated Ras signaling, a NORE1A-null, Ras-positive transgenic mouse system would be useful. This system has been created and is currently being analyzed by members of Geoffrey Clark’s laboratory.

A lesser-studied NORE1 isoform, NORE1B plays a role in immune cells and seems to be expressed more in the lymphoid compartment compared to the fairly ubiquitous expression of NORE1A. Specifically, NORE1B plays an important role in lymphocyte and dendritic cell migration and adhesion and was shown to be a crucial part of immunosurveillance [133]. Loss of NORE1B expression in a mouse model resulted in immune dysfunction, including impaired migration of lymphocytes and dendritic cells and impaired B cell maturation [133]. Like NORE1A, NORE1B associates with MST1, and the two negatively regulate T cell proliferation upon T cell antigen receptor stimulation [163]. NORE1B can also work with Ras to regulate T cell signaling by recruiting activated Ras to T cell synapses [164]. Although NORE1B can associate with Ras-related proteins and Ras, little evidence exists to show that NORE1B can act as a tumor suppressor or experiences epigenetic inactivation [165]. Thus, NORE1A will be the focus of this discussion.

NORE1A in the Cell

In cells transfected with over-expressed, GFP-tagged NORE1A, NORE1A can be seen in what appear to be small dots in the nucleus (Figure 10) [16, 17]. Upon stimulation by activated Ras, NORE1A also associates with HIPK2 and PP1A in these nuclear spots, facilitating Ras-induced senescence [16, 17]. These spots look similar to a well-known nuclear body formed by the Promyelocytic Leukemia protein, PML nuclear bodies. PML is also a well-known tumor
Figure 10. **NORE1A Forms Nuclear Spots.** When exogenously expressed in Cos-7 cells, NORE1A forms small spots in the nucleus. GFP vector is shown in the left panel, while GFP-NORE1A in the nucleus is shown in the right panel. Bar represents 40µm.
suppressor that can associate with p53 and Rb, and PML is required for Ras-induced senescence. PML will be discussed in the following section.

1.4 - The Promyelocytic Leukemia Protein (PML)

Discovery

Symptoms of acute promyelocytic leukemia (APL), the disease for which the PML protein is named, were initially observed in the 1930’s and 40’s, but the disease was not named until 1957 [166]. APL is fairly rare, accounting for about ten percent of acute myeloid leukemia cases [167-169]. When initially discovered, the disease was quite devastating for patients, with many of them presenting with hemorrhage and severe anemia at the time of presentation, and average patient survival was poor [167-169].

The PML protein was not implicated as being part of the pathogenesis of APL until 1977, when Rowley et al. described a translocation between chromosomes 15 and 17 consistently found in APL patients [170]. Once the retinoic acid receptor α (RAR\(\alpha\)) gene was mapped to the q21 locus of chromosome 17 in 1988, it was quickly identified as one of the genes that was rearranged in APL patients [171-174]. Furthermore, a chimeric transcript consisting of RAR\(\alpha\) and a previously undiscovered gene on chromosome 15, called myl for myelocytic leukemia, was discovered in 1990 [175]. Myl, later renamed PML for promyelocytes, was found to encode a protein that contained two zinc-finger-like, cystine-rich domains and a candidate leucine zipper domain [176]. These domains were thought to allow PML to bind to DNA and to form PML dimers, respectively [176]. Additionally, a proline-rich sequence in the N-terminal region of PML suggested that the protein contained a transcription activation domain [176]. Further research revealed that this novel gene sequence could undergo alternative splicing to form different protein isoforms, and the disruption of this gene was thought to play a role in the pathogenesis of APL [177].
APL is always associated with chromosomal translocations that involve the RARα gene on chromosome 17, and the t(15;17) translocation in particular has become a major disease marker and is present in over 98% of APL cases [170, 178, 179]. APL is treated with two major therapies that both induce the differentiation of promyelocytes and the remission of APL: retinoic acid and arsenic trioxide [179]. The combination of these two therapies results in the cure of virtually all APL patients by targeting the PML/RARα fusion protein, therefore promoting differentiation of promyelocytes into mature blood cells [180-184]. Treatment of APL with these reagents also led researchers to notice a strange observation: strange structures in the nucleus, simply termed “nuclear bodies” were not present in APL cells but returned upon treatment with retinoic acid [185].

PML Nuclear Bodies

For over sixty years, the nucleus has been known to be a non-homogenous organelle with obvious distinct morphological regions [186, 187]. One of these regions, the nuclear body, was first observed in the early 1960’s [186-190], but a more definitive study in 1963 found that nuclear bodies were more complex than originally believed [191]. Weber and Frommes discovered nuclear bodies ranging from 0.8 to 1.2 µM in diameter in a number of different cell types [191]. These bodies appeared to have an outer fibrillar area that surrounded an electron-dense core, which was later shown to be composed of an outer shell of PML molecules (Figure 11) [179, 191]. They also found that the nuclear bodies were more abundant or looked unique in different cell types, prompting them to draw the conclusion that these bodies may play a role in specific processes within certain cells [191].

As research on the different types of nuclear bodies progressed, one nuclear body-type structure in particular gained interest. Ronald Evan’s group discovered that certain nuclear bodies were composed primarily of PML protein molecules, prompting them to name it the PML oncogenic domain, or POD [185]. In a cell line derived from an APL patient, however, Evan’s
Figure 11. PML Nuclear Bodies. PML nuclear bodies were labeled by immunofluorescence in CHO (Chinese Hamster Ovary) cells (left image). On the right, an individual PML nuclear body (NB) is analyzed by electron microscopy, in which gold-labeled anti-PML antibodies were used to show PML distribution in the shell of the electron-dense NB core. Bar represents one micrometer.
group noticed that PML did not form large, noticeable PODs; instead, it formed small, numerous speckles throughout the nucleus [185]. The same was also true for bone marrow samples from an APL patient [185]. Further work revealed that the PML-RARα fusion protein does not enter the nucleus effectively, although the presence of wild-type PML did somewhat enhance this translocation, suggesting that PML forms a heterodimer with PML-RARα to enhance its localization to the PODs [185].

In addition to defining the POD as a novel nuclear structure, Evan's group provided evidence that POD disruption in APL patients was an oncogenic event [185]. Treatment of an APL cell line with all-trans retinoic acid, a known treatment to promote differentiation of promyelocytes at the time, restored the organization of PODs [185] (Figure 12). Cells that were known to be resistant to all-trans retinoic acid treatment, however, did not exhibit POD reorganization upon treatment, suggesting that the loss of PML NBs is linked to the maturation arrest of blood cells seen in APL [185]. This disruption of PML NBs by the PML-RARα fusion protein and restoration upon treatment soon became a frequently observed phenomenon in studies examining the results of the treatment of APL [185, 192-195]. Indeed, PML NBs are restored by two APL therapies, retinoic acid treatment and arsenic trioxide treatment, both of which induce degradation of the PML-RARα fusion protein [196, 197]. Later studies showed that PML NBs are not just disrupted in APL patients; they can also be altered under conditions of cellular stress, such as viral infection, heavy metal exposure, and heat shock [198-200].

PML nuclear bodies (NBs), or PODs, were also found to be multi-protein complexes [201-203]. Later work revealed that PML itself is the major organizer of these bodies, as a number of PML NB-associated proteins are found dispersed throughout the cell in PML−/− cell lines, and that PML post-translational modifications, notably sumoylation, are crucial for the formation of PML NBs [204-206]. Protein sumoylation refers to the post-translational modification involving the covalent attachment of a small ubiquitin-like modifer (SUMO) to a protein. In the case of PML, sumoylation is required for the formation of PML NBs, and PML is capable of binding directly to both SUMO and UBC9, a SUMO-conjugating enzyme [204, 206, 207]. Additionally, many of the proteins associated with PML NBs are sumoylated [21, 179, 205].
Figure 12. PML Nuclear bodies Are Reorganized Upon Treatment with All-trans Retinoic Acid. This model depicts the loss of PML NB, or PML Oncogenic Domain (POD), structure in APL cell. Treatment with all-trans retinoic acid restores PML NBs and allows myeloid differentiation, effectively treating Promyelocytic Leukemia induced by the t(15;17) PML-RARα translocation.
PML NBs regulate a number of different processes within the cell and play a role in the response to cellular stress. These processes include transcriptional regulation, the response to DNA damage, regulation of apoptosis (both p53-dependent and –independent [208]), regulation of senescence, and regulation of angiogenesis [208-210] (Figure 13). PML regulates these processes by recruiting protein partners along with protein-modifying enzymes, mediating protein posttranslational modifications, protein sequestration, and protein degradation [21].

**PML Isoforms**

The PML gene can undergo alternative splicing of its C-terminal region to form seven different PML protein isoforms, designated PMLI through PMLVII [211]. Variations in this C-terminal region give different isoforms unique functions and allow them to interact with a unique subset of proteins. PML is a member of the tripartite motif family of proteins (TRIM family), and all isoforms contain a N-terminal RBCC/TRIM motif that consists of a RING (Really Interesting New Gene) finger domain, two B-boxes, and an α-helical coiled-coil domain (Figure 14) [212, 213]. This motif allows PML to form NBs and allows PML to homodimerize [211].

PML isoforms do share some common functions through their N-terminal RBCC motif, but the different C-terminal segments allow each isoform to mediate specific functions and interact with unique proteins (Reviewed in [211]). Additionally, there are reports of differential expression levels of different PML isoforms: for example, PMLI and PMLII are expressed in much higher quantities than PMLIII, PMLIV, or PMLV [214]. All of the PML isoforms can also be found in the nucleus, with the exception of PMLVIIb, which lacks the nuclear localization sequence and is therefore found exclusively in the cytoplasm [211]. When examined separately, each PML isoform appears to localize to a specific area of the nucleus (Figure 15), yet when isoforms are co-expressed, they co-localize in the nuclear bodies, probably due to heterodimerization through their RBCC motif [214].
Figure 13. PML Nuclear Bodies Mediate Numerous Processes Within the Cell. Different processes that PML NBs mediate are shown and can be classified into three major groups, as indicated by color. PML NBs play a role in the identification and storage of proteins, protein post-translational modifications, and transcriptional processes. The major cellular processes that PML NBs mediate include the DNA damage response, apoptosis, senescence, and angiogenesis.
Figure 14. **PML Gene Structure.** The PML gene contains 9 exons that undergo alternative splicing to form seven different PML isoforms. The major conserved region of all PML isoforms contains a RBCC motif, consisting of a RING finger domain (R), two B boxes (B), and an α-helical coiled-coil domain (CC). Most PML isoforms also contain a nuclear localization sequence (NLS).
**Figure 15. Different PML Isoforms Exhibit Differential Nuclear Localization.** Mouse embryonic fibroblasts knocked down for all PML expression were stably transfected with specific PML isoforms, and those isoforms were labeled using a Pan-PML antibody to determine the specific nuclear organization of each isoform. Bar: 3µm.
PML as a Tumor Suppressor

After studies showed the importance of PML NBs in APL, researchers began to hypothesize that PML was a tumor suppressor. Indeed, PML NBs were found to mediate a number of important growth-inhibitory processes in the cell, and PML itself appeared to be a critical mediator of apoptosis. Early studies on PML−/− mice showed that PML knockdown inhibited the induction of apoptosis upon treatment with various agents known to induce lethal apoptosis, such as γ irradiation and TNF [215]. These mice lacked activation of caspases 1 and 3, and furthermore, the authors found that the PML-RARα fusion protein acted as a dominant negative PML product, conferring cells expressing the fusion protein resistance to apoptosis induced by Fas, TNF, or IFN [215]. PML−/− Mouse Embryonic Fibroblasts (MEFs) also grow faster in culture than their PML+/− or PML+/+ counterparts, suggesting that PML may also mediate growth suppression [215, 216]. PML over-expression can also induce a caspase-independent form of apoptosis in cells, although the mechanism of this process has been debated [210, 217].

One major mechanism by which PML acts as a tumor suppressor is through its interaction with p53. p53 is a regulator of cell growth and survival and acts as a tumor suppressor through multiple mechanisms, including the induction of apoptosis, senescence, and cell cycle arrest [218]. In cells lacking PML, p53 activation results in a decreased amount of senescence and apoptosis compared to wild-type cells, indicating that PML is a mediator of p53 activity [219, 220]. PML can mediate p53 activity through a number of mechanisms, including the stabilization of p53 protein by inhibiting its ubiquitination and by promoting various pro-apoptotic or pro-senescent post-translational modifications, such as acetylation, phosphorylation, and sumoylation [19, 20, 221-223]. Although the relationship between PML and p53 is complex—PML can promote different modifications of p53 under different circumstances—the general consensus for PML’s activation of p53 is that PML recruits p53 to the nuclear bodies, especially under conditions of cellular stress or DNA damage, where PML can then promote p53’s association with modifying proteins and/or protect p53 from degradation [210, 224].
PML can also promote senescence through its interaction with Rb. PML physically interacts with Rb and can form a complex with Rb and a number of proteins that mediate tumor suppression [225, 226]. PML can also promote Rb-mediated transcriptional repression, and the PML-RARα fusion protein disrupts this process [227]. Additionally, the ability of Rb to repress transcription of target proteins in PML−/− MEFs is also impaired [226, 227]. Finally, more recent work shows that Rb is able to cooperate with PML to promote senescence in cells, showing that the two interact with one another in this process [228]. Overall, PML acts as a tumor suppressor by promoting apoptosis and senescence through various pathways, including p53 and Rb (Figure 16).

