NUCLEAR RECEPTOR 4A1 (NR4A1): A NOVEL DRUG TARGET FOR TREATMENT OF RHABDOMYOSARCOMA

A Dissertation

by

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ABSTRACT

Nuclear receptor 4A1 (NR4A1) is an orphan receptor that is overexpressed in several cancers and is known for its pro-oncogenic activities in solid tumors. NR4A1 plays a crucial role in multiple cancers and regulates expression of important genes that are required for growth and proliferation (EGFR), migration and invasion (integrins), and survival (survivin) of cancer cells including rhabdomyosarcoma (RMS). Furthermore, NR4A1 also suppresses expression of interleukin-24 (IL-24) in RMS cells and IL-24 is an important gene that induces apoptosis and inhibits epithelial to mesenchymal transition (EMT). Thus, NR4A1 is an important target gene for cancer therapeutics and studies in our laboratory have developed a series of bis-indole derived molecules (CDIMs) that bind NR4A1 and act as NR4A1 antagonists in cancer cells. These antagonists inhibit NR4A1-regulated metabolic pathways in RMS and also inhibit the expression of NR4A1-regulated pro-oncogenic genes in RMS including the fusion oncogene PAX3-FOXO1. This makes NR4A1 a potential drug target in RMS chemotherapy. My studies were focused on the role of NR4A1 in regulating pro-oncogenic pathways and genes and on the mechanisms of these responses in RMS cells. I initially investigated the role of NR4A1 in TGFβ-induced embryonal RMS (ERMS) cell invasion and showed that NR4A1 antagonists inhibit invasion, which was due to a Bcl-2-NR4A1 complex activation responsible for mitochondrial disruption, IL-24 induction and β-catenin downregulation. I also examined the tumor promoting role of an important pro-oncogene G9a in alveolar RMS (ARMS) and showed that G9a is regulated by NR4A1 in ARMS. Furthermore, my studies have shown that NR4A1 antagonists are a novel class of G9a inhibitors. In addition, my studies have also characterized natural flavonoids kaempferol and quercetin as novel NR4A1 ligands and they inhibit NR4A1-regulated genes and pathways in RMS, and this includes PAX3-

FOXO1 and G9a. These results suggest that flavonoids can be repurposed for RMS cancer therapy and thereby enhance the efficacy of current treatments and decrease their toxicity. Overall, I have identified novel NR4A1-regulated pro-oncogenic pathways in RMS and have shown that these pathways can be inhibited by treatment with NR4A1 antagonists that include flavonoids such as kaempferol and quercetin.

DEDICATION

I would like to dedicate this dissertation to my father Ram Bahadur Shrestha and to my mother Ram Devi Shrestha, who have done everything that they could to make sure that I achieve my educational aspirations. Their hard work, support and desire to see me succeed are the reasons that I am here in this position today. I also want to thank my brother Deepesh Shrestha who has always been there for me, supported me, and has been a constant source of encouragement in my life. Thanks to all three of them for their continued love, patience and support.

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INTRODUCTION AND LITERATURE REVIEW

CANCER OVERVIEW

Cancer is caused when normal cells become highly dysregulated and exhibit enhanced growth, survival, migration and invasion. While there are several factors that cause cancer such as somatic and a limited number of inherited gene mutations, a number of other genomic and epigenomic abnormalities are key factors in carcinogenesis including the steps associated with cancer initiation, promotion and progression (1). Cancer is a complex, heterogeneous and a multifaceted disease. The phenotypic and functional heterogeneity of cancers occurring in several organs is what makes this disease unique (2). Because of its complexity and heterogeneity, only a fraction of the characteristic markers of specific tumor types and potential molecular targets have been identified in tumors, even though billions of dollars are being spent on cancer research every year. This disease has also been prevalent among ancestors of modern humans for over a million years and has been affecting multicellular organisms for more than 200 million years (3). It is interesting that the oldest surgical treatment of cancer was reported in Egypt in 1600 BC, where eight cases of breast tumors were treated by cauterization (2).

Cancer is one of the most feared diseases in both 20^{th} and 21^{st} centuries (4). The prevalence of this disease is so high that one out of five people possesses a lifetime risk of developing cancer and one out of ten will die from this disease (5). There is a 20% probability of developing cancer before the age of 75 and 10% probability of dying from it (5). Cancer is the second leading cause of mortality in both men and women worldwide after ischemic heart disease (6). In 2020, 19.3 million new cases of cancer were diagnosed and 10 million deaths were observed (7). Cancer is

also projected to become the leading cause of mortality worldwide by 2060 with an estimated 18.63 million deaths per year (6). In the United States, 21% of the 2,813,503 recorded deaths in 2018 were from cancer (8). It is also estimated that a total of 1,898,160 new cases of cancer (equivalent to 5200 new cases each day) will be diagnosed in 2021 and 608,570 deaths will be observed in this country (8). This estimation of total number of new cases of cancer and mortality may be modified because of the delays in important cancer diagnoses due to the 2020/2021 COVID-19 pandemic and this will also be the case in several other countries (5). The global pandemic in 2020/2021 has not only delayed the registration of cancer patients, but also has increased the risk of detecting cancers in patients at a more advanced stage due to low rates of routine cancer screening (5).

Cancer is diagnosed in both men and women however, there are some cancers that are more prevalent in males vs females and vice versa. For example: breast cancer is the most commonly diagnosed cancer in females worldwide, followed by colorectal and lung cancer and the leading cause of cancer related deaths in women worldwide is due to breast cancer followed by lung and colorectal cancer (7). In contrast, the most frequently diagnosed cancer in men is lung cancer, followed by prostate and colorectal cancer. When it comes to mortality rates, lung cancer is the leading cause of cancer related deaths in men, followed by liver and colorectal cancer (7). In 2021, in the United States, the estimated most common cancer related deaths in both men and women will be due to lung and bronchus cancer. However, the estimated highest new cases of cancer in men and women are prostate and breast cancers respectively (8). The projected new cases of cancers and cancer related deaths in men and women by ten leading cancer types in the United States in 2021 are shown in Table 1.

Males

Type of Cancer	Estimated New Cases	
	Total	**(%)**
Prostate	248,530	26%
Lung & bronchus	119,100	12%
Colon & rectum	79,520	8%
Urinary bladder	64,280	7%
Melanoma of the skin	62,260	6%
Kidney & renal pelvis	48,780	5%
Non-Hodgkin lymphoma	45,630	5%
Oral cavity & pharynx	38,800	4%
Leukemia	35,530	4%
Pancreas	31,950	3%
All Sites	**970,250**	**100%**

Type of Cancer	Estimated Deaths	
	Total	**(%)**
Lung & bronchus	69,410	22%
Prostate	34,130	11%
Colon & rectum	28,520	9%
Pancreas	25,270	8%
Liver & intrahepatic bile duct	20,300	6%
Leukemia	13,900	4%
Esophagus	12,410	4%
Urinary bladder	12,260	4%
Non-Hodgkin lymphoma	12,170	4%
Brain & other nervous system	10,500	3%
All Sites	**319,420**	**100%**

Females

Type of Cancer	Estimated New Cases	
	Total	**(%)**
Breast	281,550	30%
Lung & bronchus	116,660	13%
Colon & rectum	69,980	8%
Uterine corpus	66,570	7%
Melanoma of the skin	43,850	5%
Non-Hodgkin lymphoma	35,930	4%
Thyroid	32,130	3%
Pancreas	28,480	3%
Kidney & renal pelvis	27,300	3%
Leukemia	25,560	3%
All Sites	**927,910**	**100%**

Type of Cancer	Estimated Deaths	
	Total	**(%)**
Lung & bronchus	62,470	22%
Breast	43,600	15%
Colon & rectum	24,460	8%
Pancreas	22,950	8%
Ovary	22,950	5%
Uterine corpus	12,940	4%
Liver & intrahepatic bile duct	9,930	3%
Leukemia	9,760	3%
Non-Hodgkin lymphoma	8,550	3%
Brain & other nervous system	8,100	3%
All Sites	**289,150**	**100%**

Table 1: Estimated new cases of cancers and cancer related deaths in men and women by ten leading cancer types in the United States in 2021 (8). Basal cell skin cancers, squamous cell skin cancers and in situ carcinoma except urinary bladder are excluded in this estimation. Recreated with permission (Siegel et al., Cancer Statistics, 2021, *CA Cancer J Clin.*, 2021).

The cruelty of a cancer diagnosis is not only limited to men and women, but it also is observed in children and adolescents. It is estimated that 10,500 children below 14 years of age and 5090 adolescents aged 15-19 years will be diagnosed with cancer in 2021 and 1190 children and 590 adolescents will die from this disease (8). The most common cancer in children is leukemia and it is responsible for 28% of total childhood cancers. Several other cancers such as neuroblastoma that are related to the brain and other central nervous system cancers account for another 27% of childhood cancer. Statistics show that there has been an increase in the overall cancer incidence rate in children in recent years (8). Since the total number of cancer cases and its mortality rate is alarmingly high, it has been projected that the total cost of cancer care in the United States in 2020 will be $173,000,000,000, which is 27% higher than the cost in 2010 (9). The goal of both basic and clinical cancer research is to detect cancer early, stratify tumors to guide therapy, and develop effective treatments to prevent cancer. Unfortunately, despite years of scientific and clinical studies and the investment of funds supporting cancer research, this disease still remains as one of the primary causes of mortality and will remain so until more effective therapies are discovered.

Studies on cancer biology show that tumor formation is a multistep process and each of these steps contribute to genetic events leading to changes in the genome that induce transformation of normal cells into malignant tumor cells. This transformation involves mutations and silencing of tumor suppressor genes that control cell proliferation and activation of oncogenes that promote cell proliferation and survival (10). Errors in DNA replication, free radical-induced chemical instability of DNA bases are few examples of processes that can lead to genome damage and gene mutations (11). These genetic alterations accumulate in a tumor-specific stepwise fashion and facilitate malignant tumor progression (12). Therefore, discovering therapeutic strategies that

inhibit the progression of normal and preneoplastic cells into malignant cells has been important in development of treatments for early-stage cancer (2). The process of transforming normal cells into malignant cells requires six essential alterations in cell physiology which have now been defined as hallmarks of cancers (11). It includes sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting apoptosis (Fig. 1). These trademark biological capabilities of cancer cells have provided scientists a foundation for understanding the biology of tumorigenesis, as the combinations of these acquired properties allow cancer cells to grow, proliferate, invade, and metastasize at various steps of tumorigenesis. A decade later, two additional alterations have been added to the hallmarks of cancer and these include deregulating cellular energetics and avoiding immune destruction (13) and these are discussed below.

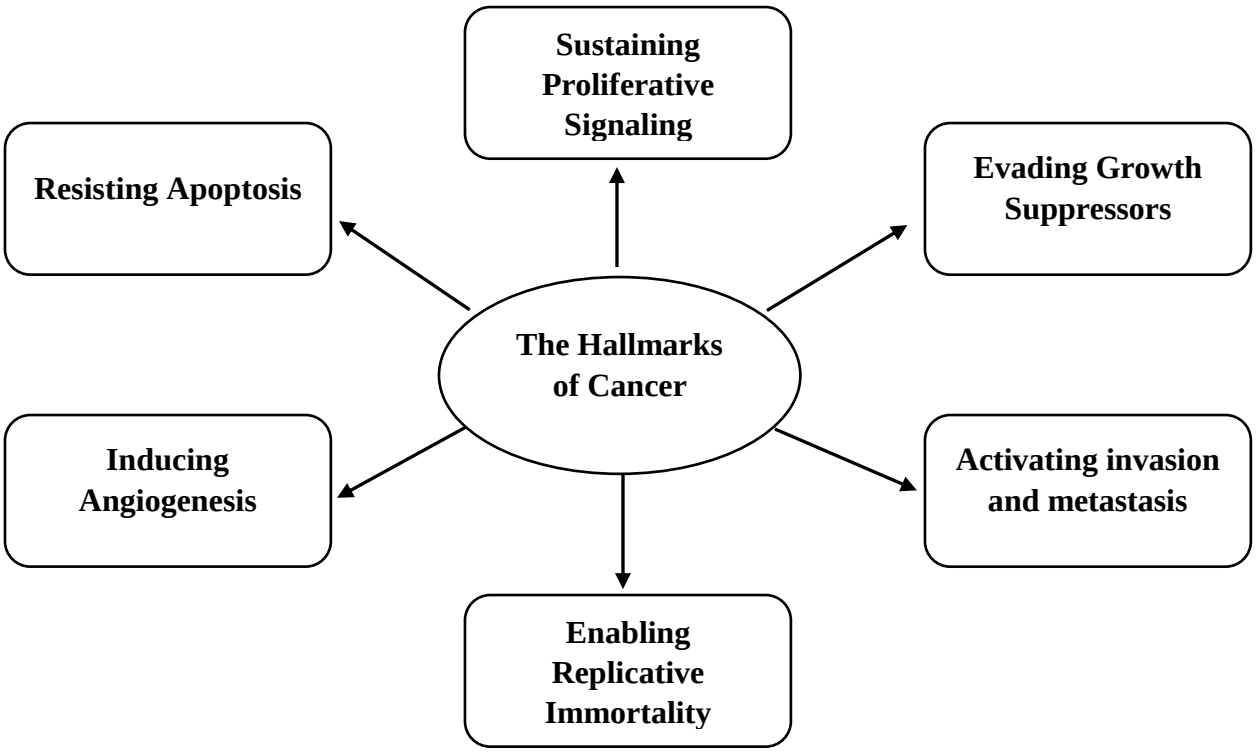

Figure 1: The hallmarks of cancer: Normal cells acquire six essential alterations to become cancerous which have been designated as the hallmarks of cancer by Hanahan and Weinberg (11, 13). Recreated with permission (Hanahan and Weinberg, The Hallmarks of Cancer, *Cell*, 2000).

Sustaining proliferative signaling

Normally, inactive cells in a quiescent state (G_0 phase) turn into an active proliferative state when they receive mitogenic growth signals (11). A few examples of such signaling molecules are major extracellular matrix proteins (fibronectin, laminin, vitronectin, and collagen), diffusible growth factors and cell-to-cell interaction molecules. It is crucial for cells to engage with these molecules for activation of several growth-promoting pathways (11, 14). This process is initiated with binding of growth factors to their cognate high-affinity transmembrane receptors that subsequently undergo rapid phosphorylation to transmit early growth signals into the cell. Shortly after this signal is propagated into the nucleus, multiple cellular and molecular responses are activated leading to DNA synthesis and cell division, turning cells into active proliferative state (15). Interestingly, when normal cells become malignant, they develop the ability to initiate and maintain proliferative signaling in other ways. Firstly, they acquire an ability to generate their own growth signals to stimulate self-proliferation (16). Hence, they acquire mitogenic growth signal autonomy and therefore no longer depend on the cellular microenvironment for growth signals. Secondly, the receptor molecules that transmit the extracellular signals into the cell are overexpressed in cancer, and thus cancer cells become highly responsive to growth signals. Fibroblast growth factor receptors, epidermal growth factor receptors, G protein coupled receptors, transferrin receptors are a few of many examples of receptors that are overexpressed in wide range of cancer cells (17) and these receptors help them transmit pro-growth signals (11). In addition, some mutations in cancer cells activate multiple signal transduction pathways such as Wnt, PI3K/Akt, NF-kB and Notch that promote uncontrolled cell proliferation (18). Therefore, when normal cells become malignant, they acquire capability of sustaining proliferative signaling, which is arguably one of the most fundamental traits of cancer cells (13).

Evading growth suppressors

In addition to developing self-sufficiency in growth signals, cancer cells also acquire an ability to become insensitive to antigrowth signals and thereby remain in an active proliferative state. There are several tumor suppressor genes, which upon activation can switch actively proliferating cells into quiescent G_0 phase or even force them to undergo terminal differentiation to post-mitotic state. These tumor suppressors can also induce senescence, cause cell cycle arrest or even stimulate apoptosis in these active cells (19). Two well-known tumor suppressors that are responsible for anti-proliferative signals are RB (retinoblastoma associated) and TP53 proteins and they complement each other's functions to determine whether cell growth should be suppressed. The RB protein transduces anti-growth signals predominantly outside of the cells whereas the TP53 protein integrates the signals in the intracellular environment (13). RB proteins function as a crucial gatekeeper for inhibiting cell-cycle progression and therefore halt persistent cell proliferation when necessary. RB proteins block proliferation by directly affecting the function of E2F transcription factors, which are responsible for regulating the expression of several genes that are essential for cells to progress from G_1 into S phase (20). The hypo-phosphorylated RB protein binds E2F transcription factors and inhibits the transcription of multiple E2F-regulated genes including c-myc, B-myb, cdc2, dihydrofolate reductase and thymidine kinase, which are important for the control of cell growth (20). In addition, signaling molecules such as transforming growth factor β (TGFβ) keep RB proteins activated by inhibiting its phosphorylation by cyclin:CDK complexes by inducing p21 and p15 CDK inhibiting proteins (21) resulting in cell cycle arrest at G_1 phase (22). On the other hand, TP53 protein induces apoptosis and also terminates the cell-cycle progression as an intracellular modulator. When normal cells become cancerous, they acquire the capabilities for avoiding these signals from growth suppressors. TGFβ-induced growth

inhibition is silenced by mutations that allow cancer cells to grow and proliferate without resistance (22). These mutations can lead to loss of function or decreased production of transmembrane TGFβ receptors and downstream signaling molecules (Smad2 and Smad4) that transmit TGFβ signal to downstream targets (22). Mutations in TGFβ regulated pathways leads to phosphorylation of RB proteins that decreases its functional interactions with E2F transcription factors and this activates transcription of several pro-growth genes (20). In addition, expression of integrins and other cell adhesion molecules is inhibited in cancer cells and this allows them to evade the antigrowth signals (11).