A study investigating the status of PML expression in various cancers and in cancer progression found that PML expression, while present in all normal tissues, was lost in a number of different cancers, including prostate, breast, colon, and lung cancer, along with some lymphomas and CNS tumors [229]. Moreover, the loss of PML expression was associated with an enhanced tumor grade and/or tumor progression in some cancers, including prostate and breast cancer and CNS tumors [229]. Multiple studies indicate that PML appears to be targeted for ubiquitination and is subsequently degraded in the proteasome in multiple cancer types [230]. This evidence verifies that PML is indeed an important tumor suppressor.

**PML and Ras**

PML was first linked to Ras in 2000 when two independent groups published studies linking PML to oncogene-induced senescence. Pearson et al. showed that PML knockdown abrogates senescence induced by oncogenic Ras and found that the activation of p53 by acetylation of Lys382 plays a crucial role in PML-induced senescence [20]. Indeed, studies using PML knockdown cell lines showed that Ras was unable to fully promote p53 acetylation at Lys382 or fully activate p53 in the absence of PML [20]. They also showed that oncogenic Ras expression increases overall PML expression via an increase in size and number of PML NBs.
Figure 16. PML Nuclear Bodies Regulate the Formation of Complexes Involved in Tumor Suppression. PML recruits p53 under conditions of cell stress and can regulate its post-translational modifications and/or stability within the cell. PML can interact with the transcription corepressor DAXX in the NBs, where DAXX can modulate apoptosis. PML can also recruit the Rb tumor suppressor and a number of other proteins to the NBs to regulate cell growth, apoptosis, and senescence.
and found that expression of either oncogenic Ras or PML induced NB localization of PML, p53, and CBP, an acetyltransferase [20].

In a paper published only a month after Pearson et al.’s, Scott Lowe’s group also explored the relationship between oncogenic Ras and PML. They too found that the expression of oncogenic Ras increased the size and number of PML NBs in IMR90 human fibroblast cells, and furthermore, they showed that PML expression is actually induced during Ras-stimulated premature senescence [19]. Providing evidence that over-expression of PML can also induce senescence, this group also showed that PML expression increases the intracellular levels of both the p53 and Rb tumor suppressors, both of which were already linked to Ras-induced senescence [9, 19, 231, 232]. Similar to Pearson et al., Lowe’s group also saw the PML NB localization of p53 and Rb upon oncogenic Ras stimulation and hypothesized that post-translational modifications of these proteins were occurring in the PML NBs [19]. Overall, these two studies laid the groundwork for establishing the relationship between Ras and PML and hinted at the complexity of the regulatory network involved in oncogene-induced senescence, areas that have been further explored in great detail in the past fifteen years.

Following the publication of Ras’s interaction with PML, a plethora of papers in the early 2000’s worked to further explore the interaction of these two proteins and to further explain PML’s involvement in senescence. Several of these studies pointed to PML’s involvement in activating p53. For example, two proteins, SIRT1 and MageA2, were found to work to inhibit p53 activation in PML NBs, mostly by antagonizing p53 acetylation there [233, 234]. Other studies indicated that oncogenic Ras actually induces PML protein up-regulation, not just PML transcription, via the Ras/MEK1/mTOR/elf4E signaling axis, a system that works independently of p53 [235].

A groundbreaking study during this time, however, revealed the importance of different PML isoforms in cellular signaling. Bischof et al. showed that although all PML isoforms can recruit p53 and CBP to PML NBs, only the PMLIV isoform is capable of inducing premature senescence in cells, and PMLIV promotes p53 acetylation on Lys382 and phosphorylation on S46, factors that may lead to p53 activation and senescence induction [22]. This study was the first to explore distinct functions of PML isoforms and was the first to identify that a single isoform,
PMLIV, is specific for senescence induction. However, the complex interplay between activated Ras and PMLIV-induced senescence remains to be fully elucidated.

1.5 - NORE1A: Is Location Critical for Ras-induced senescence?

Since 2000, Ras has been known to drive senescence through PML, but the exact mechanism of how this process occurs has not been fully elucidated [19, 20]. Two recent papers show that NORE1A is a critical mediator of Ras-induced senescence and that Ras promotes the scaffolding of NORE1A with both HIPK2 and PP1A, proteins that modify p53 and Rb, respectively, to promote senescence [16, 17]. NORE1A appears to be interacting with both HIPK2 and PP1A in small spots in the nucleus; these spots appear very similar to PML NBs. We hypothesized that Ras may be promoting the association of NORE1A with PML, specifically the PMLIV isoform, to promote senescence. The interaction of NORE1A with PMLIV would therefore provide an explanation for how Ras is able to induce senescence via both PMLIV and NORE1A: it promotes the interaction of the two, and together, they scaffold molecules that modify p53 and Rb to promote senescence.

This dissertation will show that NORE1A does indeed co-localize with PMLIV in PMLIV NBs, and the two proteins form a complex in cells that is enhanced by activated Ras. A collaborator further verified this data by showing that NORE1A endogenously interacts with PML in human liver tissue. NORE1A promotes the acetylation of PMLIV and promotes the recruitment of tumor suppressor proteins that are involved in activating senescence, namely p53, to PMLIV NBs and enhances the association of PMLIV and p53. Preliminary results show that NORE1A also enhances the association of PMLIV with Rb. These functions of NORE1A have numerous implications for how Ras may mediate protein function and activity in the cell, as PML NBs are centers of protein regulation and post-translational modifications [21]. NORE1A is a critical mediator of Ras-induced senescence through its promotion of p53 acetylation and Rb dephosphorylation [16-18], so it is possible that NORE1A must recruit these proteins to PML NBs in order to promote these modifications. Indeed, the knockdown of PML drastically reduces
NORE1A-induced senescence in lung cancer cells, suggesting that the interaction of NORE1A with PMLIV and NORE1A’s localization to PMLIV NBs are critical steps in the process of Ras-induced senescence.
2.1 – Overview

Designing experiments to explore the interactions of various tumor suppressors and their biological consequences on the cell presents numerous challenges, as most signaling pathways are not simple, “one way streets” and instead have extensive crosstalk and overlap with one another. The work presented in this dissertation involves a number of different molecular biology and cell culture techniques, and experiments were designed to isolate specific aspects of various pathways to provide insight into how NORE1A and Ras interact with and influence PML. Overall, this work aims to reveal novel functions of NORE1A as a tumor suppressor through its interaction with PML, specifically the PMLIV isoform, and to determine if localization to PML nuclear bodies is an essential component of NORE1A-mediated senescence. This section reviews the materials and methodology used in this process in detail and groups the methods used into different classes, including molecular biology, cell culture, and biological assays.

2.2 – Molecular Biology

*Polymerase Chain Reaction (PCR):* For subcloning purposes, DNA was amplified using a standard thermocycler from Bio-Rad (Model T100). PCR reactions were performed using standard PCR master mixes (Invitrogen) with Platinum Taq Polymerase (Invitrogen). Reaction parameters were as follows: an initial five minutes at 95°C to melt DNA, followed by 35 cycles of 95°C for one minute, annealing temperatures between 56°C and 64°C, and 72°C for one minute.
per one kilobase to be amplified. Annealing temperatures varied depending on the predicted annealing temperatures for specific primers. After cycling, reactions were given an elongation time of seven minutes at 72°C, and reactions were held at 4°C until needed for further processing.

**TA/TOPO Cloning:** Amplified DNA fragments were subcloned into pCR2.1-TOPO (Invitrogen) or pGEM-T-EASY (Promega) depending on the specified protocol. After TA/TOPO cloning, all PCR reactions were sequenced to ensure that the Taq polyerase did not introduce any errors during PCR amplification.

**Restriction Enzyme Digestion:** In order to alter recombinant DNA plasmids, DNA subcloning requires digestion by restriction enzymes. All restriction enzymes used were from New England Biolabs (NEB®, Inc. Restriction enzymes were used in reactions consisting of DNA, the required buffers for each specific restriction enzyme, and 1x BSA (if needed). Restriction digests were incubated for one to two hours at 37°C to allow for complete digestion of the DNA.

**DNA Ligation:** DNA ligation reactions were used to recombine two DNA fragments together. In order for this to occur, DNA fragments must first be digested with restriction enzymes to create compatible overhangs for ligation. A 3:1 molar ratio of vector DNA backbone fragment to the DNA fragment containing the gene of interest was used to maximize the efficiency of ligations. After digestion, these DNA fragments are added to the ligation reaction along with 10x ligation buffer (NEB®, Inc.) that is diluted to a final concentration of 1x. T4 DNA ligase (NEB®, Inc.) was then added to the ligation reaction mix, and the reaction was incubated overnight at 4°C.

**Agarose gel electrophoresis:** 1% agarose gels were used to separate linearized DNA fragments from restriction enzyme digests and PCR reactions by size. 1% agarose gels were made by mixing powdered agarose (SeaKem LE agarose, Lonza, Cat. number 50004) with a 1x TAE (Tris-Base, Acetic Acid, EDTA) solution, which was diluted from a 10x TAE solution (MediaTech, Cat.
number 46-010-CM). Solutions were heated in a microwave until the powdered agarose was completely dissolved, and Ethidium Bromide (Invitrogen) was added to the gel solution before casting the gel. Ethidium Bromide allows the DNA to be visualized under UV light. Gels were then exposed to DC electric current at approximately 80mA, or 110V, to allow the separation of DNA fragments. Gels were visualized using UV light. In some cases, DNA bands needed to be retrieved from the agarose gel. In these cases, the DNA bands were excised and placed into a GenElute Spin Column (Sigma, Cat. Number 56500) and were processed as specified in the protocol.

**Bacterial Transformation:** Chemically competent bacteria were obtained from Invitrogen, and different strains with different competency levels were used, including subcloning efficiency DH5α, Max Efficiency DH5α, and Chemically and Electrically Competent TOP10 E. coli (Invitrogen, Cat. numbers 18265-017, 18258-102, C5050-03, and K4850-01, respectively). Subcloning efficiency DH5α cells were used for standard, whole plasmid transformations, but for plasmid ligations or TA/TOPO cloning, higher competency bacterial cells were needed, such as Max Efficiency DH5α or TOP10 E. coli, as the total mass of a complete plasmid ligation reaction is much lower than that of a whole plasmid transformation reaction. To chemically transform plasmids into chemically competent bacteria, the bacteria cells were mixed with between 200ng and 1µg of plasmid DNA and incubated on ice for 30 minutes. After 30 minutes, the mixture was heat shocked in a 42°C water bath for 40 seconds and was then placed on ice for two minutes. 200µL of SOC media (Corning Cellgro, Cat. number 46-003-CR) was added to the mixture and placed in a 37°C water bath for one hour for outgrowth, after which the mixture was plated onto LB agar plates containing either Ampicillin (100µg/mL) or Kanamycin (50µg/mL), depending on the resistance markers on the transformed plasmid. The plates were incubated at 37°C overnight. The next day, colonies were selected and grown in LB broth media supplemented with the appropriate antibiotic (using the same concentrations as those listed earlier) and were again incubated overnight at 37°C in a shaker set at 300 RPM. After the colonies were grown and
expanded in the LB broth media, the cultures were processed, and the plasmids were extracted and purified as described below.

Plasmid Purification (Preparation): DNA plasmids were extracted and purified from E. coli cultures using Mini-Scale and Midi-Scale plasmid kits (Qiagen, Cat. number 27106 and Sigma, Cat. number NA0200-1KT, respectively). Mini scale plasmid preparations required a 5mL culture isolated from a single bacterial colony from the plasmid transformation. This culture was grown overnight in LB broth media at 37°C on a shaker set at 300RPM in the presence of the antibiotics required by the plasmid resistance marker. The 5mL culture was then pelleted using a bucket centrifuge at 4000 RPM for 15 minutes. After centrifugation, the broth media was decanted, and the bacterial pellet was resusupended in the resusupension buffer provided by the kit. Plasmid extraction and purification were then performed using the manufacturer’s protocol. For Midi-Scale plasmid preparations, a single colony was isolated as described above and grown for eight hours at 37°C in 5mL of LB containing the proper antibiotics. The 5mL culture was then added to 50mL of LB media with the proper antibiotics, and the entire mixture was incubated overnight at 37°C on a shaker set at 300 RPM. The next day, the bacterial culture was centrifuged for 15 minutes at 4000 RPM, after which the supernatant was decanted. The bacterial pellet was then resuspended in the resuspension buffer provided by the kit and was processed according the Midi-Scale kit manufacturer's protocol.

2.3 – Plasmids

pCR2.1-TOPO: Purchased from Invitrogen, this plasmid is part of a TA cloning system and has a TOPO-Isomerase attached to both ends of the pCR2.1 plasmid. This vector is designed for the insertion of PCR fragments, as it has “T” overhangs that allow base pairing with polymerized DNA fragments, which have “A” overhangs. To determine which colonies have successfully taken up the complete vector during transformations, colonies can be selected with Blue/White X-gal screening.
**pGEM-T-Easy:** Purchased from Promega, this vector system works similarly to the pCR2.1-TOPO vector, except a T4 DNA ligase must be used in the reaction instead of the vector having a bound isomerase. This vector system was used when cloning DNA fragments that were digested with the enzyme BglII, as the pCR2.1 plasmid has an internal BglII site that interferes with cloning DNA digested with that enzyme.

**pCDNA3-HA and pCDNA3-Flag:** The pCDNA3 expression vector was purchased from Invitrogen and contains a CMV promoter upstream of its multiple cloning site. Unfortunately, this vector does not contain an epitope tag, so a tag needed to be added in order for it to be useful for biological studies. Dr. Geoffrey Clark generated pCDNA3 expression vectors containing fused HA or Flag epitope tags. Thus, inserts in this vector result in the expression of proteins with either HA or Flag tags on their N-terminus.

**pEGFP-C1:** Purchased from Clonetech, this expression vector is driven by a CMV promoter. cDNAs inserted into the multiple cloning site have a fused N-terminal Green Fluorescent Protein (GFP) tag.

**pmKate2-C:** Purchased from Evrogen, this expression construct is very similar to the pEGFP-C1 vector backbone, except this construct expresses a Red Fluorescent Protein (RFP) tag that is fused to proteins generated from inserts in the multiple cloning site. pEGFP-C1 and pmKate2-C are often used in biological assays to examine protein localization by fluorescent microscopy and for co-immunoprecipitation experiments (Section 2.6).

**NORE1A:** Full-length human NORE1A was purchased from Origene and was sub-cloned into various expression constructs by Dr. M. Lee Schmidt. Dr. Schmidt PCR amplified the NORE1A cDNA clone with the following primers: "hNore1a5," with the sequence 5’ – GCAGATCTATGGCATGGCTCCCAGCCATC – 3’ and “hNore1a3," with the sequence 5’ –
This yielded a 1,273 base pair DNA fragment containing 5'-BglII and 3'-EcoRI restriction enzyme sites. The fragment was TOPO cloned into pCR2.1-TOPO for sequencing and sub-cloning. After analysis to verify the proper insertion of the complete, correct DNA sequence, 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 (Invitrogen) 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 pEGFP-C1 (GFP) and pmKate2-C (Kate) constructs that were digested with BglII and EcoRI.

PMLIV: PMLIV cDNA was a generous gift from Dr. David Bazett-Jones at the Hospital for Sick Children (Toronto, Canada). The cDNA was amplified with the following primers: “PMLF JM” 5’ – GCGGATCCATGGAGCCTGCACCCGCCCGATCT – 3’ and “PMLR JM” 5’ – GCGAATTCCTACTACTAAATTAGAAAGGGGTGGGGGTAGC – 3’. This yielded a 1,899 base pair DNA fragment containing 5'-BamHI and 3'-EcoRI restriction enzyme sites. This fragment was cloned into the pGEM vector by pGEM-T-Easy cloning for sequencing and sub-cloning. After analysis to verify the correct insertion of the complete, correct DNA sequence, the pGEM-PMLIV plasmid was digested with BamHI and EcoRI and ligated into a BamHI/EcoRI digest of a pCDNA3 mammalian expression construct (Invitrogen) containing an in-frame 5'-HA tag. The BamHI/EcoRI digested PMLIV fragment was also ligated into pEGFP-C1 (GFP) and pmKate2-C (Kate) constructs that were digested with BglII and EcoRI.

PML isoforms I-VI: Kate-tagged PML isoforms were a generous gift from Dr. David Bazett-Jones at the Hospital for Sick Children (Toronto, Canada).

H-Ras-12V: This constitutively activated H-Ras was expressed with the pCGN and Kate tags. pCGN-HA-H-Ras-12V was generated by cloning the full-lenth H-Ras-12V cDNA as a BamHI/EcoRI fragment into the pCGN-HA vector [236]. The H-Ras-12V cDNA fragment was also
sub-cloned into the pmKate2-C construct using pmKate2-C’s BglII/EcoRI restriction sites, resulting in the pmKate2-C-H-Ras-12V expression construct.

**shPML:** Four unique 29mer HuSH shRNA constructs designed to target PML (TR302401) were purchased from Origene, along with a non-effective 29mer scrambled shRNA control plasmid (TR30012). All constructs were in an untagged retroviral plasmid, pRS.

**siPML:** Control ON-TARGETplus Non-targeting pool (D-001810-10-05) and SMARTpool ON-TARGETplus siPML (L-006547-00-0005) siRNA constructs were purchased from GE Dharmacon. Sequence information for these products was not disclosed by GE Dharmacon, but both the non-targeting pool and PML SMARTpool contain four different siRNAs. The siRNAs designed against human PML are designed to knock down the gene expression of all PML isoforms.

### 2.4 – Cell lines

**HEK-293:** Obtained from ATCC, this cell line was originally derived from human embryonic kidney cells. These cells have been immortalized by exposure to sheared DNA fragments of adenovirus type 5 [237]. HEK-293 cells are useful for many experiments because they are very easy to transfect and present a functional platform for transgenic experiments. These cells are maintained in DMEM (described in the next section) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Pen-Strep antibiotic.

**HEK-293T:** Obtained from ATCC, these cells are modified HEK-293 cells that stably express the SV40 Large T Antigen, which binds SV40 enhancers and is commonly found in expression vectors to increase the production of proteins. The Large T Antigen also makes HEK-293T cells useful for transgenic studies because it suppresses the tumor suppressor activity of the p53 and Rb tumor suppressors, making the cells more tolerant to transfection with various growth
suppressive constructs. These cells are maintained in DMEM supplemented with 10% FBS and 1% Pen-Strep.

**A549:** A549 cells are human adenocarcinoma alveolar based epithelial cells derived from cancerous lung tissue by ATCC. These cells contain a KRAS point mutation (G12S) that results in the constitutive activation of KRAS signaling. A549 cells were ideal for cellular senescence studies, as they have wild-type p53 and Rb signaling pathways, which are the two main elements involved in the induction of cellular senescence. These cells are maintained in DMEM supplemented with 10% FBS and 1% Pen-Strep.

**COS-7:** Purchased from ATCC, COS-7 (CV-1 in Origin with SV40 genes) cells are a fibroblast-like cell line derived from African green monkey (*Cercopithecus aethiops*) kidney cells. This cell line was developed by immortalizing CV-1 cells with a mutant SV40 virus that produces Large-T antigen but has a defect in genomic replication [238]. Although this is not a human cell line, COS-7 cells are very useful for studying fluorescently labeled protein localization and co-localization, as they have a flat morphology. This morphology allows for optimal high resolution, live-cell imaging microscopy with reduced background fluorescence. These cells are maintained in DMEM supplemented with 10% FBS and 1% Pen-Strep.

**HBEC-3KT:** These cells are immortalized, non-transformed Human Bronchial Epithelial Cells and were a generous gift from Dr. Jerry Shay (UT Southwestern, Dallas, TX). These cells were established by infecting primary human bronchial epithelial cells with human telomerase (hTERT) and mouse cyclin dependent kinase (CDK4) retrovirus constructs and selecting with puromycin and G418 [239]. Dr. Howard Donninger created a matched pair of HBEC cell lines stably knocked down for NORE1A expression [17]. These cells are maintained in keratinocyte serum-free medium containing bovine pituitary extract and recombinant epidermal growth factor (Invitrogen).
2.5 – Cell Culture and Transfections

All cell culture was performed using a laminar flow biological safety cabinet per protocols from the University of Louisville, the Department of Environmental Health and Safety, and the Occupational Safety and Health Administration. Cells were cultured on T-25 and T-75 cell culture flasks (TPP), 100mm and 60mm cell culture dishes (Greiner Bio-One), and 6 and 12 well cell culture dishes (Greiner Bio-One). Descriptions of the media used and the different solutions used in cell culture and for transfections are listed below:

**DMEM:** Dulbecco’s Modified Eagle’s Medium was purchased from Corning Cellgro (Cat. number 10-013-CV) and is composed of 4.5g/L glucose supplemented with L-glutamine and pyruvate. The DMEM also had 10% FBS (Valley Biomedical) and 1% Penicillin-Streptomycin Antibiotic (Corning Cellgro, Cat. number 30-002-CI).

**Keratinocyte-SFM:** HBEC cells require a specific growth media formulation. Obtained from Invitrogen (Cat. number 17005-042), this medium is designed for keratinocytes and comes in a kit with Keratinocyte-SFM medium (Cat. number 10724-011) supplemented with 25mg Bovine Pituitary Extract (Cat. number 13028-014), 2.5µg human recombinant EGF (Cat. number 10450-013), and 1% Pen-Strep antibiotic (Corning Cellgro).

**Phosphate Buffered Saline (PBS):** PBS was purchased from Corning Cellgro (Cat. number 21-040-CV and #46-013-CM). PBS was used to wash cells as required.

**Trypsin EDTA 0.25%:** Trypsin (Corning Cellgro, Cat. number 25-053-CI) is used to remove adherent cells from culture vessels. Media was removed from cell culture dishes by aspiration, and cells were washed once with 1x PBS. PBS was then aspirated, and enough trypsin-EDTA solution was added to cover the bottom of the dish with a thin layer of fluid. Dishes were then placed back into the 37-degree incubator for 2-4 minutes to allow the trypsin to remove the
adherent cells from the dish. After cells were removed, the trypsin-EDTA was neutralized with supplemented culture medium, and cells were then pelleted by centrifugation for 5 minutes at 1,500 RPM. Cell pellets were resuspended in culture medium and plated as required for various applications.

Puromycin: For the selection of cells that expressed a puromycin resistance marker, Puromycin dihydrochloride (Sigma Aldrich, Cat. number P8833-10MG) was added to cell culture media at a final concentration of 1µg/mL.

RIPA Buffer: RIPA buffer (Sigma Aldrich, Cat. number R0278) was used to lyse cells when protein/protein complexes were not being investigated by co-immunoprecipitation. This buffer provides an efficient lysis of cells with excellent protein stabilization for analysis by Western blot. RIPA buffer is composed of 150mM NaCl, 1.0% IGEPAL® CA630, 0.1% SDS, 0.5% sodium deoxycholate, and 50mM Tris pH 8.0.

Cell Transfections: Cells were transfected using several different transfection reagents and methods. HEK-293 and HEK-293T cells were transfected with the JetPRIME™ (Polyplus) transfection reagent, while A549 cells were transfected with DNA-In™ (VitaScientific), a transfection reagent designed specifically for A549 cells, which can be difficult to transfet with JetPRIME™. siRNA transfections were performed using the DharmaFECT 2 (GE Dharmacon) transfection reagent. Transfection reagent details and methodology used are described below.

JetPRIME™: The JetPRIME™ (VWR, Cat. number 89137-972) DNA transfection reagent is designed to ensure high DNA transfection efficiency and is described by the manufacturer as being a novel and versatile cationic polymer-based reagent. Using the “proton sponge mechanism,” JetPRIME™ forms positively charged complexes with DNA that penetrate cell membranes through endocytosis, releasing DNA into the cytoplasm. DNA plasmids enter the nucleus when the nuclear envelope disappears during mitosis. For transfections, 1-2µg of
specific DNA plasmids were mixed in JetPrime™ Transfection Buffer. Different amounts of JetPrime™ Transfection Buffer were used depending on the size of the cell culture dish. 60mm dishes require 200µL JetPrime™ Transfection Buffer, while 35mm dishes require 100µL. 2µL of JetPRIME™ per 1µg DNA was then added to the DNA-Buffer mixture. The mixture was then vortexed for 10 seconds, briefly centrifuged, and incubated for 10 minutes at room temperature. The mixture was then added to the cell culture dish and incubated for 6-8 hours, after which culture media was removed, and fresh media was added to reduce cytotoxicity.

**DNA-In™:** The DNA-In™ A549 Transfection Reagent (VitaScientific, Cat. number MTIC3002X-73771) offers maximum transfection efficiency in A549 cells, which can be difficult to transfect with JetPRIME™. A549 cells were transfected according to the manufacturer’s protocol. Briefly, 200,000-300,000 cells were plated in 35mm dishes. Cells were transfected with plasmid DNA diluted in 100µL OptiMEM per 1µg of DNA. 3µL of the DNA-In™ reagent were added per 1µg DNA, and the solution was briefly vortexed. The solution was incubated for ten minutes before being added to the cells, which were cultured in DMEM free of antibiotics. Cell media was changed the following day to reduce cytotoxicity.