Activating invasion and metastasis

Cancer cells acquire the capability to invade neighboring tissues and travel from the primary cancer site to develop new secondary malignant tumors in distal sites. This fundamental capability of cancer cells to disseminate to distant organs and further adapt in a foreign tissue microenvironment to grow uncontrollably is called metastasis (23), which is responsible for 90% of cancer-related deaths in patients (24). The ability to invade and metastasize also allows cancer cells to find new habitats where nutrients and space are not limiting (11). An example of a physiological process that cancer cells utilize to invade and metastasize during tumor progression is epithelial-to-mesenchymal transition (EMT), in which cells in the epithelial state elongate, become motile, and acquire mesenchymal cells like properties. During EMT, the epithelial cell-to-cell as well as cell-to-extracellular matrix adhesion is disrupted resulting in their conversion into a mesenchymal cell-like phenotype (25). EMT not only enhances cancer cell motility but also makes them more aggressive, resistive to drugs and stresses, and facilitates their evasion from senescence and anoikis (26). Transformation of normal cells into malignant cancer cells is accompanied by changes in expression of several EMT-regulated genes which includes decreased

expression of E-cadherin and increased expression of N-cadherin, Snail, and Zeb1 (27-30). Downregulation of the tumor suppressor E-cadherin in cancer cells facilitates tumor progression, invasion, and metastasis and contribute to the poor patient prognosis (27). Upregulation of genes such as N-cadherin, Snail, and Zeb1 also promotes tumor progression, invasion, metastasis and angiogenesis but suppresses cellular senescence, anoikis, and apoptosis (28-30). In addition, cancer cells also alter expression of other cell-to-cell and cell-to-extracellular matrix molecules including integrins and several protease inhibitor genes, and this enhances cell penetration, invasion, and metastasis (31, 32).

Enabling replicative immortality

In order to form macroscopic tumors, cancer cells acquire limitless replicative potential and therefore multiply uncontrollably which contrasts to the intrinsic cell-autonomous programs that restrict cell replication (11, 13). The intrinsic limitation of somatic cells to proliferate is strictly regulated, and cells undergo 50-70 cell divisions after which, they enter a phase called replicative senescence (33). In this phase, cell division ceases and cell growth is arrested (13, 34). Senescence is specifically triggered by shortening of telomeres, which can further force cells into a crisis phase that stimulates cell death by end-to-end fusion of chromosomal DNA in non-immortalized cells (13, 33). Interestingly, cancer cells express a significant level of a DNA polymerase enzyme called telomerase, which prevents telomere attrition and these cells escape both senescence and crisis phase to become immortalized cells. Research shows that more than 85% of cancer cells exhibit significant telomerase activity, which is absent in normal somatic tissues (33, 35). High expression of telomerase enables cancer cells to gain replicative immortality and hence they develop unlimited dividing potential. Since high levels of telomerase activity are essential for cancer cells, this enzyme has been an important drug target for anticancer therapies.

Inducing angiogenesis

Angiogenesis is a dynamic and complex physiological process in which new capillaries are developed from pre-existing blood vessels (36). It plays a crucial role in delivery of required nutrients and oxygen to tissues and also in excretion of metabolic waste and carbon dioxide in both normal and tumor tissues (13). During embryogenesis in normal tissues, vasculogenesis is followed by angiogenesis that lead to formation and further development of blood vessels from pre-existing ones. Angiogenesis is then switched off and is only turned on temporarily during processes such as wound healing and female reproductive cycling. This switch is regulated by the combination of pro-angiogenic signaling molecules/proteins such as vascular endothelial growth factor-A (VEGF-A) and anti-angiogenic molecules/proteins such as thrombospondin-1 (TSP-1). When pro-angiogenic molecules like VEGF-A are more highly expressed than anti-angiogenic molecules (e.g. TSP-1), the angiogenesis switch is turned on (36). In normal tissues, when angiogenesis is necessary, VEGF-A and related ligands bind transmembrane VEGF receptors in endothelial cells to stimulate angiogenesis. When this process is complete, anti-angiogenic molecules bind to transmembrane receptors to stimulate signals that downregulate angiogenesis (13, 37). In cancer cells, angiogenesis generates irregular blood vessels with dead ends that cause abnormal blood flow and high permeability. This results in an increase in hypoxia, which in turn stimulates VEGF production, creating a positive feedback loop (38). In many tumors, expression levels of VEGF are increased and expression of TSP-1 related anti-angiogenic molecules are suppressed, allowing cancer cells to maintain angiogenesis and generate new vasculature as they grow and metastasize (11, 39-41).

Resisting apoptosis

Cell death or cell suicide, is as important as cell division in maintaining normal cell homeostasis. Since over a million cells are produced in our body every second, programmed cell death pathways including apoptosis, necrosis, and autophagy maintain acceptable cell numbers (19, 42). Apoptosis is a cell death program that is stimulated by both internal and external stimuli and causes cell death by a series of precisely programmed steps. Induction of apoptosis results in disruption of cell membranes, disintegration of the cell skeleton, cytosolic shrinkage, and fragmentation of nucleus, followed by engulfing of dead cells by neighboring tissues (11, 43). Cancer cells resist apoptosis and this contributes towards their proliferative and invasive phenotype. Intrinsic apoptosis involves initial release of mitochondrial cytochrome c and this is accompanied by activation of pro-apoptotic (Bax, Bak, Bid and Bim) and inhibition of pro-survival or anti-apoptotic (e.g. Bcl-2) genes which are members of the Bcl-2 protein family. In cancer cells, these processes are reversed and pro-survival pathways are dominant. The extrinsic apoptosis pathway involves extracellular signals (e.g. death ligands) that activate cell death through caspase 8 in normal cells whereas this pathway is inhibited in cancer cell lines. The two other mechanisms of cell deaths are a) autophagy, which is a recycling program where cells are degraded to fuel biosynthesis pathways for energy production, and b) necrosis, which is defined as "uncontrolled" cell death. Studies show that both autophagy and necrosis can inhibit as well as promote tumor progression.

Emerging Hallmarks: Deregulating cellular energetics and avoiding immune destruction

Deregulating cellular energetics and avoiding immune destruction are two additional trademarks of cancer and are classified as "Emerging Hallmarks of cancer" because they are not yet fully validated (13). The hallmark "deregulating cellular energetics" depicts the capability of

cancer cells to reprogram the cellular metabolism in order to facilitate cancer proliferation. Cancer cells alter the process of glucose metabolism and energy production by switching to glycolysis even during aerobic condition and this is called “aerobic glycolysis” (13). Aerobic glycolysis is considered to be the metabolic adaptation of cancer cells known as Warburg effect (44) and there is evidence suggesting that the Warburg effect occurs in rapidly dividing embryos (45-47). One recent study concluded that reduced oxygen levels creates a favorable environment for embryo culture and these embryos have development potential due to the Warburg effect (45). It has also been suggested that although glycolysis produces less ATP than oxidative phosphorylation, it only becomes a problem when resources are scarce and this is not observed in cancer cells (48). Instead, the increased glycolysis in cancer cells allows utilization of glycolytic intermediates for other biosynthetic pathways and this facilitates biosynthesis of macromolecules and organelles, which are subsequently used for cancer cell proliferation (13, 48, 49).

Avoiding immune destruction is the second emerging hallmark of cancer. It recognizes the ability of cancer cells to escape destruction by the immune system. It is still not fully understood how tumor formation and progression go unnoticed by the immune system, although this system is considered to be alert at all times and it constantly monitors cells and tissues in the body (13). Studies show that cancer cells can disable or modify immune system components of the tumor microenvironment and develop mechanisms to interact with the immune system to protect the tumor cells from immune-surveillance and help them evade destruction (50, 51).

The characteristics of cancer cell are consistent with the initial formation and growth at the site of the tumor in a specific tissue and the subsequent metastasis to distal sites. Tumors can be classified based on their site of origin such as prostate cancer, lung cancer, breast cancer, cervix cancer, brain cancer, and renal cancer. Cancers are also classified based on the type of tissue from

which they originate and this classification is histology-based. The first category of cancer cells are carcinomas, which originate from epithelial cells. The epithelial cells are derived either from ectoderm or endoderm however, a few epithelial tumors like renal cell carcinoma, adrenal cortical adenoma and papillary carcinomas of ovary are derived from mesoderm (52). Since epithelial cells form the internal and external linings of all body organs, carcinomas are the most common type of cancer and account for almost 80% to 90% of all cancer cases according to the National Cancer Institute. Carcinomas can arise in epithelial tissue of skin as well as in the lining of organs such as liver or kidney. Based on different types of epithelial tissues, carcinomas are further classified into adenocarcinomas, basal cell carcinomas, squamous cell carcinomas and transitional cell carcinomas. Sarcomas are the second histology-based category of cancer and they originate in connective tissues throughout the body. Connective tissues are derived from mesenchymal cells which comes from mesoderm and all the sarcomas are mesoderm-derived (52). This type of cancer is common in young adults, and it develops in connective tissues such as muscle, bones, tendons, cartilage etc. There are several types of sarcomas and these include osteosarcoma (bone), chondrosarcoma (cartilage), leiomyosarcoma (smooth muscle), liposarcoma (adipose tissue) and rhabdomyosarcoma (skeletal muscle). The other categories of cancer based on their tissues of origin are myeloma, leukemia, and lymphoma, which arise in plasma cells of bone marrow, bone marrow, and nodes and glands of lymphatic tissues respectively. There are also mixed cancer types and all of these tumors arise from a multistep process known as carcinogenesis. This thesis focuses on rhabdomyosarcoma, a deadly pediatric cancer and our approach for designing novel drugs for the treatment against this devastating disease.

RHABDOMYOSARCOMA (RMS)

Introduction and Epidemiology

Rhabdomyosarcoma (RMS) is a pediatric cancer in which the tumor cells undergo muscle cell-like myogenic differentiation (53). RMS arises in mesenchymal cells, which are derived from mesoderm. RMS usually occurs in striated skeletal muscle cells however, it can also develop in other tissues such as skin, fat, and nerves (54). RMS can arise in different parts of the body such as orbit, head and neck region, parameningeal sites, trunk, extremities, reproductive organs and urinary bladder (55) and metastatic RMS is common in organs such as lungs, bone marrow, and lymph nodes. RMS is the most common sarcoma in children and adolescents and accounts for 5% of pediatric cancers and 50% of soft tissue sarcomas (55). It is also the third most common solid tumor in children that occurs outside of the cranium, after Wilms tumor and neuroblastoma (56). Most of the RMS cases are observed in children that are below six years of age and RMS incidence is slightly higher in male than in female children. In contrast, RMS accounts for less than 1% of all solid tumors in adults and less than 4% of all sarcomas (54). In the United States, approximately 350 new cases of RMS are observed annually and 50% of the cases are diagnosed in children under 10 years of age (54). The incidence rate of RMS is approximately 4.5 cases per million individuals below 20 years of age per year in this country (53). European countries have a similar RMS incidence rate as the United States but it is lower among Asian populations. Early diagnosis and advance treatments have improved prognosis of RMS patients, however children with metastatic and recurrent RMS are rarely cured and those who undergo treatment exhibit life-threatening toxicities later in life (57, 58).

Etiology and Risk factors

RMS occurs sporadically and therefore the risk factors associated with RMS are unknown. However, children with certain genetic disorders such as Li-Fraumeni, neurofibromatosis type 1, DICER1, and Costello and Noonan syndromes are of increased risk of developing RMS (53, 56). One study on a cohort of RMS patients that included both children and adolescents showed that 32% of the patients had prior congenital abnormalities (59). Besides the genetic predisposition, other factors such as X-ray exposure and recreational drug use by parents during pregnancy, accelerated in utero growth, low socioeconomic status and other environmental factors also increased the risk of developing RMS in their offspring (53, 60).

Subtypes

Based on histopathological, molecular, and clinical variations, RMS is divided into two major subtypes; Embryonal RMS (ERMS) and Alveolar RMS (ARMS).

ERMS is the most common subtype of RMS and accounts for more than two-thirds of all RMS cases (55). ERMS occurs primarily in children younger than 5 years of age and is usually observed in the head-neck region including the eye socket and the genitourinary tract of RMS patients (61). The loss of heterozygosity (allelic loss) at 11p15 locus is observed in 80% of ERMS patients (56) and it is hypothesized that this locus contains a tumor suppressor gene, which is lost in ERMS. Mutations in several genes including seven RAS family genes were observed in ERMS tumors (62). In addition, ERMS patients do not express fusion proteins because there are no translocations of chromosomes in ERMS, whereas the majority of ARMS cases express fusion proteins. Thus, ERMS is also referred to as fusion-negative RMS.

ARMS accounts for 25% of all RMS and it occurs mainly in adolescents and arises in extremities and parameningeal locations, with a very small fraction arising in head, neck or torso region. The chromosomal translocation between chromosome 2 or 1 and chromosome 13 is observed in ARMS, which are referred to as t(2;13) and t(1;13) respectively. This results in fusion of either PAX3 gene on chromosome 2 or PAX7 gene on chromosome 1 with the FOXO1 gene on chromosome 13 to generate chimeric PAX3-FOXO1 or PAX7-FOXO1 genes respectively. Therefore ARMS is also referred to as fusion-positive RMS. A combination of reverse transcription PCR and fluorescent in situ hybridization experiments showed that 60% of ARMS patients express PAX3-FOXO1, 20% express PAX7-FOXO1, and the remaining 20% do not express fusion oncogenes and show genetic variations (expression patterns and clinical outcomes) similar to ERMS patients (57). The fusion oncogenes are the major drivers of aggressiveness in ARMS. Expression of PAX3-FOXO1 or PAX7-FOXO1 oncogenes are also associated with increased metastasis in ARMS tumors and the patients expressing PAX3-FOXO1 have higher risk of metastasis than patients expressing PAX7-FOXO1 (57). Metastatic ARMS tumors can metastasize into other organs such as lungs, bone, and bone marrow. Besides ERMS and ARMS, a new subtype of RMS called spindle cell and sclerosing RMS has been identified that shows the recurring fusions involving *VGLL2* or *NCOA2*. It is a rare variant of RMS and has a favorable prognosis (63).

Diagnosis/ Histopathology

Painless masses or lumps which are normally greater than 5 centimeters in size are usually detected in the head-neck region or extremities of RMS patients during diagnosis (60). Diagnosis of RMS has been facilitated by the development of advanced techniques such as immunohistochemistry and electron microscopy, which detect skeletal-myoblast like tumor cells

in RMS patients (57). A detailed medical history, results of imaging studies, and biopsies followed by molecular histopathological analysis of tumor tissue are required for RMS diagnosis. Since RMS usually expresses gene products such as myogenin, myoglobin, desmin, and muscle specific actin, immunohistochemistry has become a useful tool in detecting skeletal muscle molecular markers in diagnosis of RMS (60, 64). Moreover, RMS usually tests negative for markers such as CD45, CD99, CK, S100, and NSE. The subtypes of RMS can be diagnosed and differentiated by the appearance of their cellular arrangements to some extent and also by the expression of fusion oncoproteins. ERMS cells are densely packed with immature rhabdomyoblasts arranged in sheets with a myxoid matrix that does not express either of the fusion oncoproteins (60). In contrast, ARMS cells often exhibit a loosely packed alveoli-like parenchymal appearance in lung tissue and express either PAX3-FOXO1 or PAX7-FOXO1 fusion oncoproteins. A few RMS cases also show mixed features of both ERMS and ARMS.

Staging/ Clinical Group

The staging of RMS is based on two systems- the TNM (tumor, nodes, and metastasis) system and the Clinical Grouping (CG) system and they complement each other (60). The TNM staging system is based on the results of imaging and physical examinations of RMS patients that are performed prior to the treatment. The TNM system classifies RMS patients into four stages depending on the size, location, degree of invasion, involvement of lymph node and the presence or absence of metastasis in the tumor as outlined in Table 2.

Stage	Site	T	Size	N	M
1	Orbit Head and neck (excluding parameningeal) GU-non-bladder/ non-prostate Biliary tract/ liver	T_1 or T_2	a or b	N_0 or N_1 or N_x	M_0
2	Bladder/ prostate Extremity, cranial Parameningeal, other (includes trunk, retroperitoneum, etc.) Except biliary tract/ liver	T_1 or T_2	a	N_0 or N_x	M_0
3	Bladder/ prostate Extremity cranial Parameningeal, other (includes trunk, retroperitoneum, etc.) Except biliary tract/ liver	T_1 or T_2	a b	N_1 N_0 or N_1 or N_x	M_0 M_0
4	All	T_1 or T_2	a or b	N_0 or N_1	M_1

Tumor
$T(site)_1$ – confined to anatomic site of origin
$T(site)_2$ – extension and/or fixative to surrounding tissue
(a) ≤ 5 cm in diameter in size
(b) > 5 cm in diameter in size
Regional nodes
N_0 region nodes not clinically involved
N_1 region nodes clinically involved by neoplasm defined as > 1 cm by CT or MRI, or 2) 18-FDG avid
N_x clinical status of regional nodes unknown (especially sites that preclude lymph node evaluation)
Metastasis
M_0 no distant metastasis
M_1 metastasis present

Table 2: The TNM staging system classification of RMS tumors (56). Recreated with permission (Dasgupta et al., Rhabdomyosarcoma, *Seminars in Pediatric Surgery*, 2016).

The CG system assigns RMS patients who have undergone surgical resection into four clinical groups, before chemotherapy is started. Classification of RMS patients in the CG system is based on the degree of completion of surgical resection and the level of metastatic dissemination of the tumor into the distal sites as outlined in Table 3.

Clinical Group I	Localized disease, completely resected, regional nodes not involved. This includes both gross inspection and microscopic confirmation of complete resection.
Clinical Group II	Total gross resection with evidence of regional spread a. Grossly resected tumor with microscopic residual disease (tumor at margin) b. Regional involved nodes, completely resected with no microscopic residual c. Regional involved nodes and microscopic residual
Clinical Group III	Incomplete resection or biopsy with gross residual disease
Clinical Group IV	Distant metastatic disease present at onset

Table 3: Classification of RMS tumors based on the Clinical Grouping (CG) staging system (56). Recreated with permission (Dasgupta et al., Rhabdomyosarcoma, *Seminars in Pediatric Surgery*, 2016).

Risk Stratification

Risk stratification helps to determine the intensity of treatment that needs to be performed in RMS patients' clinical trial. After a patient is classified into a certain stage and clinical group by the TNM and CG staging system respectively, risk stratification puts them into a certain risk group. According to the Children's Oncology Group (COG), the risk stratification classifies RMS patients into low, intermediate and high risk groups as shown in Table 4. It is interesting to note

Risk group	Histology	Primary site	Initial resection	Distant metastases	Proportion of patients (%)	EFS (%)
Low	ERMS Unfavorable	Favorable Unfavorable	Any Yes	None None	32	70-95
Intermediate	ERMS ARMS	Unfavorable Any	No Any	None None	27 25	73 65
High	ERMS ARMS	Any Any	Any Any	Present Present	8 8	35 15

Table 4: Children's Oncology Group (COG) risk stratification of RMS tumor studies. EFS, event-free survival. Recreated with permission (Dasgupta et al., Rhabdomyosarcoma, *Seminars in Pediatric Surgery*, 2016).

that the risk stratification protocol is slightly different for European countries, which classify RMS tumors into low, standard, high and very high risk groups.