**DharmaFECT 2:** The transfection of siRNA into cultured cells requires very specific transfection reagents. The DharmaFECT 2 transfection reagent was purchased from GE Dharmacon (Cat. number T-2002-02) and is optimized for the highly efficient delivery of siRNAs at low concentrations. All siRNA transfections were performed using this reagent, and siRNA transfections were performed one day prior to transfections with DNA plasmids. Transfections were performed according to the manufacturer’s protocol. Briefly, A549 cells were plated at a concentration of 200,000-300,000 per 35mm dish. The following day, two tubes were prepared: tube 1 contained the diluted siRNA, in which 5µL of a 5mM stock siRNA solution was diluted into 95µL of serum-free, antibiotic-free DMEM. Tube 2 contained 5µL of the DharmaFECT 2 reagent diluted into 95µL of serum-free, antibiotic free DMEM. Both tubes were mixed and incubated at room temperature for 5 minutes, after which the contents of tube 1 were added to tube 2, for a
final volume of 200µL. This solution was gently mixed and was incubated for 20 minutes at room temperature. After the incubation, 800µL of antibiotic-free DMEM was added to the solution for a final volume of 1mL and a final siRNA concentration of 25nM. This media was added to the cells, and cells were incubated for 6-8 hours, after which the media was removed, cells were washed with 1x PBS, and fresh media was added to reduce cytotoxicity. Cells were incubated for a minimum of 72 hours to ensure proper protein knockdown.

2.6 – Immunoprecipitation

The co-immunoprecipitation assay is one of the best ways to detect the interaction of two proteins. This assay precipitates a specific protein using an antibody that is usually conjugated to an agarose bead, and the precipitation can be analyzed for the presence or absence of other proteins that bound to the precipitated protein. This assay must be performed consistently and carefully to minimize issues that can arise, including inadequate protein purification or cell lysis and the precipitation of non-specific proteins.

Immunoprecipitation of Over-Expressed Proteins: Cells that had been transfected were lysed 24 to 48 hours after transfection in a modified RIPA, or NP-40 buffer. This buffer consists of 50mM Tris-HCl, ph 7.4, 200mM NaCl, and 1% NP-40. Modified RIPA buffer was supplemented with a protease inhibitor cocktail (Sigma Aldrich) and 1mM sodium orthovanadate. To lyse cells, 400µL of modified RIPA buffer was added to each 60mm dish of transfected cells. Cells were then scraped and moved into microcentrifuge tubes, which were placed on a rotator for four hours at 4°C. After rotation, the samples were each passed through a 25-gauge syringe needle six times. Samples were then centrifuged at 13,000 RPM for five minutes at 4°C to separate the supernatant from the insoluble pellet containing cell debris. The protein concentration of each sample was quantified using a Bio-Rad protein quantification assay (Bio-Rad, Cat. number 500-0006). Once quantified, 1mg of total protein from each sample was added into a new microcentrifuge tube, and modified RIPA buffer was added to make the sample a final volume of
1mL. Agarose beads were added to each sample to match the specific epitope tag of one the over-expressed proteins. For HA precipitations, 5µL of HA-conjugated agarose beads were used according to the manufacturer’s protocol (Sigma Aldrich, Cat. number A2095-5ML). For Flag immunoprecipitations, 5µL of Anti-Flag M2 Affinity Gel (Sigma Aldrich, Cat. number A2220-5ML) was prepared and used according to manufacturer’s instructions. For precipitation of the GFP epitope tag, 1µL GTP-Trap® beads (Allele Biotech, Cat. number ABP-NAB-GFPA100) were used according to the manufacturer’s instructions. When using any of these conjugated agarose beads, the cell lysate solution was rotated overnight at 4°C with the conjugated beads. The next morning, the agarose was pelleted by centrifugation of the entire mixture for three minutes at 3,000RPM at 4°C. Supernatant was removed by aspiration, and the agarose beads were moved to a new microcentrifuge tube and washed with 1mL of modified RIPA buffer. This process was repeated three times in the new microcentrifuge tube. After the final wash, beads were resuspended in 10µL of RIPA buffer and 5µL of 4x LDS (Lithium Dodecyl Sulfate) sample running buffer (Invitrogen, Cat. number NP0007). Samples and lysate controls were run on SDS-PAGE gels and analyzed by Western blotting.

**Immunoprecipitation of Endogenous Proteins:** Endogenous proteins do not contain an epitope tag, so they must be immunoprecipitated differently than over-expressed proteins. Cells were lysed in modified RIPA, or NP-40, buffer; only in this case, 3mg of total cell lysate was used for immunoprecipitation at a final volume of approximately 1mL. A primary antibody against the target protein was added and rotated overnight with the cell lysate at 4°C. The following morning, an agarose conjugated secondary antibody (mouse or rabbit, Rockland Cat. number 00-8800-25 or 00-8811-25) was added and rotated for four hours at 4°C to capture the primary antibody/target protein complex. After rotation, the agarose beads were processed like the agarose beads used for over-expressed co-immunoprecipitations in that the beads were washed three times with NP-40 buffer. Beads were resuspended 10µL of RIPA buffer and 5µL of 4x LDS sample running buffer. The co-immunoprecipitation, controls, and cell lysates were analyzed by Western blot.
2.7 – Western Blotting

Sample Preparation: Protein samples were mixed with a 4x LDS Sample Buffer (Invitrogen, Cat. number NP008) containing 10% β-Mercaptoethanol (Sigma Aldrich, Cat. Number M6250-100mL) to a final concentration of 1x LDS buffer. The protein sample/LDS mixture was then heated at 95°C for 10 minutes to denature the protein sample, allowing for a more consistent separation of proteins of different sizes in the polyacrylamide gel.

SDS-PAGE: Prepared protein sample/LDS mixtures were then loaded on a pre-cast 4-12% Bis-Tris Polyacrylamide Gel (Invitrogen, Cat. Number NP0321BOX), which was immersed in 1x NuPAGE® MOPS SDS Running Buffer (Invitrogen, Cat. Number NP0001). The gel was run at 120V until the loading dye reached the bottom of the gel, after which the gel was removed and prepared for transfer onto a nitrocellulose membrane.

Nitrocellulose Transfer: The protein gel was transferred onto a 0.2µm pore size nitrocellulose membrane by electrophoresis in 1x NuPAGE® Transfer Buffer (Invitrogen, Cat. Number NP0006) supplemented with 20% methanol for 3 hours at 35V DC power.

Western Blot Detection: After transfer, the nitrocellulose filter was blocked in a 5% milk in 1x TBST solution for one hour. The blot was then incubated in primary and secondary antibodies as described in following section. After the probes with primary and secondary antibodies were finished, the blots were detected using a SuperSignal™ West Pico Chemi-Luminescent Substrate solution (ThermoFisher, Cat. Number 34080) and exposed to chemiluminescent detection film.

2.8 – Antibodies
**Mouse-Anti-HA:** This antibody against the HA epitope tag was purchased from Covance (Cat. number MMS-101P). For detecting proteins on Western blots, this antibody was diluted at 1:5,000 in a 5% milk/TBST (TBS-Tween – 50mM Tris-HCL, ph 7.4, 150mM NaCl, 0.1% Tween 20) solution, and the blot was incubated in the antibody solution overnight at 4°C. The blots were then washed with 1x TBST and probed with a Sheep-Mouse-IgG-HRP secondary antibody (GE Amersham, Cat. number NA931-1ML) diluted at 1:20,000 in 1x TBST for one hour at room temperature. Blots were detected using West Pico Enhanced Chemi-Luminescence (ECL) (Pierce, Cat. number 34080).

**Mouse-Anti-GFP:** This antibody was used to detect the GFP epitope tag and was purchased from Santa-Cruz Biotechnology (Cat. number SC-9996). For detection of proteins containing a GFP epitope tag on a Western blot, this antibody was diluted at 1:500 in a 5% milk/TBST solution. The Western blot was then incubated in the antibody solution at 4°C overnight. After incubation, the Western blot was exposed to a Mouse-IgG-HRP secondary antibody (GE Amersham) at a dilution of 1:20,000 in 1x TBST for one hour at room temperature and was detected using West Pico ECL (Pierce).

**Mouse-Anti-Flag:** Used for detection of proteins with a Flag epitope tag, this Anti-Flag antibody (Sigma Aldrich, Cat. number F1804) was diluted to 1:1,000 in a 5% milk/TBST solution and incubated with Western blots overnight at 4°C. Western blots were then exposed to a Mouse-IgG-HRP secondary antibody (GE Amersham) at a dilution of 1:10,000 in 1x TBST for one hour at room temperature and were detected by West Pico ECL (Pierce).

**Rabbit-Anti-RFP (Kate):** This rabbit polyclonal Anti-tRFP antibody (Evrogen, Cat. number AB233, 234) was used to detect the Katushka (pmKate-2-C RFP) epitope tag. For detection of Kate-tagged proteins on Western blots, this antibody was diluted to 1:2,000 in a 5% milk/TBST solution and incubated overnight at 4°C. Blots were probed with a Goat Anti-Rabbit secondary antibody
(KPL, Cat. number 474-1506) at a dilution of 1:10,000 in 1x TBST for one hour at room temperature and were detected using West Pico ECL (Pierce).

*Mouse-Anti-p53:* Obtained from Santa Cruz Biotechnology (Cat. number sc-226), this mouse monoclonal antibody detects total endogenous p53. For use in Western blots, antibody was diluted to 1:2,000 in a 5% milk/TBST solution and incubated overnight at 4°C. The next day, blots were washed and probed with a Mouse-IgG-HRP secondary antibody (GE Amersham) at a dilution of 1:10,000 with 1x TBST for one hour at room temperature. Blots were detected using West Pico ECL (Pierce).

*Mouse-Anti-Rb [4H1]:* Purchased from Cell Signaling (Cat. number 9309), this antibody was used to detect endogenous levels of total Rb protein. For detection of Western blots, this antibody was diluted at 1:1,000 in a 5% milk/TBST solution, and blots were incubated overnight in antibody at 4°C. Blots were then exposed to a Mouse-IgG-HRP secondary antibody (GE Amersham) at a dilution of 1:10,000 in 1x TBST for one hour at room temperature and were detected by West Pico ECL (Pierce).

*Rabbit-Anti-PML:* This antibody detects endogenous PML protein and was purchased from Novus Biologicals (Cat. number NB100-59787). For PML detection on Western blots, this antibody was diluted at 1:2,000 in a 5% Bovine Serum Albumin (BSA, Sigma Aldrich, Cat. number A3059-100G)/TBST solution and incubated with the Western blot membrane overnight at 4°C. Blots were probed with a Goat Anti-Rabbit secondary antibody (KPL) at a dilution of 1:10,000 in 1x TBST for one hour at room temperature and were detected using West Pico ECL (Pierce). For endogenous immunoprecipitation of PML, a different rabbit polyclonal antibody was used. This antibody was purchased from Proteintech (Cat. number 21041-AP), and 5µg of the antibody was used to immunoprecipitate endogenous PML protein.
Rat-Anti-Mouse-HRP TrueBlot Secondary Antibody: A major problem for Western blots of co-immunoprecipitation studies is the detection of heavy and light IgG chain bands. These bands are especially a problem when using agarose-conjugated beads that are bound with mouse or rabbit secondary antibodies, so Rockland’s TrueBlot secondary antibody is an HRP-conjugated immunoblotting detection reagent that does not detect these heavy and light chain IgG bands, yielding much cleaner Western blot results. In some cases, this Mouse TrueBlot antibody (Rockland, Cat. number 18-8817-33) was used instead of the standard HRP-mouse secondary described above. When used, this antibody was diluted at 1:1,000 in a 5% milk/TBST solution and incubated with the Western blot for one to two hours at room temperature before being detected with West Pico ECL (Pierce).

Mouse-Anti-Rabbit TrueBlot Secondary Antibody: This Rabbit-HRP secondary antibody (Rockland, Cat. number 18-8816-33) was used in similar instances as the Mouse TrueBlot secondary antibody described above, as it also does not detect heavy and light chain IgG bands from Rabbit secondary antibody-conjugated agarose beads. This antibody was used at a 1:1,000 dilution in a milk/TBST solution and was incubated with Western blots for one to two hours at room temperature before being detected with West Pico ECL (Pierce).