Management/ Treatment

The management of RMS worldwide is coordinated by the three cooperative groups; COG Soft Tissue Sarcoma Committee (North America), the European pediatric Soft Tissue Sarcoma Study Group (EpSSG), and the Cooperative Weichteilsarkom Studiengruppe der GPOH (CWS) group. Their goal is to detect RMS tumors early, stratify risk and apply effective treatments to control the disease. The multimodal therapeutic approach of RMS treatment regimen includes the combination of surgery, chemotherapy and radiotherapy. Surgical resection and external beam ionizing radiation (depending on metastatic and recurring nature of the tumor) is performed to remove the primary tumor from its local site. Ionization radiation is primarily applied to higher stage RMS patients in order to minimize disease relapse. Chemotherapy involves multiple agents that are administered in up to 15 cycles over 6-9 months depending on disease stratification (60). In North America, the VAC treatment regimen is followed and that involves treatment with the combination of vincristine, actinomycin D and cyclophosphamide whereas in European countries, the treatment regimen IVA is followed and it includes ifosfamide, vincristine, and actinomycin D (57). The outcomes were very similar in a study that compared the effects of VAC vs IVA treatment regimens (65). Addition of doxorubicin to currently available RMS treatment regimens have been suggested since doxorubicin is used in treatment of several sarcomas. In fact, the EpSSG recently conducted a study to investigate whether addition of doxorubicin to their standard IVA regimen improves the effectiveness of RMS chemotherapy (66). However, addition of doxorubicin to the IVA regimen resulted in several adverse effects such as anemia, thrombocytopenia and gastrointestinal problems in RMS patients. In addition, there were no significant differences in

three-year event-free survival rates in between IVA vs IVA plus doxorubicin treatment groups. Moreover, two treatment-related deaths were observed in IVA plus doxorubicin group and therefore EpSSG decided to retain the IVA chemotherapy regimen as the standard care of RMS in Europe (66). Although the currently available VAC and IVA treatment regimens for RMS are effective in low-risk cases, the children with metastatic RMS have very poor survival outcome (3-year survival 25 to 30% with less than 20% event free survival) and therefore the discovery of new and more effective treatments are needed. Moreover, RMS survivors who undergo the currently available treatment regimens show severe side effects that affect the quality of their lives later in life. Adverse effects such as nausea, vomiting, cancer-related pain, genitourinary and sexual/reproductive health related problems such as enuresis and erectile dysfunction have been reported in adult RMS patients who have undergone current treatments as adolescents (57). In addition, physical impairment, performance limitations and difficulties in social adaptation have also been reported in RMS survivors (67).

MOLECULAR DRUG TARGETS IN RHABDOMYOSARCOMA

In an attempt to increase survival rates of RMS patients and to improve their quality of lives post-treatment, several targeted therapy studies are being investigated. The overall goal has been to discover critical targets in RMS carcinogenesis and design drugs to inhibit their functions and ultimately inhibit RMS tumor growth. A few examples of such targets in RMS therapy are insulin-like growth factor 1 receptor (IGF1R), anaplastic lymphoma kinase (ALK), vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 4 (FGFR4), platelet-derived growth factor receptor (PDGFR), mesenchymal-epithelial transition factor (MET), mammalian target of rapamycin (mTOR) and others which are summarized in Table 5 (68-70). Although a promising number of such targets in RMS have been

identified and their small molecule inhibitors show promising activities against RMS tumors in pre-clinical studies, they do not show the similar outcomes in clinical studies in patients and therefore are not approved for clinical applications. Table 5 lists some of the compounds and their targets that are currently being investigated for targeted therapies in RMS in preclinical and/or clinical development in both North America and Europe (70).

Molecular target	Drug	Phase
BRD4	JQ1, OTX015	Preclinical
CHD4	ED2-AD101 (SMARCA5/CHD4 dual inhibitor)	Preclinical
HDAC	Entinostat, Vorinostat	Clinical (I/II)
ALK	Critotinib	Clinical (II)
PLK1	Volasertib	Preclinicial
GSK3β	Tideglusib, LY2090314, 9-ING-41	Preclinical
PI3K/mTOR	Omipalisib, Temsirolimus	Clinical (I/II)
MEK1	Cobimetinib	Clinical (I/II)
FGFR	Erdafitinib	Clinical (II)
IGF-1R	R1507 (mAb)	Clinical (II)
VEGF	Bevacizumab (mAb)	Clinical (II)
Multi-RTKs	Regorafenib	Clinical (II)
SMO	LDE225, Erismodegib, Vismodegib, Sonidegib	Clinical (II)
NOTCH	RO4929097, MK0572, brontictuzumab (mAb), tarextumab (mAb)	Preclinical
CDK4/6	Palbociclib, Ribociclib, Abemaciclib	Preclinical
Wee1	AZD1775	Clinical (I/II)
PARP	Olaparib, Iniparib, Veliparib	Clinical (II)
Bcl-2	Venetoclax, ABT-737	Preclinical
XIAP	Smac mimetics (LCL161)	Preclinical

Table 5: Targeted therapies for RMS that are undergoing preclinical and clinical assessment in North America and Europe (70). Created from Chen et al., Current and Future Treatment Strategies for Rhabdomyosarcoma, *Frontiers in Oncology*, 2019.

Receptor tyrosine kinases (RTKs)

RTKs are transmembrane protein receptors that are possible targets in RMS chemotherapy and IGF1R, ALK, VEGFR, EGFR, and FGFR4 are a few examples of such proteins (68). IGF1R

is a receptor for a family of mitogenic growth factors known as IGFs. IGF1R is important for cell survival, which is developmentally regulated i.e. its expression is high in embryonic stages but it gradually decreases in postnatal stages. However, the level of IGF1R is upregulated in cancer cells including RMS and the growth signal is constitutively activated (71). Another important RTK is VEGFR, a receptor that binds VEGF and has been previously discussed in the hallmarks of cancer section. Overexpression of VEGF in cancers including many pediatric solid tumors activates angiogenesis constitutively, which is an important hallmark of cancer. In RMS, studies have shown that VEGF is an important therapeutic target and its expression is significantly higher in ARMS than ERMS (72). Furthermore, the transcription of RTKs such as IGF1R and ALK is enhanced by fusion protein PAX3-FOXO1 in ARMS and therefore, are potential drug targets in RMS chemotherapy (68). In addition, members of several RTK-regulated downstream pathways such as PI3K/AKT/mTOR, JAK/STAT3, and RAS/MEK/ERK, along with the enzymes, receptors and proteins in other important pathways such as apoptosis, cell cycle progression and Hedgehog signaling have been investigated as possible targets for RMS therapy (68). Although inhibition of RTKs sounds promising for RMS treatment, the clinical efficacy of RTK-inhibitors has been limited and is observed only in a very small number of RMS patients (68).

PAX3-FOXO1

Translocation of chromosome 2 and 13, t(2;13) results in the fusion of N-terminal DNA binding domain of PAX3 protein to the C-terminal transactivation domain of FOXO1 protein resulting in the formation of chimeric PAX3-FOXO1 oncoprotein (73). Both PAX3 and FOXO1 are transcription factors and the PAX3-FOXO1 fusion protein is a potent activator of gene transcription. Studies show that the expression of PAX3-FOXO1 increases the activation of downstream target genes by 10-100 fold in comparison to wild type PAX3 or FOXO1 genes (74).

Microarray hybridization studies (cDNA and oligonucleotides) have identified several potential targets of PAX3-FOXO1 and majority of them are involved in myogenic differentiation and signaling, mesodermal development, and muscle contraction (75, 76). PAX3-FOXO1 regulates transcription of these target genes and activates several downstream pathways to promote tumorigenesis. PAX3-FOXO1 also facilitates RMS tumor cell proliferation and survival, induces angiogenesis and suppresses terminal differentiation and apoptosis (75). PAX3-FOXO1 is also a major driver of aggressiveness in fusion positive ARMS. It is also responsible for unfavorable prognosis, early metastasis and more frequent post-therapy relapses in ARMS patients (73, 75, 77). PAX3-FOXO1 also promotes tumorigenesis by suppressing the antitumor activity of interleukin-24 (IL-24) and therefore it is an important drug target in RMS (77). Since expression of PAX3-FOXO1 is specific to fusion positive ARMS tumor cells, targeting PAX3-FOXO1 for ARMS treatment can be a promising therapeutic strategy with a lower risk of treatment resistance. However, targeting PAX3-FOXO1 or any other fusion proteins and testing the effects of potential drugs/inhibitors is challenging because they are expressed in a small number of patients in these disease categories (74). Moreover, PAX3-FOXO1 is a transcription factor that lacks enzymatic activity, which makes it even more difficult to target PAX3-FOXO1 directly (78). Therefore, targeting downstream effector molecules in PAX3-FOXO1 regulated pathways, transcriptional and epigenetic co-regulators and upstream activators of PAX3-FOXO1 could be promising approach in targeted therapeutic studies in RMS (73). Recent studies in this laboratory show that PAX3-FOXO1 is regulated by the orphan nuclear receptor 4A1 (NR4A1) and the effects of NR4A1 antagonists on PAX3-FOXO1 expression are discussed below (79).

Epigenome

Epigenomic alterations change the conformation of chromatin that results in activation or repression of several genes which determine how organisms respond/adapt to the environment. Regulation of transcription and gene expression is important because it controls cell division, growth, migration, and survival. Mutations in cancer cells result in epigenomic modifications that can result in repression of tumor suppressor genes and activation of tumor pro-oncogenes (80). Therefore, genes and enzymes involved in epigenetic alterations such as histone and DNA modifications are potential targets in cancer therapy including RMS (81). Histone deacetylases (HDACs) are an example of such a family of enzymes. HDACs catalyze removal of acetyl groups from histone lysine residues and this results in compact nucleosome conformation and restricted access resulting transcription silencing (82). In cancers, histone deacetylases are overexpressed and this results in repression of tumor suppressor genes expression (83, 84). Therefore, inhibition of histone deacetylases is a promising approach in cancer therapy. One study showed that histone deacetylases inhibitors induced apoptosis and suppressed growth and proliferation in RMS (85). Another study showed that histone deacetylases inhibitors induced reactive oxidative species (ROS) that inhibited Sp transcription factors (TFs) and decreased expression of Sp-regulated oncogenes resulting in decreased RMS cell and tumor growth (86). Lysine-specific demethylase 1 (LSD1) is another enzyme that is responsible for epigenetic alterations. LSD1 catalyzes demethylation of lysine 4 on histone 3 (H3K4me2) and causes transcriptional silencing. LSD1 promotes cancer cells growth and proliferation and tumor progression by inhibiting tumor suppressor p53 protein expression (87). Since LSD1 is also overexpressed in RMS (88), it is a target for RMS therapeutic studies. RMS tumor cells overexpress multiple other enzymes that are involved in epigenomic modifications, which are drug targets in RMS studies (81). This thesis

focuses on euchromatin histone methyltransferase (EHMT2/G9a), an enzyme primarily responsible for histone methylation which is discussed below.

EHMT2/ G9a

EHMT2, commonly known as G9a is a histone methyltransferase protein that primarily catalyzes dimethylation of lysine 9 residue on histone 3 (H3K9me2). G9a-mediated dimethylation of histone (H3K9me2) results in a more compact nucleosome conformation and transcriptional repression (89, 90). G9a-mediated gene repression is essential in multiple important biological processes such as embryonic development, DNA repair, gene transcription, autophagy, cognitive and adaptive behavior, and adipogenesis (91, 92). G9a-mediated transcriptional silencing is dominant and loss of G9a results in embryonic lethality in mice (90, 93). G9a also regulates transcription of several RNA polymerase I and RNA polymerase II- regulated genes (93) and G9a directly interacts with replication protein A (RPA) complex to facilitate DNA repair, and loss of G9a halts the DNA repair process (94). Moreover, G9a-mediated repression of several key genes in adult heart is necessary to maintain homeostasis in cardiomyocytes (95). In addition, G9a directly contributes the occurrence and development of tumors and is overexpressed in multiple cancer cells such as colon, ovarian, bladder, liver, bone marrow, gastric, breast, lung, pancreatic and prostate cancer (91, 96). G9a is a pro-oncogene that facilitates growth and proliferation of cancer cells. In gastric cancer cells, G9a directly activates mTOR to enhance cell proliferation and suppress autophagy (96). In breast cancer, several genes that suppress tumor growth and progression under hypoxic condition are silenced by G9a-mediated H3K9me2 and G9a is also associated with poor prognosis in breast cancer patients (97). In ovarian cancer, G9a promotes migration, invasion and peritoneal metastasis and the expression of G9a is directly correlated to the aggressiveness of the ovarian cancer (98). Expression of G9a and G9a-mediated gene

repression are correlated to tumorigenesis, and therefore G9a is an important target in cancer therapy. Recently, it was shown that G9a is also overexpressed in both ERMS and ARMS cell lines and it has pro-oncogenic functions in those cell lines and therefore targeting G9a for RMS treatment can be a promising approach (99, 100). Since G9a promotes ERMS cell proliferation by suppressing canonical Wnt pathway, treatment of ERMS cells with G9a inhibitor UNC0642 decreased cell proliferation and viability and enhanced myogenic differentiation (99). G9a also promotes growth and proliferation and suppress myogenic differentiation in ARMS cells. In addition, G9a-mediated formation of H3K9me2 silences the expression of tumor suppressor PTEN protein in ARMS cells and enhances tumor growth and progression (100). Therefore, G9a is a potential drug target in RMS. The G9a-mediated H3K9me2 silencing of PTEN and subsequent phosphorylation of Akt in ARMS is suppressed by G9a inhibitor UNC0642 and ARMS tumor growth in vivo is also inhibited by G9a inhibitor UNC0642 (100). Our recent studies in this lab show that G9a is regulated by NR4A1. Furthermore, NR4A1 antagonists inhibit the expression of G9a-mediated downstream genes as well as their functions, which are discussed below.

NUCLEAR RECEPTORS

The nuclear receptor (NR) superfamily includes several transcription factors that play important roles in maintaining cellular homeostasis associated with development, reproduction, metabolism, inflammation and are also involved in multiple disease processes (101). Forty-eight NRs have been identified in humans and cognate ligands for approximately 50% of NRs have also been identified (102). Although there is evidence that some NRs exhibit extracellular functions and ligand-independent activities, most NRs are ligand-activated transcription factors (101). The ligands associated with NRs are diverse and include vitamins, steroid hormones, retinoids, phospholipids, nutrient metabolites, and synthetic small molecules such as dexamethasone,

enzalutamide, vitamin D3, and fatty acid derivatives (103, 104). Ligand binding to NRs results in conformational changes which displace co-repressor molecules bound to the receptor. This results in recruitment of co-activator proteins that interact with the receptor and facilitates interactions of the bound NR to specific DNA sequences in promoter regions of target genes to regulate transcription (105). Since NRs bind ligands, they are also important drug targets for treating several diseases such as metabolic disorders, inflammation, osteoporosis, and cancer (103, 106). In fact, a study (107) showed that the NR family accounts for 3% of all protein drug targets in humans. Drug-NR interactions are responsible for the therapeutic effects of 16% of all available small-molecule drugs, only surpassed by G protein-coupled receptors (33%) and ion channels (18%) (107). There are currently 161 total drugs (small molecules and biologics) that target NRs (107) and they are used for the treatment of allergies, contraception, autoimmune disorders, hyperlipidemia, and cancer (103). One study reported that in 2002, the prescribed drugs that targeted NRs generated revenues of more than 9 billion USD (108).

The NRs have common structural motifs. A canonical NR has an N-terminal domain (A/B) containing AF-1, a central DNA-binding domain (DBD) (C), a hinge region (D), and a C-terminal ligand binding domain (LBD) (E) containing AF-2 (F) as illustrated in Figure 2. The N-terminal domain of a NR is highly variable and has a ligand-independent activation domain AF-1 (109), which is intrinsically disordered and is essential for transcriptional regulation (102). The central DBD contains a highly conserved double zinc-finger that allows the NRs to target and bind to specific DNA sequences. There is a hinge region in between the DBD and the LBD, which like the N-terminal domain is also intrinsically disordered. The hinge region which also contains a nuclear localization signal and sites for posttranslational modifications, plays a crucial role in allosteric regulation, inter-domain communications, and maintains the structural flexibility to

allow the receptor dimers to interact with multiple response elements (102, 109, 110). The hinge region is followed by a C-terminal LBD which is important for ligand recognition and binding, cofactor interactions as well as receptor dimerization (109). The final alpha-helix (H12) in LBD is called AF-2, which upon ligand binding undergo conformational change that releases co-repressors bound to the receptor and recruits co-activators to activate the receptor (105, 111). The NRs can also be in constitutively active state in some cases where AF-2 domains are in fixed active conformation, in which case the activity of a receptor can also be regulated by modifications such as phosphorylation or acetylation (109).

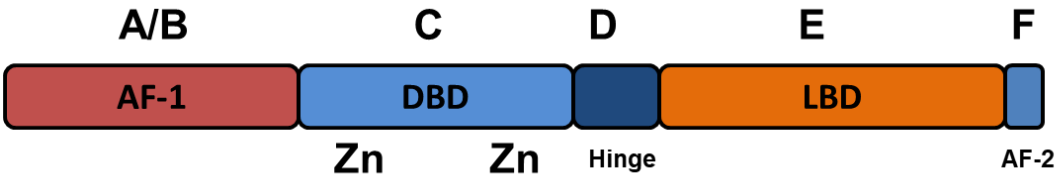

Figure 2: Schematic representation of a canonical NR containing an N-terminal AF-1 domain, a central DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD) with AF-2.

Based on the discovery of their endogenous ligands, the NR superfamily can be classified into three major families; endocrine receptors, adopted orphan receptors, and orphan receptors which are discussed below (109):

Endocrine receptors

The first two identified NRs; glucocorticoid receptor and estrogen receptor α belong to endocrine receptor family, which includes steroid hormone receptors and the heterodimeric

receptors (109, 112, 113). The endocrine receptors bind fat soluble hormones and vitamins with very high affinity (K_d values in the nM range). Steroid hormone receptors, which function as homodimers include receptors that bind to steroid hormones such as progesterone, estrogen, androgen, glucocorticoids and mineralocorticoids (114). The steroid hormone system regulates important processes associated with metabolism (carbohydrate metabolism and electrolyte balance) and development (reproduction and sexual differentiation) (115). In contrast, heterodimeric receptors include receptors such as thyroid hormone receptor, vitamins A receptor (retinoic acid receptors), and vitamin D receptor which form heterodimers with retinoid X receptor (RXR) (109). Since endocrine receptors are important for maintaining the endocrinal homeostasis, they are targeted by several clinically used drugs. For example: Tamoxifen targets the estrogen receptor and is therefore included in endocrine therapies for the treatment of estrogen receptor positive breast cancer (116).

Adopted orphan receptors

Adopted orphan receptors were designated as orphan receptors at first since they were initially identified based on sequence homology to endocrine receptors; their endogenous ligands were unknown in the beginning but were subsequently identified. Unlike endocrine receptors, adopted orphan receptors respond to dietary lipids and bind to lipid ligands with relatively low affinity (K_d values in 1- 10 µM range) (115). Adopted orphan receptors include lipid sensors and enigmatic orphans. Lipid sensors include receptors for fatty acids (PPARs), oxysterol (LXRs), bile acids (FXR) and xenobiotics (PXR) whereas enigmatic orphans include receptors for androstane (CAR), fatty acids (HNFs), phospholipids (SF-1/LRH-1), cholesterol and retinoic acid (RORs) (109, 115). Adopted orphan receptors function by forming heterodimer with the retinoid X receptor (RXR). These receptors bind lipid ligands and regulate lipid homeostasis and glucose metabolism

and are therefore an important drug targets for the treatment of metabolic diseases such as dyslipidemia, diabetes, obesity, and cardiovascular-related diseases (117) .

Orphan nuclear receptors

Orphan NRs are the third family of the NR family and are often referred to as "true orphans" since endogenous ligands for these receptors are not yet identified. Some of these receptors may not have endogenous ligands and there is also evidence that the ligand binding pocket of some orphan NRs contain bulky amino acid side chains that inhibit ligand binding. It is also possible that ligands do not induce appropriate conformational change in orphan NRs or there may be problems for effective interactions of cofactors (109, 118, 119). Therefore, it is possible that the orphan NRs are regulated by the combination of receptor expression and covalent modifications (109). Orphan NRs also regulate transcription like other NRs and therefore activate or repress expression of downstream target genes. Orphan NRs also play an important role in cellular homeostasis and are involved in several diseases including inflammation, obesity, atherosclerosis, and cancer (120, 121). This also makes orphan NRs an important drug target even though role of endogenous ligands are not defined. To understand this, several approaches such as changing the expression levels of these receptors and understanding their interactions with other co-activators, co-repressors, and synthetic ligands have been investigated (122). Studies have now identified synthetic ligands for many orphan NRs which have allowed the researchers to understand the mechanism of how orphan NRs modulate gene transcription (120, 123-125). The orphan NRs are classified into several groups based on their discovery, regulation and physiological functions (126). These groups with unknown endogenous ligands are discussed below:

NR0B Orphan Receptors

The NR0B group includes two uncharacteristic orphan NRs; dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region on chromosome X gene (DAX-1/NR0B1) and small heterodimeric partner (SHP/NR0B2). They contain a classical LBD but lack canonical DBD (126). The DBD in these receptors are replaced by repeats of 65-70 amino acid alanine/glycine-rich motif with unknown homology (127). Since their DBDs are absent, DAX-1 and SHP bind to the AF2 of other NRs and thereby prevent coactivator binding to repress transcription (126, 128). The transcriptional repression functions of DAX-1 and SHP are important in cholesterol metabolism pathways (128). DAX-1 is overexpressed in the hypothalamic-pituitary-adrenal-gonadal axis and plays an important role in steroidogenesis and reproductive development such as gametogenesis and sex determination (126, 128, 129). Unlike DAX-1, SHP is overexpressed in the liver and small intestine and is involved in maintaining cholesterol and bile acid and glucose homeostasis (126, 128). In addition, DAX-1 and SHP are also involved in development of several diseases including multiple cancers and adrenal hypoplasia (120, 128, 130).

NR1D Orphan Receptors

The NR1D group contains two nuclear orphan receptors; REV-ERBα (NR1D1) and REV-ERBβ (NR1D2). Receptors in this group are also atypical because unlike other NRs, they lack the conventional alpha-helix (H12) AF2 domain where coactivators bind, creating a hydrophobic surface (131). This results in receptor-conformations that favor corepressor binding instead and therefore these orphan receptors are dominant transcriptional repressors (131). REV-ERBs are involved in several important cellular processes such as adipogenesis, brain development, and mitochondria biogenesis. In addition NR1D orphan receptors are also known for their involvement

in maintaining the circadian clock in brain and liver tissues as they regulate the expression of molecular clock genes such as BMAL1 and CLOCK (120, 126, 131).

NR2C Orphan Receptors

Two structurally related testicular receptors TR2 (NR2C1) and TR4 (NR2C2) are included in the orphan receptor NR2C group. These receptors are widely expressed in several tissues. They bind to response elements either as homodimers or as heterodimers partnered to one another (126) and can act as both transcriptional activators and repressors. One study showed that the TR group regulates the silencing of embryonic β-type globin genes by recruiting multiple corepressors for epigenetic modifications (132). Another study also showed that the TRs are crucial in embryogenesis since TR knockout mouse embryos were developmentally defective and died in early stages (133).

NR2E Orphan Receptors

The two members of NR2E orphan receptor group are tailless homolog TLX (NR2E1) and photoreceptor-specific PNR (NR2E3) NRs. TLX receptors are expressed in brain and retinal neurogenic region and are important in brain stem cells and neocortex development and maintenance (134). However, overexpression of TLX receptors is associated with brain tumor carcinogenesis and poor survival of glioma patients (135). PNR receptors are widely expressed in cone and rod cells in retina and is important for photoreceptor development (136). Mutations in PNR resulted in enhanced S-cone syndrome in humans and retinal degeneration in mice (134, 137). A study showed that the PNR enhances the tumor suppressor p53 acetylation and therefore is a potential drug target in cancer (136).

NR2F Orphan Receptors

The NR2F group of orphan receptors includes two chicken ovalbumin upstream promoting TF (COUP-TF) receptors; COUP-TFI (NR2F1) and COUP-TFII (NR2F2) receptors and an ERBA-related protein 2 (EAR2/ NR2F6). COUP-TFs were initially identified as homodimers that bind response element in the chicken ovalbumin promoter and are known to repress transcription (126). COUP-TFI and COUP-TFII show extraordinary sequence homology of 97% and 99% in their LBD and DBD respectively (120, 138). COUP-TFs are very important for development and differentiation of tissues and they are involved in critical processes such as cell cycle regulation, cell survival maintenance and regulation of cell-specific differentiation (139). Moreover, COUP-TFs receptors are overexpressed in several cancers such as ovarian, breast, and prostate cancer and promote tumorigenesis and are therefore drug targets in these cancers (120, 140-142). Another receptor in NR2F group, EAR2 is also a transcriptional repressor which forms homodimers or heterodimers with COUP-TFs and other NRs to regulate genes involved in several biological processes. EAR2 is involved in development of locus coeruleus and deletion of EAR2 causes neural and behavioral deficiencies in mice (143). Moreover, EAR2 also suppressed lymphocyte activation and the autoimmune system in mice (144) and deletion of NR2F6 in CD8+ T cells facilitated early IFN-γ production that enhanced antigen-specific memory formation (145). In addition, EAR2 is upregulated and inhibits apoptosis in colon cancer cells and facilitates colorectal cancer development which makes it an important drug target in treatment of colorectal cancer (146).

NR6A Orphan Receptors

The NR6A group contains a single orphan NR namely germ cell nuclear factor (GCNF/ NR6A1). GCNF binds to DR0 (direct repeat elements with 0 spacing) and interacts with other

corepressors to silence transcription of several other genes. For example: GCNF recruits DNA methylation machinery to target and repress the transcription of Oct4, a gene that is essential for pluripotency in early embryonic cells (147). GCNF is highly expressed in embryos and is essential for germ cell differentiation and embryonic development and the GCN-deficient embryos undergo several embryogenic and cardiovascular-related deficiencies that result in early death (148). GCNF is also widely expressed in the developing nervous system and is involved in neurogenesis and neural differentiation (148, 149).

NR4A Orphan Receptors

NR4A Introduction

NR4A includes three orphan NRs; NR4A1 (Nur77/ TR3), NR4A2 (Nurr1) and NR4A3 (Nor1). The NR4A are early immediate or stress response genes that are induced by multiple diverse stimuli (150-152). Fatty acids, growth factors, calcium, neurotransmitters, and inflammatory cytokines are some examples of physiological stimuli and magnetic fields, mechanical agitation and membrane depolarization are physical stimuli that induce NR4As (150). Since NR4As rapidly respond to changes in the cellular environment, they are expressed in many tissues such as brain, skeletal muscle, heart, kidney, adipose tissues, T-cells, and liver, where energy demand is high (150). They are also involved in various cellular processes such as cell proliferation, differentiation, survival, apoptosis, DNA repair, angiogenesis, and fatty acid metabolism (151, 153, 154). NR4A also play major roles in hormonal, physiological, and pathological processes and are therefore highly expressed in inflammatory diseases, cardiovascular diseases, atherosclerotic lesions, neurological disorders, chondrosarcomas, metabolic diseases, psoriasis, and cancer (101, 120, 150, 152, 154, 155).

NR4A Structure

The three members of NR4A group are structurally similar and the sequence homologies in the C-terminal LBDs and central DBDs in between these receptors are 60% and 91-95% respectively, however the sequence conservation in their N-terminal domains is only 26-28% (101, 154, 156). These receptors however, are atypical of conventional NRs because they have a unique LBD. As previously discussed, ligand-binding induces conformational changes in many NRs including positional shifting of alpha-helix (H12), also known as activation helix in LBD. This results in formation of hydrophobic cleft (H12, H3 and H4 are involved) which binds several co-activators that are required to activate NRs (153). In addition, two highly conserved NR-LBD amino acid residues; a lysine in helix 3 (H3) and a glutamate residue in H12, upon conformational change induced by ligand-binding, generate a charge clamp to directly interact with the co-activator (153, 157). In contrast, the NR4As have a significantly different and an unusual LBD. The NR4As have a relatively small ligand binding pocket which hinders ligand-binding (109). The crystal structure of the LBD domain of NR4A2 and DHR38, a *Drosophila* ortholog of the mammalian NR4A1 showed that the combination of bulky aromatic and hydrophobic residues in LBD were packed in a tight conformation resulting in the absence of a ligand-binding pocket (118, 119, 153, 158). Moreover, a classical co-activator binding site is also absent in NR4A2 thus inhibiting co-activator recruitment (119). Furthermore, in NR4As, the highly conserved lysine in H3 is replaced by glutamate and the highly conserved glutamate in H12 is replaced by lysine, generating an unusual charged surface instead of a hydrophobic cleft, which precludes co-activator binding (119, 153, 156). The unconventional features of the NR4A-LBD have made it challenging to identify the endogenous ligands for these receptors and it was initially suggested that all the

NR4As are ligand-independent transcription factors (119). Studies have now identified several synthetic compounds that bind orphan NR4As and act as exogenous ligands (101, 123).

NR4As as transcription factors

The NR4As bind to different response elements on gene promoters to regulate their transcription (101, 150, 159). Studies have shown that NR4As independently or by interaction with other cis-molecules activate ligand-independent gene expression (159). NR4As bind to octanucleotide sequence known as NGFI-β response element (NBRE; AAAGGTCA) as a monomer and a Nur-responsive element (NuRE; $TGATATTTn_6AAATGCCA$) as a homodimer (124). NR4A1 and NR4A2 can also form a heterodimer with RXR and bind to DR5 motif that has two direct repeats of an NR binding motif separated by five nucleotides (101, 150, 159). In addition, NR4A also interact with DNA-bound specificity (Sp) proteins as transcriptional co-factors and regulate expression of multiple pro-oncogenic Sp-regulated genes in cancer cells (101). NR4As bind two common Sp proteins; Sp1 or Sp4 and the NR4A1-Sp complex along with other co-factors regulate the expression of some important genes such as PAX3-FOXO1, Bcl-2, and β-integrins in different cancers (79, 159, 160). Studies also show that NR4A1-knockdown and/or Sp-knockdown decrease the expression of those oncogenes in cancer. A unique pathway that primarily involves NR4A1 has shown that several apoptosis-inducing agents trigger nuclear export of NR4A1, where NR4A1 interacts with Bcl-2 protein forming a mitochondrial pro-apoptotic complex that induces cytochrome c release, disrupts mitochondria and triggers apoptosis in cells (161-163).

Role of NR4As in blood-derived tumors

NR4A1 and NR4A3 exhibit potent tumor suppressor activity in acute myeloid leukemia (AML) and pre-AML malignancies and mutant mice lacking NR4A3 and NR4A1 (double knockout) showed rapid development of AML (164, 165). Deletion of both NR4A3 and NR4A1 in mice resulted in abnormal expansion of hematopoietic stems cells and myeloid progenitors. The NR4A3 and NR4A1 double knock out mice also displayed defective apoptotic signaling, abnormal phenotype, and died within 2-4 weeks due to rapid AML development (164). Interestingly, the level of NR4A3 and NR4A1 transcripts are also downregulated in AML patients' leukemic blasts further showing that these NR4As are tumor suppressors in AML (164). A significant 71% reduction of NR4A1 and NR4A3 were also reported in chronic lymphocytic B-cell leukemia in comparison to normal cells of origin (166). In addition, dihydroergotamine, an FDA-approved drug, show anti-leukemic activity in human AML cells by inducing NR4As and NR4A-regulated genes (165). Moreover, treatment of AML cells with the histone deacetylase (HDAC) inhibitor Entinostat restored the expression of NR4A1 and NR4A3 and induced apoptosis in AML cells (167). This makes NR4As an important drug target in AML treatment.

NR4A1 and NR4A3 are also tumor suppressors in lymphoma and a study (166) showed a significant reduction in expression (both mRNA and protein) of these receptors in two aggressive B-cell lymphomas; follicular lymphoma (70%) and diffuse large B-cell lymphoma (74%) when compared to normal cells. The same study also reported that reduction of NR4A1 and NR4A3 in those lymphomas are also directly correlated with decreased apoptosis. In addition, decreased NR4A1 expression increased the aggressiveness of lymphoma tumor and correlated with poor patient survival (166). Overexpression of NR4A1 and NR4A3 induced apoptosis in aggressive lymphoma cells and reduced tumor growth in mouse xenografts bearing lymphoma cells (166,

168, 169). Moreover, treatment of aggressive lymphoma cells with NR4A1-agonist cytosporone B (101, 170) or with NR4A3-inducer thapsigargin (171) induced apoptosis showing tumor-suppressor activities of NR4A1 and NR4A3 in aggressive lymphomas (166, 168).

Role of NR4As in solid tumors

The functions of NR4As in solid tumors have been widely studied and the role of NR4A1 has been more extensively investigated compared to NR4A2 and NR4A3 (159). Interestingly, although NR4A1 has tumor suppressor-like activity in blood-derived tumors, it exhibits pro-oncogenic characteristic in solid tumors (79, 120, 159, 172). Similar to NR4A1, studies have shown that NR4A2 is also pro-oncogenic in solid tumors and plays an important role in cancer cell growth, proliferation, migration, invasion and survival (173, 174). NR4A3 is overexpressed in several carcinomas and it is pro-oncogenic in acinic cell carcinoma since it induces cell proliferation and regulates expression of downstream oncogenes (159, 175, 176). However, NR4A3 exhibits tumor suppressor-like activities in several other tumors and it has been reported that the tumor suppressor p53 protein directly binds to the promoter of NR4A3 to induce its transcription in multiple cancer cell lines, where NR4A3 induces apoptosis (177). This shows that although NR4A1 and NR4A2 are pro-oncogenic in solid tumors, NR4A3 exhibits tumor-suppressor like characteristics in multiple cancers. Therefore, the NR4As are important drug targets in treatment of different cancers. This thesis specifically focuses on the role of NR4A1 in solid tumors which is discussed below:

Role of NR4A1 in solid tumors

Although NR4A1 exhibits tumor suppressor-like activities in blood-derived cancers, NR4A1 is pro-oncogenic in solid tumors and the mechanisms of how NR4A1 exhibits pro-

oncogenic activities in solid tumors have been extensively studied. NR4A1 is overexpressed in multiple solid tumors and that includes colon, pancreatic, breast, lung, ovarian, endometrial, cervical cancers, melanomas, and RMS (79, 120, 160, 172, 178-190). Experiments such as knockdown and/or overexpression of NR4A1 have been carried out to understand the functional activity of NR4A1 in solid tumors. Knockdown of NR4A1 in multiple solid tumor-derived cell lines decreased cancer cell growth, proliferation, survival, migration, and invasion showing the pro-oncogenic role of NR4A1 in solid tumors (79, 160, 172, 180, 183, 186, 187, 189). In addition, overexpression of NR4A1 is also associated with unfavorable prognosis and decreased survival in breast, lung, colon and ovarian cancer patients (178-180, 182, 188). NR4A1 regulates several pro-oncogenic pathways and genes in cancer cells (Figure 3) and is therefore a potentially important drug target for cancer therapy. Knockdown of NR4A1 or its inhibition by treatment with CDIM/ NR4A1 antagonists (which will be discussed later) inhibit NR4A1-regulated pro-oncogenic pathways and gene expression in cancer cells. NR4A1 directly binds the tumor suppressor p53 protein, blocks its acetylation and downregulates the transcriptional activity of p53 (187, 191). In lung and colon cancer cells, NR4A1 binds and inactivates p53, thereby inhibiting sestrin-2 and AMPKα, which results in activation of mTOR signaling pathways that facilitates cancer cell growth and proliferation (180, 186). NR4A1 also regulates expression of pro-reductant genes thioredoxin domain-containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1) in cancer cells (172, 183, 186) and this decreases oxidative stress and induces activation of mTOR signaling pathways and cancer cell proliferation (172, 183, 186) . Knockdown of NR4A1 decreases expression of TXNDC5 and IDH1 and induces ROS and oxidative stress. ROS induction activates sestrin-2 and AMPKα which inhibits mTOR signaling and cell proliferation (160, 172).