2.9 – Biological Assays

Senescence Assay: A hallmark of cells that have undergone senescence is an increase in β-galactosidase activity. BioVision’s Senescence Detection Kit (Cat. number K320-250) detects β-galactosidase activity histochemically in cultured cells and tissue sections. A549 cells were plated in 35mm dishes at 200,000 cells per dish. The next day, cells were transfected with various expression constructs to analyze their effects on senescence induction. Fresh media was added to the cells every 24 hours for 72 hours post-transfection, after which, cells were processed using the Senescence Detection Kit according to the manufacturer’s protocol. To score assays, cells in five random fields of view per dish were counted, and a ratio was created of...
the number of β-galactosidase activity positive (blue) stained cells to the total number of cells per field of view. This gives a percentage of cells undergoing senescence. For cells first transfected with PML siRNAs, siRNAs were transfected first, and the transient transfection of NORE1A was performed on cells after 24 hours. Cells were then incubated an additional 72 hours before staining for β-galactosidase activity.

2.10 – Image Acquisition, Image Processing, and Statistical Analysis

*Image Acquisition and Processing:* Images were scanned and digitized using a Pharos FX Molecular Imager from BioRad prior to quantification using Quantity One software (BioRad). Figures were created using either Adobe Photoshop software (Adobe) or Powerpoint (Microsoft).

*Statistical Analysis:* All data are reported as mean +/- standard deviation. A two-sided Student’s t-test was used to determine differences between treatment groups when appropriate. Data was considered statistically significant when p<0.05. Statistical analyses were performed using Excel (Microsoft).
CHAPTER III

NORE1A INTERACTS WITH PMLIV IN A RAS-DEPENDENT MANNER

3.1 - Introduction

Ras is the most commonly mutated gene in human cancer, as Ras mutations are found in over 30% of human tumors [240]. Ras promotes cell growth, proliferation, and survival through three major mitogenic pathways, the MAP Kinase, PI3K/Akt, and Ral-GDS pathways [3, 4]. Although numerous attempts have been made to inhibit both Ras itself and downstream Ras signaling pathways in cancer, there are no effective inhibitors that completely block Ras’s effects, thus making mutationally activated Ras a major problem in cancer [5]. In addition to its growth promoting effects, however, Ras also has the paradoxical ability to inhibit cell growth and promote cell death by activating senescence and apoptosis, thus preventing tumorigenesis [8, 9]. Ras signaling thus exists as a delicate balance, one that can be easily tipped towards transformation or cell death depending on other events that occur in the cell [8]. This balance exists because Ras interacts with a large number of effector proteins that promote a wide range of functions [3, 4].

In addition to interacting with multi-member families of mitogenic signaling effectors, Ras interacts with effectors that promote anti-mitogenic effects. One of these Ras effector protein families, the RASSF tumor suppressor family, contains proteins that promote Ras-induced growth arrest, apoptosis, and senescence [3, 122]. The first member of this family identified, Novel Ras Effector 1A (NORE1A), or RASSF5, has been strongly implicated as a tumor suppressor, as it is frequently down regulated in cancer cells by epigenetic mechanisms and shows decreased expression in malignant liver tumors compared to normal liver tissue [14, 140]. Additionally, the
NORE1A gene experiences an inactivating mutation in a familial form of clear cell renal carcinoma [13].

NORE1A binds directly to Ras through its RA, or Ras Association, domain [124], and it is thought to act as a molecular scaffold, as it has no apparent enzymatic activity. NORE1A has pro-apoptotic properties and is hypothesized to connect Ras signaling to the pro-apoptotic Hippo pathway [11, 124], and NORE1A expression is often lost in tumors with increased Ras activity [14]. Knockdown of NORE1A in MEFs also greatly sensitizes them to transformation by oncogenic Ras [162], suggesting that the loss of NORE1A in premalignant or malignant cells shifts the balance of Ras signaling towards pro-growth, anti-apoptotic pathways. In terms of mediating Ras-induced anti-mitogenic effects, NORE1A is multifaceted. In addition to linking Ras to pro-apoptotic pathways, NORE1A also mediates Ras-induced senescence and promotes post-translational modifications, including acetylation, phosphorylation, and ubiquitination, of a number of proteins by promoting their association with acetyltransferases, kinases, phosphatases, and ubiquitin ligases [11, 16-18, 23, 124, 130, 141].

In the cell, NORE1A is mainly localized in the nucleus, where it forms small spots [17], but no one knows what these spots are or why NORE1A localizes in that particular nuclear component. Under Ras stimulation, NORE1A also forms complexes with tumor suppressors in these regions [16, 17], indicating that this may play an important role in NORE1A’s function as a tumor suppressor. One of the most famous proteins that forms nuclear spots, or nuclear bodies, is also an important mediator of Ras tumor suppression: the Promyelocytic Leukemia protein, or PML [19, 20].

PML forms distinct nuclear domains called PML nuclear bodies (NBs) that are highly dynamic structures composed of a number of different proteins, the primary component of which is PML [21, 198]. PML NBs can change in size and number in response to cell stress or at different stages of the cell cycle, and NBs mediate a number of critical functions within the cell, including protein post-translational modifications, apoptosis, senescence, and transcriptional regulation [21, 198]. Based on the role of PML NBs in the cell and because PML expression is often lost in tumors, PML is known as a tumor suppressor [21, 198, 208-210, 215, 224, 229, 230].
Moreover, PML is a critical mediator of Ras-induced senescence, and its presence in the cell is required for the process to occur [19, 20]. A specific isoform of PML, PMLIV, is the only PML isoform capable of promoting senescence on its own [22]. I observed that fluorescently tagged NORE1A and PMLIV co-localize in PML NBs. This evidence led to the hypothesis that PMLIV is a novel effector of NORE1A/Ras signaling. I hypothesized that NORE1A may associate with PMLIV in NBs, and the two proteins may work together to promote anti-mitogenic effects of Ras.

Data from a collaborator showing that PML interacts with NORE1A endogenously in human liver tissue supported this idea. Additionally, a yeast two-hybrid screen performed using NORE1A identified TRIM25 and two other TRIM proteins as binding partners of NORE1A (unpublished data). TRIM proteins are identified by their RBCC/TRIMs, or TRIpartite Motifs, consisting of a RING-finger, two B-boxes, and a coiled-coil domain [241]. PML contains a RBCC/TRIM motif and is also referred to as TRIM19 [211, 242, 243], so the interaction of NORE1A with other TRIM proteins could further support the interaction between NORE1A and PMLIV, or TRIM19. I expected that the presence of activated Ras would further enhance the interaction of these two proteins. PML is also heavily regulated by post-translational modifications within the cell [21, 205, 206, 209, 230, 244, 245], and NORE1A has recently been shown to affect protein stability and protein post-translational modifications [16, 17, 23, 130]. I therefore examined whether NORE1A was capable of influencing PML stability in the cell and whether NORE1A influenced PMLIV post-translational modifications.

3.2 – Results

NORE1A Co-localizes with PMLIV

NORE1A localizes to the nucleus in most studies, where it forms small spots and complexes with other tumor suppressor proteins [16, 17]. One study also reports that NORE1A appears to shuttle between the nucleus and the cytoplasm, although it is primarily detected in small spots in the nucleus [246]. PML is a protein that is well known for forming large nuclear aggregates of various proteins, the major component of which is PML, hence the term, PML
nuclear bodies (NBs) [21]. To determine if NORE1A nuclear “spots” were indeed part of PML NBs, I transfected GFP-tagged PMLIV and Kate-tagged NORE1A into Cos-7 cells (Figure 17). On its own, NORE1A formed small nuclear spots, while PMLIV formed large NBs. When the two were transfected together, however, there was an obvious co-localization of the two proteins in the nucleus in what appear to be PML NBs, and in some cells, the NBs appeared to have a smaller size than when PMLIV was expressed alone. Some co-localization also appeared to occur outside of the nucleus. PML has been implicated in playing a role in apoptosis at junctions of the endoplasmic reticulum and mitochondria called mitochondrial associated membranes (MAMs) [247, 248], so perhaps, there is some co-localization of these two proteins outside of the nucleus, although the vast majority of the co-localization appears to occur in the PML NBs.

**NORE1A Associates With PMLIV In A Ras-Dependent Manner**

In order to determine if NORE1A was indeed associating with PMLIV in a complex and not just co-localizing in cells, co-immunoprecipitation studies using over-expressed proteins were performed. Ras has been shown to promote the association of NORE1A with a number of tumor suppressors [16, 17], so it is possible that activated Ras signaling drives NORE1A to interact with PMLIV, where it scaffolds numerous proteins together to mediate anti-mitogenic effects of Ras. HEK 293 cells were transfected with PMLIV and NORE1A in the presence or absence of activated Ras. I found that NORE1A does indeed complex with PMLIV, and this interaction appears to be enhanced by the presence of activated Ras (Figure 18). Experiments were repeated in triplicate.

Additionally, a collaborator found that NORE1A endogenously associates with PML in human liver tissue (Figure 19), confirming that this interaction is physiologically relevant. Although attempts were made at obtaining an endogenous co-immunoprecipitation of these two proteins in human cancer cell lines, this was not possible because we currently lack a reliable NORE1A antibody. The NORE1A antibody used by our collaborator was generated and used many years ago, and, although it was a successful antibody at the time, it is no longer available.
Figure 17. **NORE1A Co-localizes with PMLIV in PMLIV Nuclear Bodies.** COS-7 cells were transfected for 24 hours with GFP-tagged PMLIV and Kate-tagged NORE1A in the presence or absence of one another. NORE1A and PMLIV strongly co-localize in the nucleus (bottom panel), but there also appears to be a small amount of co-localization outside of the nucleus. Representative images were captured using an IX50 inverted system microscope (Olympus) and a SPOT camera (Diagnostic Instruments, Inc.).
Figure 18. NORE1A Interacts With PMLIV in a Ras-Enhanced Manner. HEK 293 cells were transfected with HA-tagged NORE1A, GFP-tagged PMLIV, and Kate-H-Ras12V. After 48 hours, cells were lysed, and lysates were co-immunoprecipitated using anti-GFP conjugated agarose beads. A representative Western blot of experiments performed in triplicate is shown. Although NORE1A interacts with PMLIV without the presence of activated Ras, the presence of Ras greatly enhances this interaction.
Figure 19. NORE1A and PML Associate Endogenously in Normal Human Liver Tissue.

Representative co-immunoprecipitation analysis of NORE1A and PML in normal human liver tissue samples is shown. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Inc.) according to the manufacturer’s protocol. NC: negative control. For negative controls, the antibodies against NORE1A (rabbit polyclonal, Abcam) and PML (goat polyclonal, Santa Cruz Biotechnology) were neutralized prior to immunoprecipitation by a preincubation of two hours at room temperature with the respective immunogen peptide, which resulted in the inhibition of immunoprecipitation. These results were provided by Dr. Diego Calvisi, University of Sassari, Italy.
commercially, and attempts to use commercially available NORE1A antibodies were unsuccessful, as most available antibodies were not capable of detecting endogenous or over-expressed NORE1A. A rabbit polyclonal NORE1A antibody designed by Geoffrey Clark’s laboratory proved successful upon its initial use [16, 17], but it appears to have degraded over time and after being frozen, as it generates a number of non specific bands upon Western blotting that prevent the detection of endogenous NORE1A.

**NORE1A Co-localizes Specifically With The PMLIV Isoform in Nuclear Bodies**

There are six different PML isoforms, all of which have a conserved N-terminal domain that contains the RBCC motif [214]. PML isoforms undergo alternative splicing of the C-terminal region, and different isoforms mediate unique functions and can interact with different proteins in the cell [211, 214]. To determine if NORE1A co-localizes with PML in general or if this was a specific effect with PMLIV, co-transfections using GFP-tagged NORE1A and Kate-tagged PML isoforms were performed in COS-7 cells (Figure 20). NORE1A only forms obvious nuclear bodies with PMLIV. Some co-localization was observed in cells transfected with other PML isoforms, but NORE1A did not form obvious nuclear bodies in those cells, suggesting that its co-localization with PMLIV may occur specifically in the nuclear bodies.

To investigate if NORE1A can associate with different PML isoforms, co-transfections of NORE1A and the six PML isoforms were performed for co-immunoprecipitation experiments. These results clarified the co-localization data and show that NORE1A associates with multiple PML isoforms (Figure 21). It is impossible to tell if NORE1A associates with different PML isoforms more strongly than PMLIV because of variations in the total expression levels of the PML isoforms and of NORE1A itself. Additionally, the PMLV construct expressed at very low levels compared to the other PML isoforms, so NORE1A may associate with PMLV, but this could not be determined by these experiments.

The difference in the co-immunoprecipitation studies, which show that NORE1A can associate with most PML isoforms, and the co-localization studies, which show that NORE1A co-localizes specifically with the PMLIV isoform in nuclear bodies, may be explained by PML’s
Figure 20. NORE1A Co-localizes Specifically with PMLIV (continued on the next page).
Figure 20, continued. NORE1A Co-localizes Specifically with PMLIV. GFP-tagged NORE1A was co-transfected into COS-7 cells with different Kate-tagged PML isoforms. Representative images were obtained using an EVOS® digital inverted microscope (AMG). Bar represents 100µm.
Figure 21. NORE1A Associates with Multiple PML Isoforms. Co-transfections of HA-tagged NORE1A with Kate-tagged PML isoforms were performed in HEK-293T cells. Cells were lysed, and lysates were immunoprecipitated (IP) using HA-conjugated agarose beads and immunoblotted (IB) for the indicated proteins.
localization outside of the nucleus. Based on the strong co-immunoprecipitation results for NORE1A and various PML isoforms, it is likely that NORE1A may only specifically localize to PMLIV NBs, but it may interact with the other PML isoforms outside of the NBs. NORE1A and these isoforms may be interacting in more diffuse areas of the cell that are simply not detectable by fluorescence microscopy. Subcellular fractionation studies have shown that there is a small pool of intracellular PML that is located in the cytoplasmic compartment [249], and this would likely not be detectable by fluorescent microscopy. Therefore, NORE1A likely co-localizes with PMLIV in the PMLIV NBs, but it likely associates with most PML isoforms at other locations in the cell.

*NORE1A Knockdown Does Not Alter PML Protein Levels*

Recent studies show that NORE1A is able to alter the stability of some proteins in the cell [16, 23, 130]. It is thought to do so by mediating protein interactions with specific ubiquitin ligases, thus promoting the stability of these proteins by inhibiting their ubiquitination [16, 23, 130]. PML is a protein that experiences a number of post-translational modifications that promote the formation of nuclear bodies, and PML ubiquitination and degradation can be mediated by several pathways during tumorigenesis [21, 205, 206, 209, 230]. Additionally, activated Ras has been reported to increase levels of PML in the cell [19]. I hypothesized that the presence of NORE1A would increase the protein levels of PML in the cell by preventing its ubiquitination. To assess this, a matched pair of HBEC-3KT shNORE1A and scrambled vector shRNA cells was used. HBEC-3KT cells are a non-virally immortalized, non-transformed human bronchial epithelial cell line [239]. Dr. Howard Donninger created a matched pair of these cells that had either stable NORE1A knockdown or a control scrambled shRNA vector and validated the cells for NORE1A knockdown [17]. Cells were lysed and examined for PML expression levels. NORE1A did not influence the intracellular levels of PML (*Figure 22*). It should be noted, however, that there are not any commercially available antibodies specific for different PML isoforms. PMLIV is not the most abundant isoform in the cell [214], so NORE1A may specifically increase PMLIV stability, but this is not detectable at an endogenous level.
Figure 22. NORE1A Knockdown Does Not Alter PML Protein Levels, but NORE1A Does Increase PMLIV Acetylation.  

**A.** HBEC-3KT cells that have been stably knocked down for NORE1A were lysed and examined for PML expression by Western blot.  

**B.** HA-tagged PMLIV and Flag-tagged NORE1A were transfected into HEK 293 cells. After 48 hours, cells were lysed, and lysates were immunoprecipitated (IP) with acetyl-lysine conjugated agarose beads and immunoblotted (IB) for the indicated proteins. A representative Western blot is shown.  

**C.** Duplicate experiments were analyzed by densitometry to quantify PML knockdown. Error bars represent standard deviation.
However, NORE1A does appear to influence a post-translational modification of PMLIV: acetylation. Protein acetylation is an important post-translational modification that can influence a number of important biological processes, including chromatin remodeling, nuclear transport, cell cycle regulation, and DNA replication, and acetylation is an important regulatory mechanism for a large number of proteins [250]. NORE1A has recently been implicated as a novel mediator of Ras-induced protein acetylation, as NORE1A promotes acetylation of both p53 [17] and Rb (unpublished observations). Here, I show that NORE1A also increases PMLIV acetylation. PML can be acetylated on four major lysine residues, but the function of PML acetylation is largely unknown [251, 252].

HEK 293 cells transfected with NORE1A and PMLIV were lysed, and lysates were immunoprecipitated using acetyl lysine-conjugated agarose beads. The presence of NORE1A increases the amount of acetylated PMLIV (Figure 22), so this result provides another example of NORE1A promoting the acetylation of a tumor suppressor protein. Attempts to see if NORE1A influences the endogenous levels of acetylated PML were unsuccessful, probably because there are not any available isoform-specific antibodies for PMLIV. Again, PMLIV is not the most abundantly expressed PML isoform [214], so detecting changes in its acetylation at an endogenous level may not be possible if NORE1A is only effecting the acetylation of that isoform.

PML levels can also be influenced by a post-translational modification called sumoylation, in which one or multiple small ubiquitin-like modifier (SUMO) proteins are covalently attached to one of several lysine residues of PML [21]. PML sumoylation is crucial for the formation of PML NBs, and sumoylation generally promotes PML stability, although there has been a report of PML sumoylation preceding its ubiquitination and degradation [21, 210, 244, 245, 253, 254]. Since NORE1A did not influence the levels of PML protein in cells, I did not expect to see NORE1A altering PMLIV sumoylation by the SUMO-1 protein, and this was indeed the case in over-expression studies using a tagged SUMO-1 protein (results not shown). Thus, NORE1A does not appear to influence PML protein levels or sumoylation, but it does increase PMLIV acetylation.

3.3 – Discussion
NORE1A is a Ras effector, directly binding to Ras via the NORE1A RA domain and acting as a tumor suppressor by scaffolding Ras to anti-mitogenic signaling pathways [11, 16-18, 124]. NORE1A expression is also lost frequently in tumors [14], providing an explanation for what tips the balance of Ras signaling towards cell growth and proliferation in cancer. In the cell, NORE1A can be seen in small nuclear spots, and NORE1A can also be seen interacting with various tumor suppressors in these regions [16, 17]. No one has currently examined what these nuclear spots are and whether NORE1A’s localization in them is important for NORE1A tumor suppression.

I hypothesized that NORE1A’s location in the cell is a critical part of its function as a tumor suppressor, as NORE1A may scaffold different proteins in these nuclear bodies to mediate its anti-mitogenic effects. PML is a protein that forms distinct nuclear bodies (NBs) composed primarily of PML, and PML NBs mediate a number of important cellular functions, including protein post-translational modifications, transcriptional regulation, apoptosis, and senescence [21]. PML also has a connection with Ras signaling, as activated Ras induces PML expression, and PML itself is required for some anti-growth effects of Ras [19, 20]. I therefore hypothesized that NORE1A and PML, specifically the isoform PMLIV, which is the only PML isoform that plays a role in Ras-induced growth arrest [22], interact with one another. Since NORE1A is also capable of mediating the post-translational modifications and stability of several proteins [16, 17, 23], I also sought to examine if NORE1A could influence post-translational modifications or the overall protein stability of PML.

These results, in addition to the endogenous data from our collaborator, show that NORE1A and PMLIV do indeed co-localize and interact with one another, and this association is enhanced by activated Ras. This interaction has numerous implications for both proteins, as NORE1A may be recruiting proteins to PMLIV NBs to mediate its anti-mitogenic effects. This work also identifies NORE1A as a novel interaction partner of PMLIV, adding further complexity to PMLIV’s relationship with Ras. In addition to Ras enhancing overall cellular levels of PML [19], Ras also appears to increase PMLIV’s interaction with other tumor suppressors [19, 20].
NORE1A is a critical Ras effector that promotes the activation of a number of tumor suppressors, including p53 and Rb [16, 17]. This data suggests that NORE1A is acting to recruit tumor suppressors to PML NBs, where they can be activated. These results also imply that for Ras-induced NORE1A tumor suppression, NORE1A’s intracellular location may be crucial.

Additionally, NORE1A increases PMLIV acetylation, a lesser-studied post-translational modification of PML. Acetylation is an important post-translational modification that is crucial for a number of cellular processes, including DNA repair and cell cycle control [250, 255]. Two major tumor suppressors, p53 and Rb, are heavily regulated by acetylation and can be acetylated and activated by histone acetyltransferases like PCAF and CBP/p300 or deacetylated and inactivated by histone deacetylases like SIRT1 [17, 20, 255-257]. Dysfunction of acetylation has been linked to several disease states, including cancer [258]. Protein acetylation is therefore suggested to be just as important of a regulatory process as other post-translational modifications, including phosphorylation [250]. This is an important finding clinically, as histone deacetylase (HDAC) inhibitors exist and have been shown to induce apoptosis in cancer cells [259]. Recently, Ras has been implicated as playing an important role in protein acetylation, as it also mediates the acetylation and activation of p53 [17] and Rb (unpublished observations) via NORE1A, and HDAC inhibitors have been shown to activate apoptosis through activated Ras in cancer [259].

PML acetylation has not been well studied but has mainly been examined in the context of HDAC inhibitors, in which PML acetylation in HeLa cells is required for HDAC inhibitors to successfully mediate apoptosis [251]. Increased PML acetylation results in increased PML sumoylation in HeLa cells [251]; however, I did not observe NORE1A altering PMLIV sumoylation, even though it promotes PMLIV acetylation. PML sumoylation is required for PML NB formation [253], but the effects of PML acetylation on PML NB formation are unknown. Perhaps, PML acetylation does not enhance sumoylation enough to promote NB formation, or perhaps, PML acetylation does not influence PML NB formation and instead influences the PML protein interactome. This is certainly an area that warrants further investigation, especially since this provides a new example of NORE1A enhancing protein acetylation, which is a very recent and exciting area of RASSF protein research [17]. Further studies are needed to determine if
Ras also influences PMLIV acetylation and if NORE1A and Ras synergistically increase PMLIV acetylation.

NORE1A knockdown also did not alter overall PML protein levels, although it has been shown to stabilize a number of proteins in other studies [16, 17, 23]. NORE1A has been shown to interact with the E3 ubiquitin ligase MDM2 and with the recognition component of the SCF$^{β-TRCP}$ E3 ubiquitin ligase, β-TrCP [23, 130, 158]. Although PML can interact with MDM2, it does so primarily in the context of cell stress and DNA damage, in which it sequesters MDM2 in the PML NBs to promote p53 stability [222]. NORE1A may simply not affect the ubiquitin ligases that target PMLIV for degradation, or we may not be able to detect a specific effect of NORE1A on PMLIV stability without an isoform-specific PMLIV antibody. Instead of altering protein stability, NORE1A may act as a scaffolding molecule and enhance the interaction of PMLIV with various tumor suppressor proteins, an effect that will be explored in the following section.
CHAPTER IV

SPATIAL CONTROL OF RAS SENESCENCE SIGNALING ELEMENTS

4.1 – Introduction

I identified the novel interaction of NORE1A and PMLIV and found that NORE1A localizes to PMLIV nuclear bodies (NBs), and the interaction of these proteins is enhanced by activated Ras. Additionally, although NORE1A associates with multiple PML isoforms, it only co-localizes in NBs with PMLIV, suggesting that the association of PMLIV NBs and NORE1A is specific. As different PML isoforms mediate unique processes in the cell [211, 214], this is a potentially significant finding. PMLIV NBs may be required for NORE1A to mediate downstream effects of Ras signaling. Additionally, although NORE1A does not alter total PML stability or PMLIV sumoylation, it does appear to increase PMLIV acetylation. PMLIV acetylation has not been well studied, but there are some reports that indicate that overall PML acetylation is required for PML sumoylation and for PML tumor suppressor functions [251]. PML acetylation is hypothesized to be a requirement for PML NB formation because it is required for PML sumoylation, and PML must be sumoylated in order to form NBs [251]. This has not, however, been confirmed, nor did I observe NORE1A having an obvious effect on PMLIV NB numbers or on PML sumoylation. Potentially, however, NORE1A’s localization to PMLIV NBs and promotion of PMLIV acetylation has a different effect: I hypothesize that NORE1A may play a key role in recruiting pro-senescent tumor suppressors to PMLIV NBs. Additionally, NORE1A’s association with PMLIV may be a critical aspect of NORE1A and PMLIV tumor suppression, especially in the context of Ras signaling.
Ras’s ability to induce senescence, which is a state of permanent cell cycle arrest, has numerous implications for the role that Ras signaling plays in tumor development. For example, endogenous mutationally activated Ras can be found in both premalignant and malignant tumors, but senescent cells are only seen in premalignant samples [148]. Ras-induced senescence is thus a pathway that must be escaped during tumor development [148, 240]. Additionally, activating senescence in tumors presents a potential therapeutic strategy, as this would prevent the growth and progression of Ras-driven tumors [240].

PMLIV and NORE1A are both critical mediators of Ras-induced senescence. Early studies revealed that PML, specifically, the isoform PMLIV, is required for the induction of senescence [19, 20, 22], and more recent work on NORE1A shows that NORE1A mediates Ras-induced senescence through two major pathways, p53 and Rb [16-18]. Activated Ras promotes the localization of p53 and Rb to PML NBs, where PML promotes post-translational modifications, such as p53 acetylation at Lys382, that promote senescence [19, 20, 22]. Hypophosphorylated Rb can also be detected in PML NBs, and the presence of exogenous PML in cells has been shown to promote Rb hypophosphorylation, indicating that PML actives Rb [19].