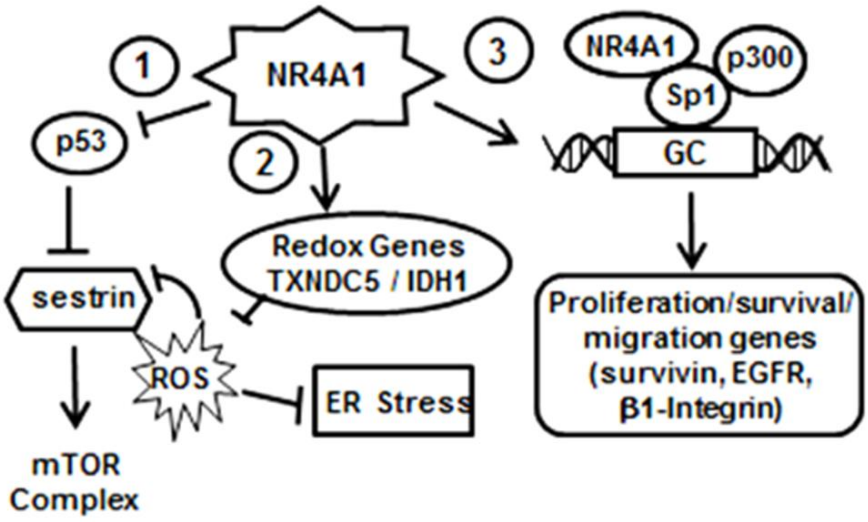

Figure 3: NR4A1-regulated pro-oncogenic pathways in cancer cells. Reprinted with permission (Lacey et al., PAX3-FOXO1A expression in rhabdomyosarcoma is driven by the targetable nuclear receptor NR4A1, *Cancer Research*, 2017).

NR4A1 binds NBRE and NuRE sequences to regulate expression of downstream target genes. In addition, it has also been reported that NR4A1 also binds DNA-bound Sp1 or Sp4 protein as a transcriptional co-factor and regulates expression of several Sp-regulated genes that facilitate cancer cell growth, proliferation, migration, and invasion (79, 192). For example, it has been reported that NR4A1 interacts with Sp4 bound to the GC rich promoter sequence of PAX3-FOXO1 and regulate the expression of this fusion oncogene (79) in ARMS cell lines. PAX3-FOXO1 is a major driver of aggressiveness and knockdown of NR4A1 decreased expression of PAX3-FOXO1 and PAX3-FOXO1-regulated downstream genes and their functions in ARMS cells. NR4A1-Sp1 complex along with p300 also bind the GC rich promoter sequence of the survivin gene and regulates expression of survivin in cancer cells (172, 186, 187). Survivin inhibits apoptosis in cancer cells and is also associated with poor clinical outcome in cancer patients (193). Studies also

show that knockdown of NR4A1 decreases expression of several other Sp-regulated genes such as anti-apoptotic Bcl-2, epidermal growth factor receptor, cyclin D1, β1 and β3 integrins, and c-myc revealing the involvement and importance of NR4A1 in Sp1-regulated gene expression in cancer cells (79, 172, 186, 187, 194).

Novel NR4A1 ligands

Cytosporone B and related compounds

Although endogenous ligands for NR4A1 have not been identified, studies in several laboratories have identified multiple small molecule compounds that bind NR4A1. Wu and coworkers screened libraries of natural products and identified the octaketide cytosporone B (Csn-B), a fungal metabolite as an NR4A1 ligand (170). They showed that Csn-B is an NR4A1 agonist that binds the NR4A1-LBD with a K_D value of 7.4×10^{-7} M and induces NR4A1-dependent transactivation (101, 170). In addition, Csn-B also induced apoptosis in cancer cells by triggering the nuclear export of NR4A1 to cytosol, where it forms pro-apoptic complex with Bcl-2 and this was accompanied by inhibition of cancer cell growth *in vitro* and tumor growth *in vivo* (170). Moreover, several analogs of Csn-B have also been characterized as NR4A1 ligands (195). PDNPA (n-Pentyl 2-[(1-nonanoyl)phenyl acetate]) is another synthetic ligand of NR4A1 which has structural similarities with Csn-B and binds NR4A1-LBD (196). Binding of PDNPA to NR4A1 specifically impedes p38α interaction with NR4A1 (101). In addition, PDNPA also binds other NR4As but without any phenotypic effects (101). Another exogenous ligand of NR4A1 with structural similarities with Csn-B is TMPA (ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl acetate]). TMPA impedes liver kinase B1 (LKB1) binding with NR4A1 and induces LKB1 nuclear export and AMPK phosphorylation (197). TMPA binds on the surface of NR4A1-LBD and is an NR4A1 antagonist. THPN (1-(3,4,5-trihydroxyphenyl)nonan-1-one) is also a Csn-B derived

analog that binds NR4A1 (198). THPN targets cytosolic NR4A1 and shuttles it to mitochondria and induces cell death via autophagy. THPN-NR4A1 binding further enhanced their interactions with the mitochondrial Nix protein and activated autophagy in melanoma cell lines (198, 199).

Celastrol and related compounds

Celastrol is an NR4A1 antagonist and binds on the surface near NR4A1-LBD with a K_D value of 0.29 μM and 0.32μM as determined by surface plasmon resonance (SPR) and fluorescence quenching assay respectively (200, 201). Celastrol inhibits NR4A1-dependent transactivation and also shuttles NR4A1 from nucleus to cytosol where it interacts with tumor necrosis factor receptor-associated factor 2 (TRAF2) to induce anti-inflammatory response (101, 200). In addition to celastrol, Csn-B and their analogs, several other biomolecules and compounds such as unsaturated lipids (arachidonic and docosahexaenoic acids), prostaglandin A_2, and IMCA (2-imino-6-methoxy-2H-chromene-3-carbothioamide) also bind NR4A1, however their receptor dependent activities and functions have not been fully investigated (101, 202-204).

Bis-indole derived (CDIM) molecules

Bis-indole derived CDIM molecules were initially identified as agonists of peroxisome proliferative-activated receptor γ (PPARγ) (205) but were later characterized as active NR4A1 antagonists that bind NR4A1 and inhibit NR4A1-dependent transactivation in several cancer cell lines (159). In addition, CDIMs inhibited most of the NR4A1-regulated pro-oncogenic pathways and downstream genes in multiple cancer cell lines including RMS (79, 160, 172, 180, 183, 186, 187, 189, 192). CDIMs also inhibited NR4A1-regulated cancer cell growth, proliferation, migration, invasion, and survival. RNA sequencing analysis along with other growth, proliferation, invasion and survival-related assays showed that similar phenotypic and genotypic effects were

observed in cancer cell lines when NR4A1 was inhibited by CDIMs or knocked down by RNA interference (RNAi) (79). 1,1-Bis(3'-indolyl)-1-(p-hydroxyphenyl)methane (DIM-C-pPhOH or CDIM8) and the corresponding p- carboxymethyl derivatives (DIM-C-pPhCO_2Me) represent a novel class of NR4A1 ligands (Figure 4A and 4B). Computational-based molecular modeling followed by fluorescence quenching and circular dichroism assays showed that the prototypical CDIM8, DIM-C-pPhCO_2Me and several other CDIM-analogs directly bind the ligand binding pocket of NR4A1-LBD and decrease NR4A1-dependent transactivation in cancer cells (186). CDIM8 binds four crucial amino acid residues Asp594, His556, Arg515 and Glu445 in NR4A1-LBD with K_D value of 0.11μM and inhibits NR4A1-regulated downstream genes and their functions (186). The K_D value for DIM-C-pPhCO_2Me was 0.25 μM (186). CDIM8 also inhibited tumor growth *in vivo* at a dose of 20-40 mg/kg/d (101). However, CDIM8 is rapidly metabolized and its blood levels are low mainly because of the hydroxyl group at C-4 position in the phenyl ring as illustrated by pharmacokinetic studies (206). Therefore, several buttressed (Figure 4C) analogs of CDIM8 with new functional groups at C-3 and C-5 positions ortho to the hydroxyl group were synthesized in this laboratory, which not only reduced the metabolic conjugation of this molecule, but also increased potency against cancer cell and tumor growth both *in vitro* and *in vivo* (190, 207). For example: DIM-C-pPhOH-3-Cl-5-OCH_3, a 3,5-substituted buttressed analog of CDIM8 inhibited breast tumor cell growth and has an IC50 of 2 mg/kg/d, which is >10 fold more potent compared to its parent molecule CDIM8 (207).

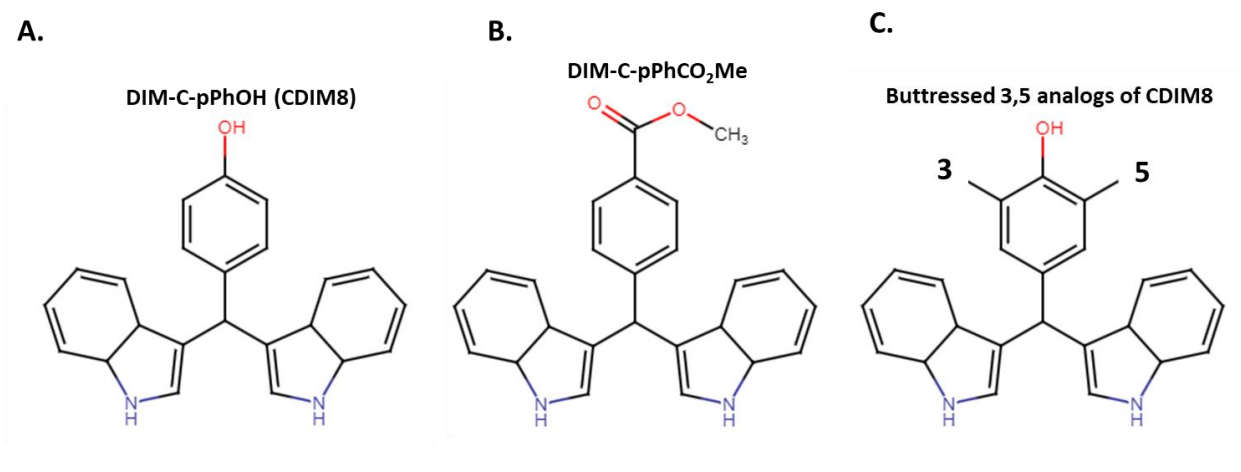

Figure 4: Bis-indole derived CDIM molecules. (A) Prototypical CDIM8 molecule. (B) p-carboxymethyl derivative of CDIM8. (C) Buttressed 3,5 substituted analogs of CDIM8.

NR4A1 is a transcription factor that binds NBRE and NuRE sequences on promoter of target genes as monomer and homodimer respectively and CDIMs directly bind NR4A1 and modulate the NR4A1-regulated gene expression (101). In addition, CDIMs also modulate the expression of genes that are regulated by NR4A1-Sp complex. CDIMs also induce and repress NR4A1-regulated genes and therefore antagonize NR4A1-regulated pro-oncogenic pathways (159). For example: the apoptosis inhibitor gene survivin is highly expressed in pancreatic cancer cells and NR4A1 knockdown or treatment with NR4A1 antagonist CDIMs decreased the expression of survivin and induced apoptosis in pancreatic cells (208). In addition, individual knockdown of NR4A1, Sp1, or p300 resulted in decreased survivin expression showing that all three of them are necessary for survivin transcription and NR4A1 acts as an essential nuclear cofactor for this Sp1-regulated gene (101). The expression of several other NR4A1-Sp regulated genes such as PAX3-FOXO1, PDL-1, Bcl-2, and integrins are also decreased in cancer cells when treated with NR4A1 antagonists and these genes are also regulated by the NR4A1/Sp complex (79, 192). TGFβ induces invasion in cancer cells by shuttling the nuclear NR4A1 into cytosol, where it interacts with proteasome complexes to degrade inhibitory SMAD-7 (178). Treatment of breast

and lung cancer cells with NR4A1 antagonists CDIMs inhibited TGFβ-induced invasion of these cancer cells by inhibiting the nuclear export of NR4A1, thereby preventing inhibitory SMAD-7 degradation (209, 210). In addition to inhibiting NR4A1-regulated pro-oncogenic pathways in solid tumors, studies show that CDIMs also enhance NR4A1-mediated glucose uptake in muscle cells, inhibit endometriosis, decrease neuronal inflammation in Parkinson's disease and enhance learning and memory in mouse models and are therefore promising pharmacologic agents for treating multiple diseases (101, 159, 189, 211-214). This thesis will specifically focus on some important pro-oncogenic pathways regulated by NR4A1 in RMS and their inhibition by NR4A1 antagonists CDIMs. Overall, this thesis has been divided into three separate chapters:

i. Chapter II will show for the first time that TGFβ induces invasion of ERMS but not ARMS cells and NR4A1 plays a crucial role in this pathway. This chapter will illustrate that TGFβ induces ERMS cells invasion through a novel pathway that involves cytosolic NR4A1 and this invasion is inhibited by NR4A1 antagonist CDIM8. Our results will also demonstrate that this is primarily due to induction of tumor suppressor-like cytokine interleukin-24 (IL-24).

ii. Chapter III will demonstrate that the histone methyltransferase gene EHMT2 (G9a) which is responsible for catalyzing methylation of histone 3 lysine 9 (H3K9) is an NR4A1-regulated gene in ARMS cells. G9a has been reported to have pro-oncogenic functions in multiple tumor types. This chapter will show for the first time that NR4A1 antagonists effectively downregulate G9a and further inhibit its downstream functions and therefore represent a novel class of G9a inhibitors.

iii. Chapter IV will identify flavonoids kaempferol and quercetin as novel NR4A1 ligands/antagonists. This chapter will illustrate that these flavonoids bind NR4A1 and decrease the

expression of NR4A1 as well other important NR4A1-regulated pro-oncogenic downstream genes. Furthermore, it will demonstrate that these flavonoids also inhibit NR4A1-regulated cell growth, proliferation, migration, and invasion and further induce apoptosis in RMS cells and also inhibit RMS tumor growth in mouse xenograft models. This discovery could provide a potential precision medicinal/nutritional approach for incorporating flavonoids in RMS therapies to improve efficacy and decrease levels of administered cytotoxic drugs.

In addition, studies on the co-regulation of various genes and responses by NR4A1and NR4A2 are ongoing but are not included in this thesis.

CHAPTER II

BIS-INDOLE DERIVED NUCLEAR RECEPTOR 4A1 (NR4A1) ANTAGONISTS INHIBIT TGFB-INDUCED INVASION OF EMBRYONAL RHABDOMYOSARCOMA CELLS*

INTRODUCTION

Orphan nuclear receptors NR4A1, NR4A2 and NR4A3 are immediate early genes induced by multiple stressors. NR4A receptors play an important role in maintaining cellular homeostasis and in pathophysiology (124). There is increasing evidence for a functional role for these receptors in metabolic, cardiovascular and neurological functions as well as in inflammation, inflammatory diseases, immune functions and cancer (124, 178-183, 215). NR4A1 is overexpressed in colon, pancreatic, breast (estrogen receptor positive and negative), and lung tumors. Moreover, high expression of NR4A1 in breast, colon and lung tumors correlates with decreased patient survival (178-182, 215). The functional activity of NR4A1 in solid tumor-derived cancer cell lines has been extensively investigated by either receptor knockdown or overexpression. The results show that NR4A1 regulates one or more of cancer cell proliferation, survival, cell cycle progression, migration and invasion in lung, melanoma, breast, lymphoma, pancreatic, colon, cervical, ovarian and gastric cancer cell lines (79, 160, 172, 178-181, 183-187, 215, 216). Studies in the laboratory have also identified and characterized a series of bis-indole derived compounds (CDIMs) including 1,1-bis(3'-indoly)-1-(p-hydroxyphenyl)methane (DIM-C-pPhOH, CDIM8) that bind NR4A1 and

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act as NR4A1 antagonists in solid tumors (79, 160, 172, 180, 181, 186, 187, 210, 215-217). In solid tumor-derived cancer cell lines, comparable results are observed after NR4A1 silencing or after treatment with the NR4A1 antagonist CDIM8. In contrast to the role of NR4A1 in most solid tumors, expression of this receptor is low in leukemia cells and patients, where there is evidence for tumor suppressor-like activity of this receptor (164, 218).

CDIM8 inhibits growth, survival and migration of RMS cells (79, 172) and these responses are associated with modulation of several genes including survivin, Bcl-2, epidermal growth factor receptor (EGFR), other receptor tyrosine kinases and several integrins. In addition, NR4A1 regulates expression of the fusion oncoprotein PAX3-FOXO1 which is a major driver of alveolar RMS (ARMS) and CDIM8 decreased expression of PAX3-FOXO1 and associated downstream genes (79). Recent studies show that NR4A1 plays an essential role in transforming growth factor β (TGFβ) induced invasion of breast and lung cancer cells (178, 210, 217) and CDIM8 inhibits this induced invasion by blocking TGFβ-induced nuclear export of NR4A1 (210, 217). TGFβ also induces growth and inhibits differentiation of embryonal RMS (ERMS) cells (219-222). However, the effects of TGFβ on invasion of ERMS or ARMS cells have not previously been investigated. Therefore, in this study, we have investigated the mechanism of action of the NR4A1 antagonist CDIM8 on TGFβ-induced invasion of RMS cells. Our results show that TGFβ induced the invasion of ERMS cells but not ARMS cells through a novel pathway that involved cytosolic NR4A1 and this invasion was inhibited by CDIM8, which was primarily due to induction of the tumor suppressor-like cytokine interleukin-24 (IL-24).

MATERIALS AND METHODS

Cell lines

RD cells were purchased from the American Type Culture Collection (Manassas, VA). SMS-CTR cells were obtained as a generous gift from Mr. Jonas Nance, Texas Tech University Health Sciences Center-Children's Oncology Group (Lubbock, TX). These cells were grown and maintained at 37^0C temperature in presence of 5% CO_2 in either Dulbecco's modified Eagle's medium (DMEM, RD cells), or Iscove's Modified Dulbecco's Medium (IMDM, SMS-CTR cells) in presence of 10% fetal bovine serum (FBS).

Reagents and Antibodies

Trypsin, FBS, DMEM, IMDM, and nuclear and cytoplasmic extraction kits were obtained from Thermofisher Scientific (Waltham, MA). TGF-β was purchased from R&D Systems (Minneapolis, MN). C-DIM8 and its analogs were synthesized in the laboratory. Primary antibodies (GAPDH, β-catenin, Non-phospho-β-catenin, p-SMAD2, SMAD2, p-SMAD3, SMAD3, Slug, ZO-1, ZEB1, Vimentin, and Bcl-2), Anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor®488 Conjugate), HRP-linked secondary antibodies, DAPI, Bafilomycin A1, and Chloroquine were purchased from Cell Signaling Technology (Danvers, MA). NR4A1, Immunofluorescent-NR4A1, IL-24, and c-Myc antibodies were purchased from Abcam (Cambridge, UK). P84 antibody was purchased from GeneTex Inc (Irvine, CA). Formaldehyde, MG-132, β-actin antibody, M2 Flag antibody, β-catenin primers for PCR, and oligonucleotides for RNAi experiments (siβ-catenin, siNR4A1, siIL-24, siBcl-2) were purchased from Sigma-Aldrich (St. Louis, MO). SMAD7 antibody was purchased from Novus Biologicals (Centennial, CO).

Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA) and human IL-24 cDNA clone in a pCMV-6 plasmid was purchased from Origene (Rockville, MD).

siRNA interference/Overexpression assay

RD or SMS-CTR cells (2.0×10^5) were seeded in medium supplemented with 10% FBS and were allowed to attach for 24 hours. The desired plasmids (siNR4A1, siβ-catenin, siIL-24, siBcl-2 or pCMV-6) were then delivered into the cells at the concentration of 100 nM using Lipofectamine 2000 (50 umol/L). The excess Lipofectamine was removed after 6 hours of treatment by replacing the media. After 48-72 hours, cells were either lysed or treated with different reagents (C-DIM8, TGF-β) prior to lysis. The oligonucleotides used were as follows: siNR4A1_A, SASI_Hs02_00333289; siNR4A1_B SASI_Hs02_00333290; siβ-catenin1, SASI_Hs02_00318698; siβ-catenin2, SASI_Hs02_00318699; siIL-24_A, SASI_Hs01_00097938; siIL-24_B, SASI_Hs01_00097940; siBcL2 (1), SASI_Hs01_00119086; siBcL2 (2), SASI_Hs01_00119087.

Boyden Chamber Invasion Assay

Attached cells (2.0×10^5) were treated with DMSO or with C-DIM8 prepared in medium supplemented with 2.5% charcoal-stripped FBS, for 24 hours, or were transfected with desired siRNAs/plasmids as outlined in siRNA interference assay. Cells were then co-treated with or without TGF-β (5 ng/ml) for 5 hours, trypsinized, and counted. One hundred thousand cells from each treatment condition were allowed to invade through the Boyden Chamber for 24 hours. Cells that invaded into the Boyden Chamber were fixed using formaldehyde, stained, and then counted. At least 3 replicates were performed for each treatment group.

Immunofluorescence

Cells (2.0×10^5) were seeded and allowed to attach for 24 hours. They were then treated with DMSO or C-DIM8 for 24 hours (with or without 5 hours co-treatment with TGF-β), washed with PBS, fixed with 37% formaldehyde, permeabilized with 0.03% Triton, blocked with 5% BSA, and then treated with fluorescent-NR4A1 antibody for 24 hours. They were then washed with PBS and then treated with Anti-rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor®488 Conjugate) antibody for an hour. They were then washed again with PBS and treated with 300 nM DAPI. After washing to remove excess DAPI, the location of NR4A1 (green) and nucleus (blue) in those cells were visualized using a fluorescent microscope (EVOS Cell Imaging Systems).

Nuclear/Cytosolic Extraction

Cells (2.0×10^5) were seeded in media supplemented with 10% FBS and were allowed to attach for 24 hours. They were then treated with DMSO or C-DIM8 prepared in 2.5% charcoal-stripped media (with or without 5 hours co-treatment with TGF-β). The nuclear and cytoplasmic extraction kit was then used according to the manufacturer's protocol to obtain cytosolic and nuclear fractions, which were further analyzed by western blots.

Western blot analysis

Cells (2.0×10^5) were seeded and after various treatments, whole cell lysates were obtained by treating them with high salt lysis buffer that contained protease and phosphatase inhibitors. The total protein in the lysates were quantified by Bradford assay. Equal amounts of protein from each lysate were then loaded on SDS polyacrylamide gel. The proteins on the gel were transferred to a PVDF membrane, which was blocked for an hour using 5% skimmed milk. The membrane was then incubated with primary antibody for 24 hours. It was then washed with Tris-buffered saline

and Polysorbate 20 (TBST) and incubated with HRP-linked secondary antibody for 2 hours. The membrane was further washed with TBST and treated with Immobilon western chemiluminescence HRP-substrates to detect the protein bands using Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR, USA).

Polymerase chain reaction (PCR)

Cells (2.0×10^5) were seeded in a medium containing 10% FBS and were allowed to attach. After 24 hours, the medium was replaced with a fresh medium supplemented with 2.5% charcoal stripped FBS that also contained the desired compounds (DMSO, CDIM8, MG132 or their combination). The RNA was extracted from them after 24 hours using Zymo Research Quick-RNA Miniprep kit (Irvine, CA) by following the manufacturer's protocol. The Bio-Rad (Richmond, CA) iTaq Universal SYBER Green 1-step kit was then used and the manufacturer's instruction was followed to quantify the total β-catenin mRNA relative to TATA binding protein (TBP) mRNA which was used as a control. The β-catenin primer sequence used was: F: 5'-TCTGAGGACAAGCCACAAGATTACA-3' (sense)

R: 5-TGGGCACCAATATCAAGTCCAA-3' (antisense).

Transmission Electron Microscopy

Cells (2.0×10^5) were seeded and were allowed to attach overnight on 35 mm Permanox petri dishes. They were then treated with either DMSO, or with C-DIM8 for 24 hours, and were rinsed with serum free media and then with 0.1 M cacodylate buffer, pH 7.2. They were then fixed with 2.5% paraformaldehyde, 2% glutaraldehyde, and 0.1 M cacodylate buffer, and stained with 1% osmium tetroxide, 0.2% ferrocyanide, and 0.1 M cacodylate buffer, followed by an hour of incubation with saturated uranyl acetate. The cells were then dehydrated using ethanol in an

ascending series of concentration, and then infiltrated by incubation in 100% propylene oxide, followed by 1:1 propylene oxide:resin. They were then incubated in 100% resin for 16 hours, and were baked at 65^0C for 48 hours. After that, blocks of the samples were cut and mounted to resin bullets, which were then cut into a 100 nm ultrathin sections using a Leica EM U6 ultramicrotome and were then placed on a copper grid (200 lines/inch hexagonal mesh). The sections were then post-stained with uranyl acetate for 6 minutes and then with Reynolds lead citrate for 45 seconds. These were then visualized using an FEI Morgagni 268 transmission electron microscope (operated by the Morgagni User Interface software), with a Megaview III CCD camera. The images were collected using iTEM software.

ROS Measurements

Cells (2.0×10^5) were seeded in a medium supplemented with 10% FBS and were allowed to attach for 24 hours. They were then treated with DMSO or CDIM8 prepared in 2.5% charcoal stripped medium for 24 hours or transfected with the desired siRNAs, followed by the compound treatment for 24 hours. The levels of ROS were then measured with Accuri flow cytometer using the cell permeable H2DCFDA reagent as outlined in the manufacturer's instruction (Life Technologies Inc.).

Statistical Analysis

Student's *t*-test was used to determine the statistical significance of differences between the treatment groups. Each experiment was repeated three times and the results were presented as means with error bars representing 95% confidence intervals. Data with a P value of <0.05 were considered statistically significant.

RESULTS

TGFβ induces invasion of ERMS cells which is inhibited by NR4A1 antagonists.

TGFβ enhances invasion of late stage tumors (223, 224), induces growth and inhibits differentiation of ERMS cells (219-222). Previous studies in breast and lung cancer cells show that this response was NR4A1-dependent and could be inhibited by bis-indole derived NR4A1 antagonists (210, 217). Results illustrated in Figure 5A show that TGFβ induces invasion of RD and SMS-CTR ERMS cells whereas it did not affect invasion of Rh30 ARMS cells (data not shown). The effects of the NR4A1 antagonist CDIM8 on TGFβ-induced invasion was determined and CDIM8 alone inhibited invasion as well as TGFβ-mediated invasion of RD and SMS-CTR cells (Fig. 5A). These results demonstrate the effects of TGFβ on ERMS (but not ARMS) cell invasion and therefore TGFβ-induced responses in ERMS cells include induction of cell growth and invasion, and inhibition of differentiation.

NR4A1 is extranuclear in RD and SMS-CTR cells and does not affect TGFβ-induced SMAD signaling.

TGFβ-induced invasion of lung and breast cancer cells resulted in nuclear export of NR4A1 and subsequent degradation of SMAD7 (210, 217), however, results in Figure 5B demonstrate that NR4A1 protein is primarily cytosolic in RD and SMS-CTR cells and TGFβ does not affect NR4A1 protein levels whereas these are decreased after treatment with CDIM8. Moreover, treatment with TGFβ, CDIM8 or their combination did not affect the intracellular location of NR4A1 which remained extranuclear. We further confirmed the location of NR4A1 in RD (Fig. 5C) and SMS-CTR (Fig. 5D) cells by immunostaining and showed that in untreated or treated cells, NR4A1 remained extranuclear and exhibited perinuclear staining. TGFβ induced nuclear export of NR4A1 in breast and lung cancer cells (210, 217) and this receptor formed part of a proteasome complex

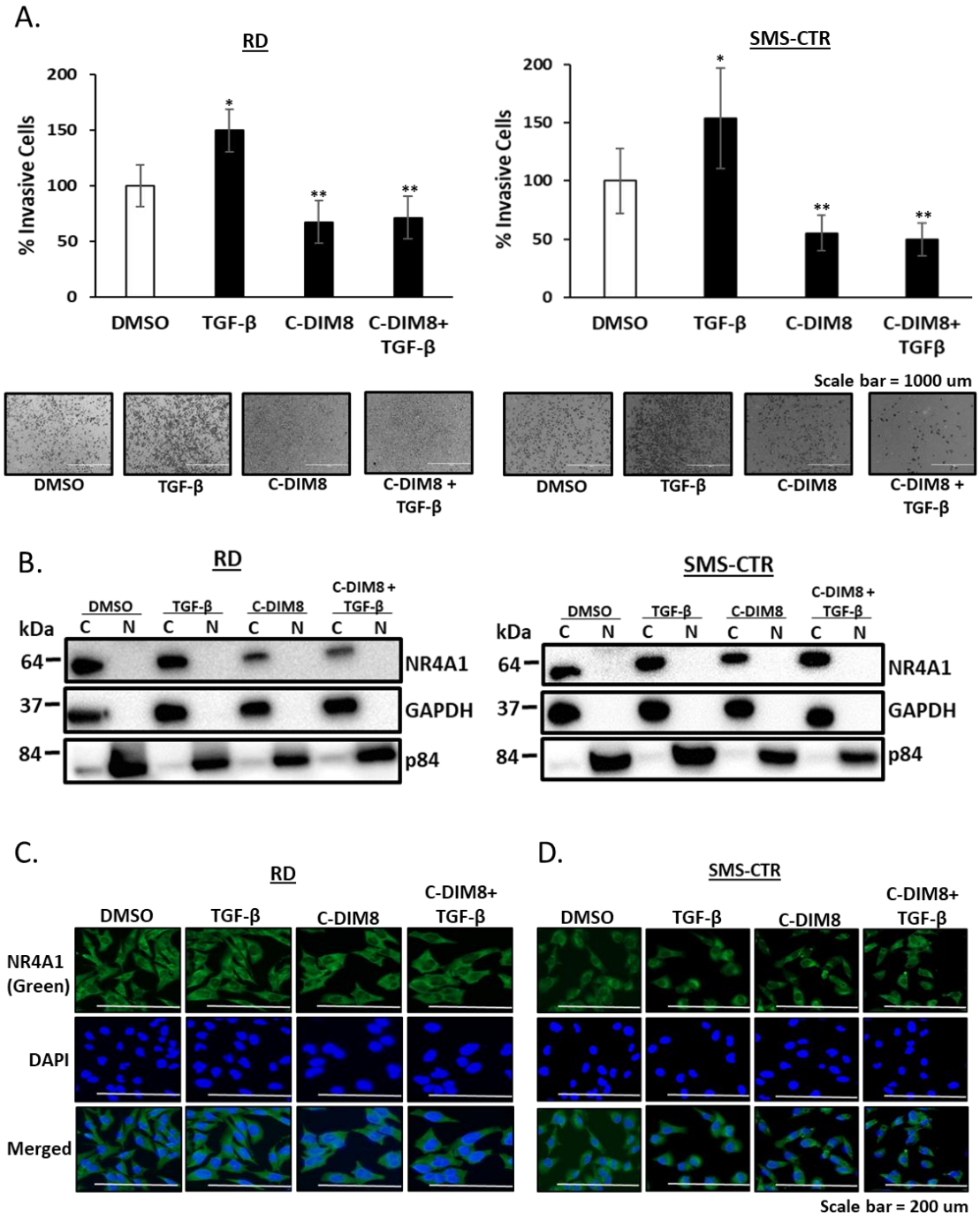

Figure 5: Modulation of TGFβ-induced invasion of RMS cells and subcellular localization of NR4A1 in ERMS cells. A. RD and SMS-CTR cells were treated with 5 ng/ml TGFβ, 20 μM CDIM8, or their combination and cell invasion was determined in a Boyden chamber assay as outlined in the Methods. B. RD and SMS-CTR cells were treated with DMSO, TGFβ, CDIM8 and their combination and cytosolic (C) and nuclear (N) fractions were separated and analyzed by western blots as outlined in the Methods. RD (C) and SMS-CTR (D) cells were treated as described above (A/B) and cellular location of NR4A1 was determined by DAPI (for nuclear staining) and NR4A1 antibody staining by immunofluorescence analysis as outlined in the Methods.

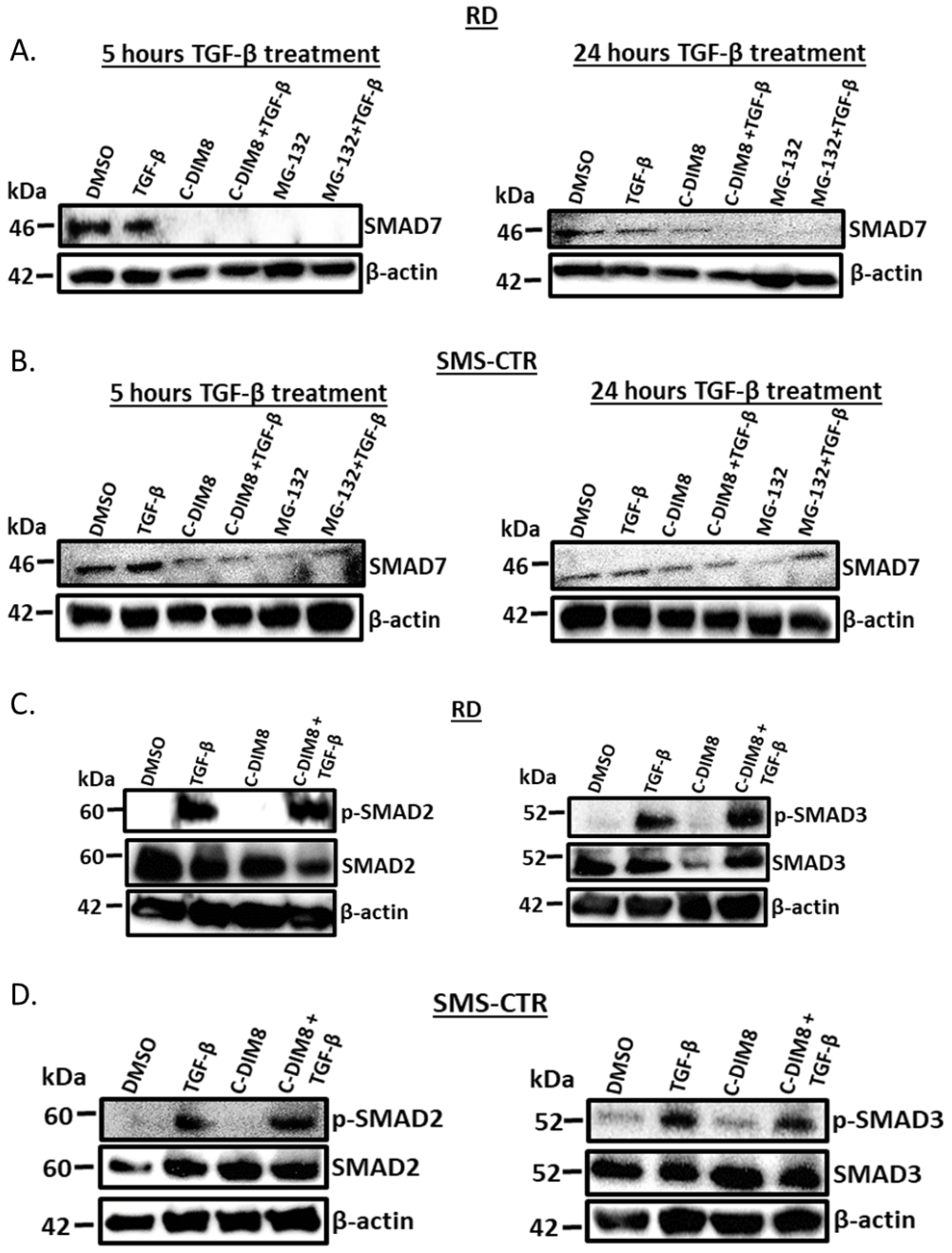

Figure 6: Effects of TGFβ/CDIM8 on SMADS. RD (A) or SMS-CTR cells (B) were treated with DMSO or 5 μM MG132 for 3 hours (alone or in combination with 20 μM CDIM8 or with 5 ng/ml TGFβ for either 5 or 24 hours and whole cell lysates were analyzed by western blots as outlined in the Methods. RD (C) and SMS-CTR (D) cells were treated with DMSO, 5 ng/ml TGFβ, 20 μM CDIM8 alone or in combination for 24 hours and whole cell lysates were analyzed by western blots as outlined in the Methods.

that degraded inhibitory SMAD7. This response was inhibited by CDIM8, as it blocked the nuclear export of NR4A1. SMAD7 plays an inhibitory role in TGFβ-induced responses by enhancing degradation of the TGFβ receptor (225). In contrast, the treatment of RD cells with CDIM8 alone, CDIM8 plus TGFβ, MG-132 (proteasome inhibitor) alone, and MG-132 plus TGFβ had minimal effects on expression of SMAD7 as the protein levels of SMAD7 were either unchanged or decreased after the treatment (Fig. 6A). Similar results were observed in SMS-CTR cells (Fig. 6B) demonstrating that the role of NR4A1 and effects of the NR4A1 antagonists on TGFβ-induced invasion was largely independent of their effects on expression of inhibitory SMAD7. These results indicate that in ERMS cells, CDIM8 did not enhance nuclear retention of NR4A1 or decrease SMAD7 degradation suggesting that the TGFβ-NR4A1-SMAD7 pathway observed in breast and lung cancer cells is inoperative in ERMS cells. TGFβ-dependent activation of SMAD2/SMAD3 can also play a role in enhanced invasion however, although TGFβ activated (phosphorylated) SMAD2 and SMAD3 in RD (Fig. 6C) and SMS-CTR (Fig. 6D) cells, CDIM8 alone affected neither the SMAD phosphorylation, nor the TGFβ-induced responses, indicating that these effects are NR4A1-independent.