NORE1A’s mediation of Ras-induced senescence also involves the activation of p53 and Rb [16, 17]. Upon stimulation by activated Ras, NORE1A complexes with HIPK2 [17], a protein kinase that can indirectly promote p53 acetylation on Lys382 by promoting its association with the acetyl transferases CBP/p300 and PCAF [256, 260]. Intriguingly, HIPK2 forms a complex with p53 and CBP/p300 in PML NBs [260]. Work by Donninger and colleagues showed that NORE1A co-localizes with HIPK2 in small aggregations in the nucleus [17], and I have shown that these nuclear aggregations appear to be PML NBs. NORE1A may therefore be a critical scaffold that localizes tumor suppressors to PML NBs. NORE1A also mediates Rb activation by dephosphorylation by promoting its association with the phosphatase PP1A, and again, NORE1A co-localizes with PP1A in small nuclear aggregations that are likely PML NBs [16]. By mediating senescence through both the p53 and Rb tumor suppressor pathways, which are the two main pathways involved in senescence, NORE1A thus acts as a double barreled Ras senescence effector [18]. Based on this information, I hypothesized that the spatial localization of NORE1A is
critical for its tumor suppressor function and that NORE1A acts as a scaffold to mediate the recruitment of various tumor suppressors involved in Ras-induced senescence, specifically p53 and Rb, to PMLIV NBs. This implies that the presence of PML and PML NBs may be critical for NORE1A tumor suppression, as NORE1A may require localization to PML NBs in order to scaffold Ras to downstream signaling pathways. Without PML NBs as centers of organization and protein interactions and modifications, NORE1A may not be capable of inducing senescence.

4.2 – Results

NORE1A Increases PMLIV’s Association With p53 and Increases PMLIV/p53 Co-localization in Nuclear Bodies

Previous studies have shown that NORE1A increases endogenous nuclear levels of p53 [123]. Here, I show that this p53 is localizing to PMLIV NBs. GFP-tagged p53 and Kate-tagged PMLIV were transfected into COS-7 cells with and without NORE1A and activated H-Ras. p53 could be seen in the nucleus in the presence of PMLIV, but this result was not the case in every cell, and p53 transfected with just vector was distributed in both the nucleus and cytoplasm. When NORE1A was co-transfected with these proteins, however, there was an obvious shift in p53 localization. PMLIV and p53 co-localized in PMLIV nuclear bodies, and NORE1A appeared to increase the size of PMLIV NBs. NORE1A is likely promoting PML aggregation with a number of proteins. When activated Ras was added to the cells with NORE1A, there was an even more obvious co-localization of p53 in PMLIV NBs, with the majority of the intracellular p53 co-localizing with PMLIV (Figure 23).

To confirm if NORE1A influences PMLIV’s association with p53, co-immunoprecipitations were performed using HA-tagged PMLIV with or without Flag-tagged NORE1A and Kate H-Ras12V. PMLIV was immunoprecipitated using anti-HA-conjugated agarose beads (Sigma Aldrich), and the co-immunoprecipitation of endogenous p53 protein was analyzed by Western blot. PMLIV did form a weak association with p53 without the presence of NORE1A or activated Ras, and activated H-Ras increased the interaction of PMLIV with p53. Activated Ras is known
Figure 23. NORE1A and Ras Enhance the Co-localization of PMLIV with p53. GFP-tagged p53 and Kate-tagged PMLIV were co-transfected into COS-7 cells with or without HA-tagged NORE1A and HA-tagged H-Ras12V. The presence of NORE1A and H-Ras12V enhances the localization of p53 into PMLIV NBs. Representative images were captured using an IX50 inverted system microscope (Olympus) and a SPOT camera (Diagnostic Instruments, Inc.).
Preliminary Results Indicate that NORE1A Increases PMLIV’s Association with Rb

NORE1A is a double barreled Ras senescence effector in that it mediates Ras-induced senescence through both the p53 and Rb pathways [16-18]. I generated strong evidence that NORE1A promotes the co-localization of p53 with PMLIV in PMLIV NBs, suggesting that NORE1A may interact with both p53 and Rb in these structures, thereby promoting post-translational modifications that induce senescence. To test this hypothesis, GFP-tagged PMLIV and HA-tagged NORE1A were co-transfected into HEK 293 cells. Cell lysates were immunoprecipitated with GFP-Trap® agarose beads (Allele Biotech), and co-immunoprecipitation of endogenous Rb was analyzed via Western blot. Preliminary results show that NORE1A does indeed enhance the association of PMLIV with endogenous Rb, supporting the hypothesis that NORE1A may be promoting the localization of p53 and Rb to PMLIV NBs (Figure 25). Future studies are needed to determine effect of Ras on this interaction.

This also shows that NORE1A promotes the localization of known tumor suppressors previously identified to interact with PML to PML NBs. NORE1A may therefore be acting as a molecular scaffold, enabling the association of PML, specifically PMLIV, with proteins involved in senescence and potentially apoptosis. To determine if NORE1A simply increases the interaction
Figure 24. NORE1A Promotes the Association of PMLIV with p53. Co-transfections of NORE1A, PMLIV, and activated Ras were performed in HEK-293 cells. Cells were lysed, and lysates were immunoprecipitated (IP) using HA-conjugated agarose beads and immunoblotted (IB) for the indicated proteins. A representative Western blot is shown. Experiments were repeated in duplicate.
Figure 25. **NORE1A Promotes the Association of PML IV and Rb.** GFP-tagged PML IV and HA-tagged NORE1A were transfected into HEK 293 cells. Cells were lysed, and lysates were immunoprecipitated (IP) with GFP-Trap® agarose beads and immunoblotted (IB) for the indicated proteins.
of PMLIV with proteins non-specifically, I examined NORE1A’s effects on the association of PMLIV with another known PMLIV interaction partner, the Yes-associated protein, or YAP.

**NORE1A Does Not Effect PMLIV’s association with YAP**

The Yes-associated protein, or YAP, is complex in that it can be classified as an oncogene but also has pro-apoptotic effects through its interaction with PML. YAP is classically known as an oncogene and is tightly regulated by the Hippo tumor suppressor pathway [261]. Originally identified in *Drosophila*, the mammalian Hippo pathway is normally involved in organ growth and development but can be dysregulated in cancer [261-263]. The Hippo pathway consists of a core kinase cassette of the MST1/2 and LATS1/2 kinases that, upon stimulation by upstream factors like cell density, promote the phosphorylation of the downstream effector YAP [261, 264]. This results in the cytoplasmic retention and subsequent ubiquitination and degradation of YAP [264]. When YAP is not inhibited by phosphorylation, it translocates to the nucleus, where it promotes the transcription of a number of genes involved in promoting cell growth and preventing apoptosis [261]. YAP expression is elevated in some cancers, and it has been linked to more malignant tumors and to the promotion of metastasis [263, 264].

Although NORE1A has not been shown to bind YAP, it does bind MST1, a negative regulator of YAP [124, 141]. NORE1A scaffolds MST1 to Ras, and MST1 phosphorylation is increased when bound to activated Ras via NORE1A [141]. NORE1A is therefore hypothesized to interact with YAP via MST1 and the Hippo pathway, but this has not been confirmed.

YAP is not always anti-apoptotic, however, as it can activate the p53 homolog p73 to induce apoptosis [265, 266]. YAP is able to do this through its interaction with PML [266]. YAP requires PML and requires localization to PML NBs in order to activate p73 in response to DNA damage [266]. In fact, YAP, PML, and p73 form a pro-apoptotic, autoregulatory feedback loop in which DNA damage promotes the association of the three proteins, upon which YAP and p73 promote the transcription of PML and a number of other pro-apoptotic proteins [265]. In turn, PML promotes YAP stabilization by promoting its sumoylation, which protects YAP from ubiquitination and stabilizes it within the cell [265]. Given that YAP is a known interaction partner
of PML that is involved in mediating pro-apoptotic effects of PML [265, 266], I wanted to see if NORE1A’s effects on promoting the association of tumor suppressor or anti-growth proteins with PMLIV was specific for p53 and Rb, or if it was a more general effect on the PMLIV protein interaction spectrum.

Co-immunoprecipitation experiments were performed in HEK-293T cells to examine if NORE1A could effect PMLIV’s association with YAP1. Exogenous expression of these proteins showed that the presence of NORE1A with or without activated Ras does not influence the association of YAP1 with PMLIV (Figure 26). A close homolog of NORE1A and fellow RASSF family member, RASSF1A, was also used in these studies, as it has a more well defined role in Hippo signaling than NORE1A and can also bind both the MST1 and MST2 kinases [127, 267, 268]. Like NORE1A, RASSF1A did not influence the association of YAP1 with PMLIV. These results are significant because they highlight the fact that NORE1A appears to be increasing the localization of specific proteins to PMLIV NBs; this is not a general effect. This makes the data obtained for p53 and Rb more interesting, as it may be that NORE1A is only specifically up-regulating the interaction of pro-senescent proteins with PMLIV.

**PML Expression is Required for NORE1A-Induced Senescence**

In order to determine if PML is a critical mediator of NORE1A-induced senescence, PML knockdown cells were needed. My first goal was to generate a stably knocked down system using shRNAs to PML. A549 cells were an ideal system for this study, as they express activated Ras, and studies by Geoffrey Clark’s group show that exogenous NORE1A expression induces senescence in these cells [16, 17, 23]. Four shRNA plasmids targeting PML were purchased from Origene, along with a scramble control shRNA. A549 cells were transfected with each of these constructs and were selected in puromycin. Upon Western analysis to determine if the PML knockdown was successful, only one shRNA construct provided some PML knockdown (Figure 27). Quantification revealed that this knockdown was less than 40%, however, making these cells useless for biological assays. Attempts to use the shRNA constructs in other cell lines produced similar results (data not shown). Because of this, siRNAs for PML were purchased.
**Figure 26. NORE1A Does Not Effect the Interaction of PMLIV with YAP.** Co-transfections of GFP-tagged YAP1, HA-tagged PMLIV, and HA-tagged RASSF1A and NORE1A with or without activated Ras were performed in HEK-293T cells. Cells were lysed, and lysates were immunoprecipitated (IP) with GFP-Trap® agarose beads and immunoblotted (IB) for the indicated proteins. Experiments were performed in triplicate.

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Figure 27. **PML Knockdown Analysis in A549 Cells.** A549s were transfected with shRNAs targeting PML and with a scrambled shRNA control. Cells were selected in puromycin until the only cells remaining were cells that had taken up the shRNAs. **A.** Cells were lysed and examined for PML knockdown via Western blot. A representational blot is shown. β-actin was used as a loading control. **B.** Duplicate experiments were analyzed by densitometry to quantify PML knockdown. Error bars represent standard deviation.
from GE Dharmacon. Testing revealed that these provided 70-90% transient PML knockdown in multiple cell lines, so these siRNAs were used for biological assays.

A549s were transfected with a pool of four PML siRNAs (GE Dharmacon). After 24 hours, the cells were then transfected with GFP-tagged NORE1A or GFP vector. All experimental conditions were performed in duplicate. Cells were then incubated 72 hours to allow for complete knockdown and to give NORE1A time to induce senescence. After 72 hours, one dish of cells for each experimental condition was lysed, and lysates were analyzed for PML knockdown via Western blot (Figure 28B). The second dish of each treatment condition was stained for β-galactosidase activity, a marker of senescence, using a Senescence Detection Kit (BioVision).

I found that the loss of PML greatly diminishes NORE1A's ability to induce senescence in A549s (Figure 28A). Consistent with previous experiments, the presence of NORE1A increased senescence in these cells [16, 17], but the transient knockdown of PML expression dropped senescence levels back to baseline levels. One caveat of this experiment is that cells treated with PML siRNAs had lower levels of over-expressed NORE1A, even though the same amount of NORE1A was transfected into the siPML and scrambled control cells (Figure 28B). NORE1A over-expression was not altered by the control siRNAs, suggesting that this effect could be the result of several possibilities. One, PML may be necessary for NORE1A stability. As I have not observed PMLIV increasing NORE1A levels in over-expression studies, this seems like an unlikely, but not impossible, possibility. The second possibility is that the loss of PML expression may shift NORE1A from being pro-senescent to being pro-apoptotic. PML knockdown would therefore result in more cell death in cells transfected with NORE1A.

NORE1A can normally be seen in nuclear aggregations [17], and I did not observe many cells with GFP-tagged NORE1A nuclear aggregations in cells treated with the PML siRNAs (data not shown). In fact, the NORE1A in these cells localized more diffusely. Under certain conditions, NORE1A can actually promote apoptosis, and one study indicates that NORE1A must be able to localize in the cytoplasm in order to successfully induce apoptosis [11, 124, 162, 269].
Figure 28. PML Knockdown Reduces NORE1A-Induced Senescence. A. A549 cells were transiently knocked down for PML expression as previously described and transfected with GFP-NORE1A. After 72 hours, 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. Experiments were repeated in duplicate. B. Cells were immunoblotted (IB) for PML and NORE1A expression. A representative Western blot is shown. β-actin was used as an internal loading control. C. Quantification of PML knockdown is shown. Error bars represent standard deviation.
If the loss of PML and PML nuclear bodies prevents NORE1A from localizing in nuclear bodies, it may shift NORE1A signaling from being pro-senescent to being pro-apoptotic. This should be examined in future studies.

Overall, this indicates that for NORE1A to successfully mediate Ras-induced senescence, it requires the presence of PML. NORE1A's ability to induce senescence is partially diminished when either p53 or Rb expression is lost [16, 17], and the loss of PML expression appears to have an even greater impact on NORE1A-induced senescence. This implies that NORE1A must associate with PML NBs, specifically PML IV NBs, in order to successfully induce senescence.

4.3 – Discussion

The discovery that NORE1A and PML IV co-localize in PML IV NBs and that NORE1A and PML IV form a Ras-enhanced association has numerous implications for the role these two proteins play in Ras tumor suppression. First, NORE1A and PML IV are both critical mediators of Ras-induced senescence, and previous studies showed NORE1A co-localizing with HIKP2 and PP1A, two tumor suppressors involved in the activation of p53 and Rb, in structures that appear to be PML NBs [16, 17]. This work reveals that NORE1A is indeed promoting the complex formation of PML IV with p53 and Rb, suggesting that NORE1A is acting as a molecular scaffold to recruit these proteins to PML IV NBs, which are centers of protein regulation and post-translational modifications in the cell [21]. When stimulated by activated Ras, PML can promote senescence by activating p53 and Rb by acetylation and dephosphorylation, respectively, in PML NBs [19, 20], so it is likely that the observed effects of NORE1A on p53 and Rb modifications and function occur in PML NBs. This further supports the hypothesis that for NORE1A, intracellular location may be a critical aspect of its tumor suppressor function.

NORE1A’s effect on PML IV’s association with tumor suppressors has a degree of specificity, as it does not promote the association of PML IV with its pro-apoptotic binding partner, YAP. Perhaps, NORE1A only increases the association of PML IV with NORE1A binding
partners, as NORE1A’s interaction with YAP has not been confirmed. NORE1A is a protein with a large number of binding partners, and it mediates important downstream processes of Ras, including apoptosis and senescence [11, 16-18, 23, 123, 124, 130, 141, 158, 162]. While NORE1A may only specifically promote the association of PMLIV with pro-senescence Ras effectors, the possibility of a NORE1A/PMLIV complex with other Ras effectors cannot be eliminated, especially given the large protein interaction spectrum of both proteins.

PML knockdown studies have previously shown that the loss of PML, and specifically the isoform PMLIV, inhibits Ras-induced senescence [22, 211, 214]. The loss of NORE1A has similar effects [17]. Knockdown studies presented in this work reveal, for the first time, that the loss of PML expression inhibits NORE1A-induced senescence. This result suggests that NORE1A’s localization to PML NBs is a crucial step in the induction of Ras-induced senescence, and PMLIV NBs may be required as centers of protein post-translational modifications, since NORE1A also promotes the association of PMLIV with p53 and Rb. This led to the development of a model in which Ras stimulates the association of PMLIV with NORE1A in PMLIV NBs (Figure 29). NORE1A acts as a molecular scaffold and promotes the recruitment of p53 and Rb to PMLIV NBs, and the two proteins are likely activated by post-translational modifications in the PMLIV NBs. All of this is a critical process for Ras-induced senescence to occur, and the loss of either NORE1A or PML would therefore prevent Ras-induced senescence.

Interestingly, the PML knockdown greatly diminished NORE1A nuclear aggregations, and PML knockdown cells transfected with NORE1A appeared to have less NORE1A expression than cells transfected with control siRNAs. Potentially, the loss of PML shifts NORE1A signaling towards apoptosis, as it cannot assemble the necessary machinery to induce senescence without PML. This would provide a potential back up mechanism for NORE1A/Ras tumor suppression in the event of PML down regulation in premalignant tumors.
Figure 29. NORE1A Mediates Ras-Induced Senescence Through Its Interaction with PMLIV. In the proposed model, NORE1A forms a Ras-enhanced complex with PMLIV and promotes the recruitment of p53 and Rb to PMLIV nuclear bodies, where they undergo post-translational modifications and activation to promote Ras-induced senescence. Without PML, NORE1A is unable promote these processes, and without NORE1A, this process would also be hindered.
CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 – Overview

Work presented in this dissertation reveals the novel, Ras-enhanced association of two tumor suppressor proteins, PMLIV and NORE1A. For the first time, NORE1A’s intracellular localization in nuclear aggregations is shown to coincide with the location of PMLIV NBs, which are centers of protein regulation and post-translational modifications in the cell. Much like a restaurant or business searches for the perfect location to maximize customers and profit, NORE1A needs PML in order to successfully mediate Ras-induced senescence, suggesting that its intracellular location is a critical aspect of NORE1A tumor suppression. This highlights the need to investigate the role of how NORE1A localization shifts its function from promoting senescence to promoting apoptosis, and observations reported here present the question of why a small pool of NORE1A and PMLIV appear to be interacting outside of the nucleus.

5.2 – Conclusions

The results presented in this dissertation reveal the importance of NORE1A localization in the cell. Work from a collaborator showed that NORE1A forms an endogenous complex with PML in human tissue, and I found that NORE1A co-localizes specifically with the isoform PMLIV and forms a Ras-enhanced complex with PMLIV in cells. Additionally, NORE1A promotes PMLIV acetylation, a process that has not been well studied but is hypothesized to modulate PML NB formation [251]. The recent discovery of NORE1A and Ras working together to promote protein
acetylation is a novel finding [16, 17], and NORE1A’s effect on PMLIV acetylation could, instead of promoting NB formation, alter PMLIV’s protein interaction spectrum. Indeed, the presence of NORE1A strongly increased PMLIV’s association with p53, and NORE1A also appears to promote PMLIV’s association with Rb. Both of these proteins are important tumor suppressors that play a role in Ras-induced senescence through their localization in PML NBs, and NORE1A has been shown to act by promoting the activation of both of these proteins in nuclear aggregations [16-18].

Additionally, NORE1A’s promotion of the association of PMLIV with p53 and Rb appears to be specific, as it does not effect PMLIV’s complex formation with YAP. YAP is a protein involved in PML and p73-mediated apoptosis [265, 266], but YAP is only hypothesized to interact with NORE1A because NORE1A binds MST1, a negative regulator of YAP [124]. Perhaps, NORE1A only promotes PMLIV’s association with certain NORE1A binding partners, or perhaps NORE1A only promotes PMLIV’s association with proteins that promote senescence and not those that promote apoptosis. This could be examined in future studies.

The presence of NORE1A in a complex with PMLIV also provides a mechanistic explanation for the connection between PML, p53, and Rb. PML is an upstream component of both the p53 and Rb pathways during Ras-induced senescence, and the loss of one of these pathways does not completely override senescence [270]. This phenomenon is also seen with NORE1A, as the loss of either p53 or Rb does not fully abrogate NORE1A-mediated senescence [16, 17]. NORE1A knockdown, however, significantly reduces Ras-induced senescence and promotes Ras transformation [17]. This suggests that the interaction of NORE1A with p53 and Rb in PML NBs is likely a critical step in Ras-induced senescence, and the loss of either NORE1A or PML is enough to shift the balance of Ras signaling towards transformation. Indeed, work presented in this dissertation shows, for the first time, that the loss of PML expression reduces NORE1A’s ability to induce senescence, providing evidence that supports this concept.

The interaction of NORE1A with PMLIV thus identifies a novel aspect of NORE1A/Ras signaling: NORE1A’s location in the cell plays a critical role in NORE1A-mediated tumor suppression. As Ras remains a difficult target in cancer treatment, perhaps focusing on
reactivating tumor suppressors is a viable goal. Both NORE1A and PMLIV are down regulated in certain cancers, and NORE1A is mostly down regulated by promoter hypermethylation. A class of drugs called DNA methyltransferase inhibitors exist that inhibit promoter methylation, but they have a wide range of potential side effects, as they inhibit global DNA methylation [271]. More specific inhibitors that target single DNA methyltransferase enzymes have been developed, and I have shown that one of these, Nanaomycin A, can efficiently promote the re-expression of a RASSF protein in melanoma cells [125]. Thus, using therapies that specifically target NORE1A re-expression could provide a novel treatment approach for Ras-driven cancers, especially if PML is present in the cells, as this could shift the balance of Ras signaling from transformation to tumor suppression.

5.3 – Future Directions

The original goal of this work was to determine if PML was a critical component of NORE1A-mediated, Ras-induced senescence. Knockdown studies showed that the transient loss of PML expression inhibited NORE1A-induced senescence and led to lower overall expression levels of NORE1A compared to cells transfected with control siRNAs. These results suggest that localization to PML NBs is a critical aspect of NORE1A-mediated senescence, and they raise the question of what NORE1A does when it cannot localize to the PML NBs. NORE1A was originally discovered as a pro-apoptotic molecule, and there are reports that NORE1A must be able to be exported from the nucleus in order to induce apoptosis [11, 124, 162, 269]. The loss of PML may therefore force NORE1A to promote apoptosis. This should be investigated in future work.

Future studies should also be directed at examining an interesting phenomenon noticed in the NORE1A/PMLIV co-localization studies. While NORE1A and PMLIV strongly co-localized in PMLIV NBs, there was also a small pool of PMLIV and NORE1A that co-localized outside of the nucleus. PMLIV has been implicated in playing a role in apoptosis at junctions of the ER and
mitochondria called mitochondrial-associated membranes, where it plays a role in mediating calcium release and apoptosis by interacting in part with the IP$_3$ receptor [248].

Preliminary studies using a mitochondrial tracker showed that NORE1A expression pushes a small pool of PMLIV outside of the nucleus, but this does not directly co-localize with mitochondria (Figure 30). Instead, it appears to be adjacent to the mitochondria. An ER marker should be used to determine if NORE1A is instead pushing PMLIV to the ER, as I suspect PMLIV is localizing to the ER side of the mitochondrial-associated membranes (MAMs), given that PMLIV appears to be directly next to the mitochondria in the mitochondrial co-localization study. Initial studies on PML at these MAMs also showed that PML exists specifically at the MAMs or in the ER; it is not found in pure extracts of mitochondria [248]. This could provide evidence that links NORE1A to a novel role in apoptosis: the control of mitochondrial calcium levels through its interaction with PMLIV.

An even more intriguing possibility lies in the fact that MAMs are known sites of lipid metabolism [272]. Recent evidence generated by Geoffrey Clark's group shows that heterozygous NORE1A transgenic mice do not develop cancer by one year of age, but many develop extensive fatty liver disease (unpublished observations). This suggests that NORE1A may play a novel role in lipid metabolism and accumulation. Moreover, a study also shows that PML knockout mice, which exhibit normal development, develop accelerated subcutaneous fat accumulation when fed a high-fat diet, and the loss of PML leads to the dysregulation of processes that inhibit adipogenesis, or the differentiation of fat cells [273]. Although PML knockout mice do not exhibit fat accumulation in the liver [273], obesity and excess lipids are often linked to cancer development [274]. A key player in lipid biosynthesis that is also connected to Ras signaling is the mammalian target of rapamycin, or mTOR kinase [275]. PML can bind mTOR and inactivate it by sequestering it in the PML NBs, thus reducing lipid biosynthesis [276].

NORE1A may thus play a role in lipid biosynthesis through its association with PML in two ways: it may influence lipid metabolism by promoting the localization of PML at the mitochondrial-associated membranes, and NORE1A and PML may work together to regulate lipid
Figure 30. Extra-nuclear Localization of PML Is Increased by NORE1A. GFP-tagged PMLIV was transfected into COS-7 cells in the presence of a Mitotracker fluorescent molecule. In the presence of NORE1A, a large pool of PML can be seen outside of the nucleus. Although these structures do not directly co-localize with mitochondria, a large portion of the extra-nuclear PML appears to be directly adjacent to mitochondria, suggesting that it may be at sites called mitochondrial-associated membranes (MAMs).
accumulation, thus preventing the negative side effects of lipid accumulation, like inflammation, which can promote cancer development [274]. At the moment, PML’s function at the MAMs has only been linked to apoptosis and calcium release [248], so if NORE1A and PML work together to regulate lipid metabolism at these sites, this would be an unexpected and novel finding for both proteins. Future studies on the interaction of these proteins could therefore reveal a novel role of RASSF proteins in lipid biosynthesis and metabolism.
REFERENCES


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