β-catenin is downregulated by CDIM8.

NR4A1 interacts with β-catenin (226, 227) and in breast cancer cells, CDIM8 blocks TGFβ-induced nuclear localization of β-catenin resulting in accumulation of β-catenin in the cytosol, which then undergoes proteasome-dependent degradation (218). Since β-catenin is also involved in epithelial-mesenchymal transition (EMT) and cell invasion, we transfected RD and SMS-CTR cells with siNR4A1 to silence NR4A1 (Fig. 7A) and also treated these cells with CDIM8 (Fig. 7B) and showed that levels of total β-catenin were decreased by both NR4A1 knockdown and the NR4A1 antagonist (Relative band intensity quantification is illustrated in

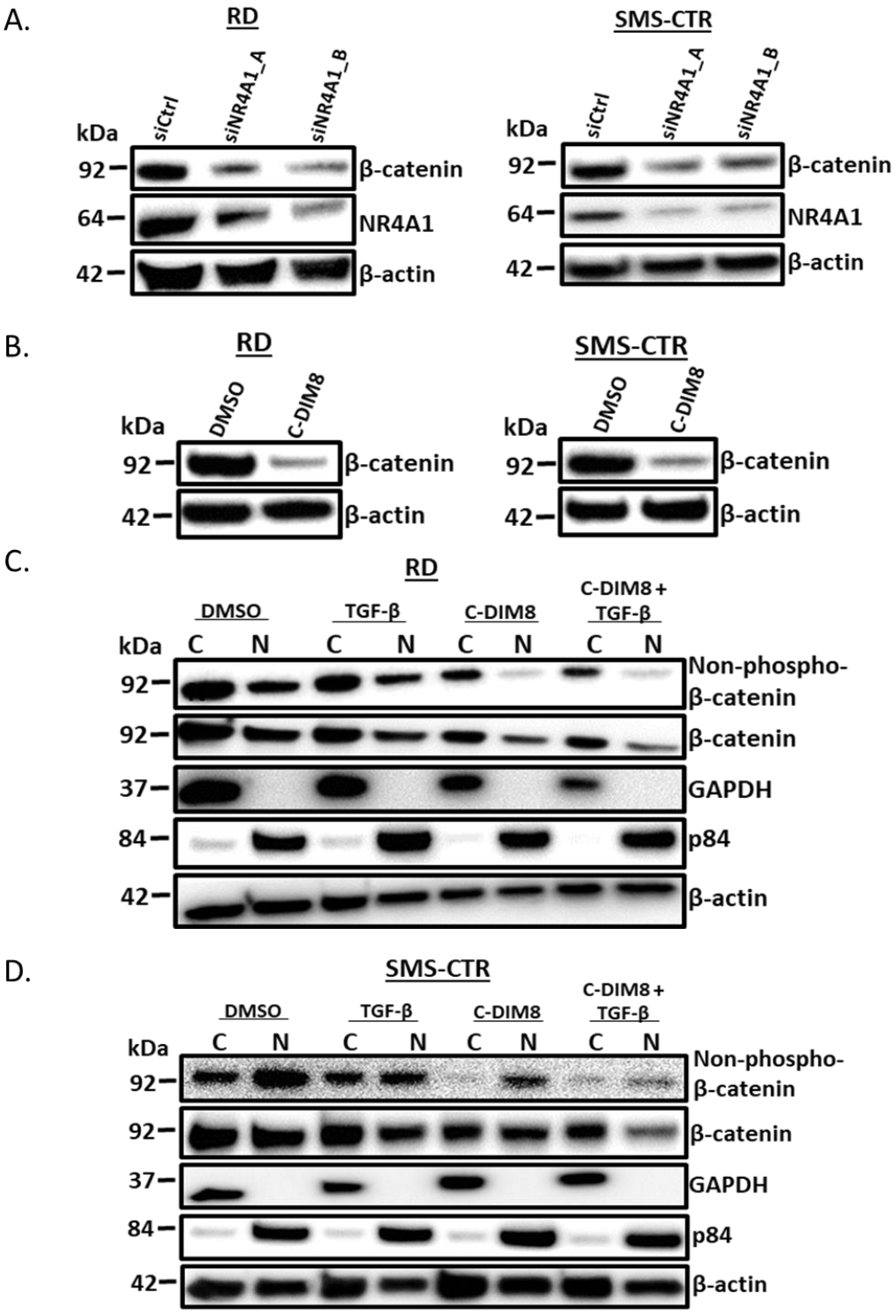

Figure 7: Effects of NR4A1 and TGFβ on β-catenin expression. RD and SMS-CTR cells were treated with siNR4A1 (A) or treated with CDIM8 for 24 hours (B) and whole cell lysates were analyzed by western blots as outlined in the Methods. RD (C) and SMS-CTR (D) cells were treated with 5 ng/ml TGFβ and various drug combinations and cytosolic and nuclear extracts were obtained and analyzed by western blots as outlined in the Methods.

Supplemental Figures 1A and 1B). Similar effects on β-catenin were previously observed in breast cancer cells when NR4A1 was silenced or inactivated by CDIM8 (217). Western blot analysis of the subcellular location of β-catenin showed that in untreated RD (Fig. 7C) and SMS-CTR cells (Fig. 7D), β-catenin was located in both the cytosolic and nuclear fractions of both cell lines with higher cytosolic and nuclear β-catenin levels in RD and SMS-CTR cells respectively. TGFβ alone did not decrease or change the subcellular location of β-catenin or its active non-phosphorylated form. CDIM8 alone or in combination with TGFβ decreased expression of cytosolic and nuclear β-catenin (total and non-phosphorylated) in both cell lines however, in SMS-CTR cells treated with CDIM8, the non-phosphorylated form was primarily nuclear indicating some differential loss of cytosolic β-catenin.

Further examination of the effects of CDIM8 on β-catenin and downstream targets was investigated and in RD and SMS-CTR cells (Fig. 8A & 8B), CDIM8 alone or in combination with TGFβ decreased expression of β-catenin (total and non-phosphorylated) and its downstream targets (c-Myc and Slug) and the EMT marker ZEB1. We also observed that TGFβ alone had minimal effects on c-Myc, Slug, ZO-1 and ZEB1 in ERMS cells (Fig 8A & 8B) and this may be due to the high endogenous levels of these gene products. These results demonstrate that NR4A1 silencing or ligand-induced inactivation of NR4A1 downregulated β-catenin (Relative band intensities are quantified and illustrated in Supplemental Figs. 1C and 1D). However, in contrast to a similar response in breast cancer cells (217), decreased expression of β-catenin due to CDIM8 was proteasome-independent and was not affected by co-treatment with the proteasome inhibitor MG132 (Fig. 8C and Supplemental Fig. 2A). Furthermore, this response was also lysosome-independent and remained unaffected by co-treatment with the lysosome inhibitors Bafilomycin A1 and Chloroquine (Supplemental Fig. 2B). Since β-catenin plays a role in EMT and cell

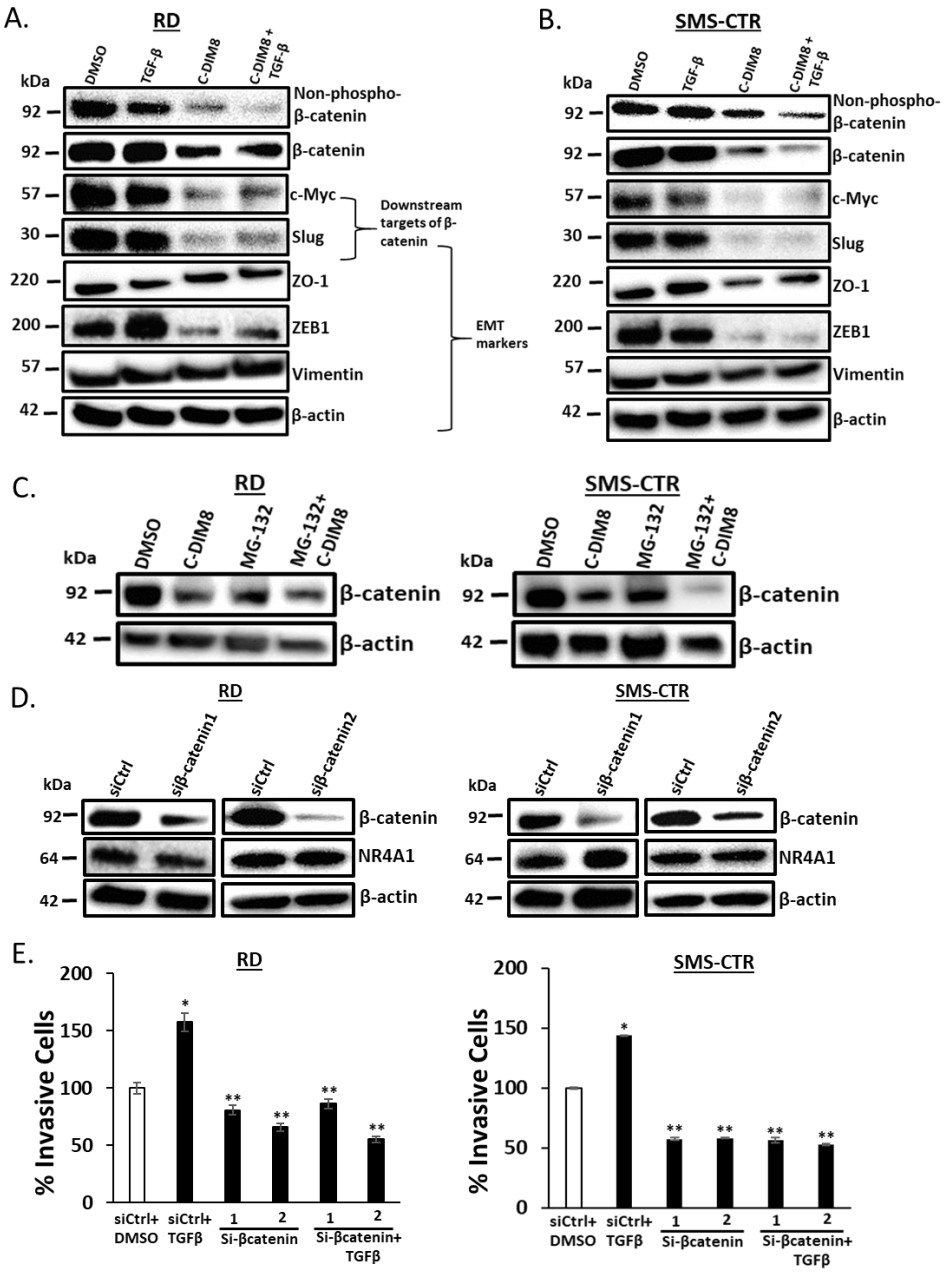

Figure 8: **Regulation of β-catenin expression by TGFβ and NR4A1 antagonists.** RD (A) and SMS-CTR (B) cells were treated with DMSO, TGFβ, CDIM8 or their combination for 24 hours and whole cell lysates were analyzed by western blots as outlined in the Methods. C. RD and SMS-CTR were treated with DMSO, 20 μM CDIM8, 5 μM MG132 (3 hours) or their combination for 24 hours and whole cell lysates were analyzed by western blots as outlined in the Methods. RD and SMS-CTR cells were transfected with siβ-catenin alone (D) or in combination with TGFβ (E) and whole cell lysates were analyzed by western blots (D) or effects on cell invasion (E) were determined in a Boyden chamber assay respectively as outlined in the Methods. Results (E) are means ± SD for at least 3 separate determinations as significant ($p<0.05$) induction (*) or inhibition (**) of the induced response are indicated.

invasion, we investigated the effects of β-catenin silencing (Fig. 8D) on basal and TGFβ-induced invasion of RD and SMS-CTR cells (Fig. 8E). The results show that β-catenin silencing results in decreased basal and TGFβ-induced invasion of RD and SMS-CTR cells suggesting that CDIM8-induced downregulation of β-catenin expression contributes to the inhibitory effects of CDIM8 on RMS cell invasion.

Role of IL-24 and the NR4A1-Bcl-2 complex.

A recent study reported that CDIM8 induced the tumor suppressor like cytokine IL-24 in RMS (RD and Rh30) cells (77) and IL-24 exhibits a diverse spectrum of anticancer activities including inhibition of cancer cell invasion (77, 228-232). Since knockdown of β-catenin inhibits ERMS cell invasion (Fig. 8E), we investigated whether induction of IL-24 by CDIM8 was associated with RMS cell invasion and if this was due, in part, to down regulation of β-catenin. Results in Figure 9A and 9B show that in RD and SMS-CTR cells treated with TGFβ, CDIM8 alone, or CDIM8 plus TGFβ, knockdown of IL-24 (siIL-24) enhanced invasion in all 3 treatment groups suggesting a role for IL-24 as an inhibitor of invasion. Western blot analysis of the various treatment groups shows that CDIM8 and CDIM8+ TGFβ-induced IL-24 and decreased β-catenin expression (Fig. 9C) and this correlated with decreased basal and TGFβ-induced invasion (Fig. 9A and 9B). In contrast, western blot analysis of cell lysates from these same treatments (CDIM8 and CDIM8+TGFβ) in combination with IL-24 silencing showed that β-catenin levels were not decreased in the absence of IL-24 (Fig. 9D) and this correlated with enhanced invasion after IL-24 silencing (Fig. 9A and 9B). Relative band intensities for Figures 9C and 9D are quantified in Supplemental Fig 3. These results suggest that induction of IL-24 by CDIM8 was important for inhibiting ERMS cell invasion and for downregulation of β-catenin, and this complements the data showing that β-catenin silencing inhibits TGFβ-induced invasion of ERMS cells (Fig 8E).

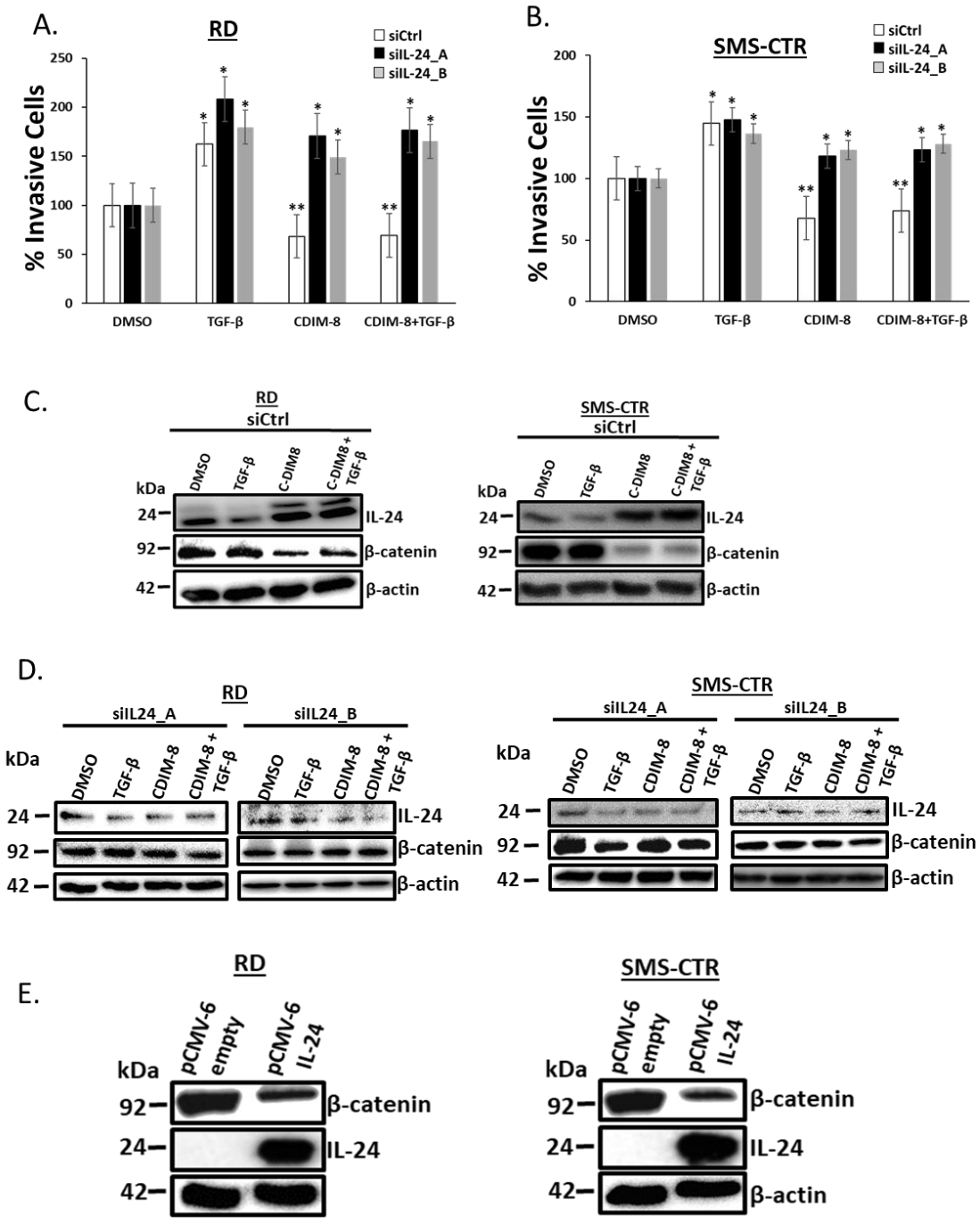

Figure 9: **Role of IL-24 in the inhibition of TGFβ-induced invasion by NR4A1 antagonists.** RD (A) and SMS-CTR (B) cells were transfected with siCtrl (non-specific oligonucleotide) or siIL-24 and treated with TGFβ, CDIM8 or their combination and effects on cell invasion were determined in a Boyden chamber assay as outlined in the Methods. RD and SMS-CTR cells were transfected with a control oligonucleotide siCtrl (C) or siIL-24 (D) and whole cell lysates were analyzed by western blots. E. RD and SMS-CTR cells were transfected with an IL-24 expression plasmid and after 48 hours whole cell lysates were analyzed by western blots. Results (A/B) are means ± SD of at least 3 separated determinations and significant ($p<0.05$) induction (*) or inhibition (**) of invasion is indicated.

Previous studies in pancreatic and breast cancer cells show that overexpression of IL-24 decreased expression of β-catenin protein (233, 234) and using a similar approach, we also demonstrate that overexpression of IL-24 in ERMS cells decreased β-catenin expression (Fig. 9E).

Thus, our results indicate that inhibition of basal and TGFβ-induced invasion of RD and SMS-CTR cells is due to induction of IL-24 and its subsequent downregulation of β-catenin. IL-24 is induced by ROS in cancer cell lines (235) and several studies show that treatment of some cancer cell lines with apoptosis-inducing agents results in nuclear export of NR4A1 and interaction with Bcl-2 to form a complex that disrupts mitochondria and induces ROS (161-163, 198, 236-238). Thus, we hypothesized that induction of IL-24 by CDIM8 in RD cells was may be due to activation of the extranuclear Bcl-2-NR4A1 complex and this was further investigated using RD cells as a model. Figure 10A shows that oligonucleotides that target Bcl-2 decrease expression of the target gene product in RD cells. Treatment of RD cells with CDIM8 induces IL-24 and this response is blocked after co-treatment with GSH thus confirming a role for ROS in the induction of IL-24 in RD cells (Fig. 10B). The role of Bcl-2 in this process is supported by results in Figure 10C showing that induction of IL-24 by CDIM8 in RD cells is decreased after knockdown of Bcl-2. Using the same treatment protocol, we also observed that CDIM8 induces ROS compared to treatment with solvent (DMSO) or control oligonucleotide, however, in Bcl-2-silenced RD cells induction of ROS by CDIM8 is abrogated (Fig. 10D). The results suggest that CDIM8-mediated induction of IL-24 and ROS are Bcl-2 dependent and therefore we examined the effects of CDIM8 on mitochondria by electron microscopy. Control RD cells exhibit the typical mitochondrial structure whereas after treatment with CDIM8 we observed extensive evidence of misshapen mitochondria and mitochondrial damage (Fig. 10E) and this was similar to previous studies using apoptosis-inducing agents. Thus, in RD cells where NR4A1 is extranuclear, CDIM8 activates the

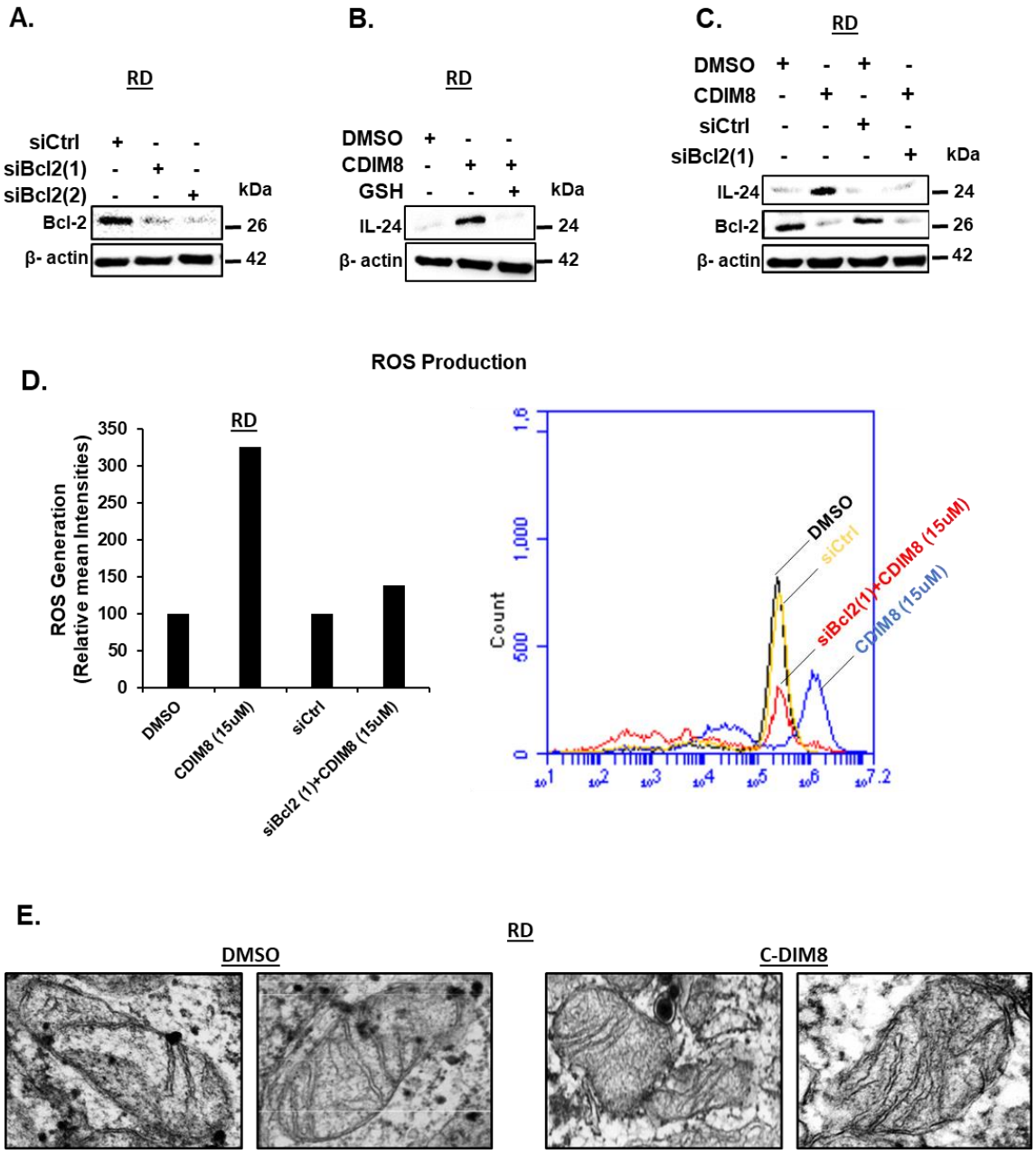

Figure 10: **NR4A1-Bcl-2 interactions and mitochondrial damage.** RD cells were transfected with siCtrl/siBcl-2 oligonucleotides(A), treated with DMSO, CDIM8 alone or in combination with GSH (5 mM) (B) or transfected with siCtrl/siBcl-2 and treated with DMSO or CDIM8 (C) and at the end of the treatment period whole cell lysates were analyzed by western blots D. RD cells were treated with DMSO, CDIM8 (15 µM), siCtrl or siBcl-2/CDIM8 and the levels of ROS were determined as outlined in the Materials and Methods. E. Electron microscopic analysis of mitochondria in RD cells treated with DMSO or 15 µM CDIM8 was determined as outlined in the Methods.

Bcl-2-NR4A1 complex to induce mitochondrial damage, ROS and IL-24 which downregulates β-catenin thereby inhibiting basal and TGFβ-induced invasion of RMS cells.

DISCUSSION

TGFβ plays a paradoxical role in cancer where many early stage tumors are inhibited by this growth factor whereas it enhances migration and invasion of late stage cancers (77, 226, 227). The mechanisms associated with TGFβ-induced cancer cell invasion are complex and can involve multiple pathways including formation of activated SMAD2/SMAD3-SMAD4 complexes. Another mechanism linked to TGFβ-induced invasion is associated with degradation of the inhibitory SMAD7 which inhibits TGFβ signaling by induction of TGFβ receptor 1 degradation (225). Recent studies have shown that NR4A1 plays an important role in TGFβ-regulated SMAD7 degradation in breast and lung cancer cells (178, 210, 217). TGFβ induces phosphorylation of NR4A1 which is necessary for nuclear export of NR4A1 and this receptor forms a complex with Arkadia, RNF12, Axin2 and SMAD7 resulting in proteasome-dependent degradation of SMAD7 (178, 210, 217). In this study, we initially showed that TGFβ also induces invasion of RD and SMS-CTR (ERMS) but not Rh30 (ARMS) cells and this is consistent with previous studies showing that TGFβ primarily enhances carcinogenesis of ERMS cells (219-226). Moreover, we also show that the NR4A1 antagonist CDIM8 inhibits basal and TGFβ-induced invasion of ERMS cells (Fig 5). However, although CDIM8 inhibits TGFβ-induced invasion in breast and lung cancer cells and also in ERMS cells (Fig. 5), the mechanism of the inhibitory effects in ERMS cells is distinct from that previously observed in lung and breast cancer cells (178, 210, 217). A major difference involves the intracellular location of NR4A1 in ERMS cells; the receptor is cytosolic/perinuclear and treatment with CDIM8, TGFβ alone or in combination with CDIM8 does not induce changes in the intracellular location of NR4A1 (Fig. 5C and 5D). Moreover, CDIM8

does not block TGFβ-induced degradation of SMAD7 or affect activation of SMAD2 or SMAD3 (Fig. 6). This indicates that other pathways are involved in TGFβ-mediated ERMS cell invasion.

Previous reports have demonstrated interactions between NR4A1 and β-catenin (226, 227) and β-catenin is involved in epithelial to mesenchymal transition (EMT) and cell invasion (239-241). Our results indicate that β-catenin is highly expressed and distributed in the cytosol and nucleus of ERMS cells and although TGFβ has minimal effects on β-catenin levels, the NR4A1 antagonist CDIM8 alone or in combination with TGFβ decreases expression of β-catenin and downstream genes (Fig. 7 and 8). A previous study showed that NR4A1 activated proteasome-dependent degradation of β-catenin (226); however, we did not observe any protection from CDIM8-induced downregulation of β-catenin by the proteasome inhibitor MG132 (Fig. 8C and Supp. Fig 2A). It was also reported that NR4A1 interacted with β-catenin in colon cancer cells and this resulted in proteasome-dependent degradation of β-catenin (226) whereas in ERMS cells, both NR4A1 antagonists and NR4A1 silencing decreased β-catenin expression (Figs. 7A and 7B) and this was proteasome independent (Fig. 8C).

A recent study in this laboratory (77) reported that NR4A1 silencing or antagonism by CDIM8 induced the tumor suppressor-like cytokine IL-24 in RMS cells and it has previously been reported that IL-24 inhibits cancer cell migration, invasion and metastasis (228-232). In ERMS cells, inhibition of TGFβ-induced invasion by CDIM8 is accompanied by induction of IL-24, and decreased expression of β-catenin; moreover, the effects of the NR4A1 antagonist on invasion, IL-24 expression and β-catenin degradation were attenuated by IL-24 silencing (Fig. 9). These results, coupled with the effects of IL-24 on β-catenin expression (decreased) (Fig. 9E) suggest that the inhibition of basal and TGFβ-induced ERMS cell invasion by CDIM8 is due, in part, to the induction of IL-24 and IL-24-mediated downregulation of β-catenin (Fig. 9E). In addition, we

show that CDIM8 activates the NR4A1-Bcl-2 complex to disrupt mitochondria and induce ROS and IL-24 (Fig. 10) and this complements previous studies showing the effects of this complex on mitochondria (228-232). This represents a novel pathway for inhibiting late stage TGFβ-induced cancer cell invasion and current studies are focused on the mechanism of β-catenin downregulation by IL-24 and potential clinical applications of drug-induced IL-24 for inhibiting invasion of ERMS and other cancer cell lines and their associated tumors *in vivo*.

In summary, this study demonstrates for the first time that TGFβ induces invasion of ERMS cells and this response is inhibited by the NR4A1 antagonist CDIM8. In contrast to other solid tumors, NR4A1 in ERMS cells is extranuclear and TGFβ-NR4A1 crosstalk is independent of nuclear export of the receptor or modulation of SMAD signaling. The cross talk between NR4A1 and other growth factors/cytokines and effects of CDIM8 in ERMS cells has not been determined and is currently being investigated. In ERMS cells, NR4A1 regulates expression of β-catenin and therefore NR4A1 silencing or treatment with an NR4A1 antagonist decreases β-catenin expression and β-catenin-linked EMT and invasion. We also observed that β-catenin downregulation by NR4A1 inactivation involves induction of the tumor suppressor-like cytokine IL-24. Thus, our results demonstrate that basal and TGFβ-induced invasion of ERMS cells are targetable by NR4A1 antagonists which inactivate the pro-invasion TGFβ-NR4A1-β-catenin pathway by induction of IL-24. Thus, the bis-indole derived NR4A1 antagonist inhibits TGFβ-induced and basal ERMS cell invasion through a novel pathway involving extranuclear NR4A1/Bcl-2 which induces ROS and IL-24. IL-24 decreases β-catenin expression and β-catenin-dependent invasion. Previous studies show that CDIM8 and related NR4A1 antagonists bind nuclear NR4A1 and either inactivate NR4A1-regulated pro-oncogenic genes pathways (228-232) or inhibit the effects of TGFβ by blocking nuclear export of NR4A1 (228-232). In contrast, this study shows that in ERMS

cells, where NR4A1 is extranuclear, CDIM8 activates the pro-apoptotic NR4A1-Bcl2 pathway which also induces IL-24 indicating the anticancer activities of CDIM8 are linked to targeting of either the nuclear or the extranuclear NR4A1 receptor.

CHAPTER III

THE HISTONE METHYLTRANSFERASE GENE G9A IS REGULATED BY NUCLEAR RECEPTOR 4A1 (NR4A1) IN ALVEOLAR RHBDOMYOSARCOMA CELLS*

INTRODUCTION

Covalent modifications of histones by acetylation, ubiquitination and methylation play a pivotal role in epigenetic modifications of gene expression required for maintaining cellular homeostasis and for abnormal pathophysiology (91, 242). Histone H3 and histone H4 are common methylation sites and the methyl transferase G9A (EHMT2) gene primarily catalyzes methylation of histone 3 lysine 9 (H3K9) (243, 244) and this determines some of its cellular functions and role in multiple diseases (91). G9A forms a heterodimeric complex with GLP/Eu -HMT-ase to catalyze mono- and dimethylation of H3K9 (244) and this significantly contributes to the reported pro-oncogenic functions of G9A in multiple tumor types. For example; G9A promotes liver cancer by epigenetic silencing of RARRES3 and also inhibits expression of multiple tumor suppressor genes in aggressive ovarian cancers. In breast cancer, expression of G9A enhances hypoxia and related genes and in endometrial cancer, G9A increases invasion by repression of E-cadherin (89, 97, 98, 245, 246). There are several other examples of G9A-mediated gene suppression playing a key pro-oncogenic role in other tumor types and this includes regulation of p21, p53 and mTOR gene expression (96, 247-250). In addition, G9A also promotes gastric cancer metastasis through its activity as a nuclear cofactor in combination with p300 and the glucocorticoid receptor to activate expression of β3-integrin (251). A recent study also reported that G9a epigenetically regulated

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PTEN in alveolar rhabdomyosarcoma (ARMS) cells thereby activating Akt and downstream pro-oncogenic pathways (100).

These data illustrate the importance of G9A-dependent gene repression and in some cases, gene activation in enhancing carcinogenesis in multiple tumor types and not surprisingly, G9A inhibitors have been developed as potential cancer chemotherapeutic agents (91). Compounds that competitively bind to the substrate binding site of G9A, and the S-adenosyl methionine G9A co-factor binding site, along with G9A inhibitors with nuclear mechanisms of action, have been developed (91). The pro-oncogenic activity of G9A has been associated with cell context-specific repression of diverse genes however, the functional properties of G9A after knockdown or inhibition are similar across cell lines. For example, in ARMS cells, and in fifteen ARMS patient samples, G9A is overexpressed compared to normal muscle cells and knockdown of G9A inhibits ARMS cell growth, differentiation and migration (100). Studies in this laboratory have reported that the orphan nuclear receptor 4A1 (NR4A1, Nur77) is also overexpressed in ARMS cells and knockdown of NR4A1 or treatment with bis-indole derived NR4A1 antagonists (C-DIMs) inhibit ARMS cell growth and migration, and induce apoptosis (77, 79). These responses have been linked to modulation of NR4A1 regulated genes including the PAX3-FOXO1 fusion oncogene that plays an important role in ARMS carcinogenesis (79). Thus, both G9A and NR4A1 regulate comparable pro-oncogenic responses in ARMS cells and therefore we hypothesized that NR4A1 may also regulate G9A expression. Our results now demonstrate that G9A is an NR4A1 regulated gene in ARMS cells and C-DIM/NR4A1 antagonists effectively downregulate G9A and represent a novel class of G9A inhibitors. Moreover, we also show similar effects on NR4A1 regulation of G9a in a panel of cancer cell lines derived from multiple tumors.

MATERIALS AND METHODS

Cell lines, reagents and antibodies

Rh30 (RMS), MDA-MB-231 and MDA-MB-468 (breast cancer), A549, H1299 and H460 (lung cancer), SNU449, HUH7, and HepG2 (liver cancer), Ishikawa (endometrial cancer), HCT116 (colon cancer), and PC3 (prostate cancer) cell lines were purchased from American Type Culture Collection (Manassas, VA). Rh41 (RMS) was a generous gift from Mr. Jonas Nance, Texas Tech University Health Sciences Center- Children's Oncology Group (Lubbock, TX). Human mammary tumor Sum159PT and HS578T cell lines were generously provided by Dr. Weston Porter, Texas A&M University (College Station, TX). Mouse mammary tumor 4T1 cell line was kindly provided by Dr. Mien-Chie Hung, MD Anderson Cancer Center (Houston, TX). Hec-1B cell line was a generous gift from Dr. Russell Broaddus, MD Anderson Cancer Center (Houston, TX). Rh30, MDA-MB-231 and A549 cells were authenticated by Biosynthesis. All tumor cells used in these studies were *Mycoplasma* negative. Rh30, H1299, H460, SNU449 and HCT116 cells were maintained in RPMI medium. Rh41 cell line was maintained in IMDM medium. HS578T, MDA-MB-231, MDA-MB-468, A549, HUH7, HepG2 and PC3 cells were maintained in DMEM medium. Sum159PT, 4T1, Ishikawa, Hec-1B cells were maintained in (DMEM)/Ham's F-12 50/50 mix containing 2.5 mmol/L L-glutamine. All of these media were supplemented with 10% fetal bovine serum (FBS) and these cells were maintained at 37^0C temperature in presence of 5% CO_2. All the reagents/antibodies and the oligonucleotide sequences that were used are summarized in Supplemental Table 1 and 2 respectively. Analysis of the expression of G9a (EHMT2) in sarcomas was generated from the UALCAN database (http://ualcan.path.uab.edu/index.html). The new buttressed CDIM analogs 1,1-bis(3'-indolyl)-1-(3,5-dimethyl-4-hydroxyphenyl)methane [3,5-$(CH_3)_2$] and 1,1-bis(3'-indolyl)-1-(3-bromo-4-

hydroxy-5-methoxyphenyl)methane (3-Br-5-OCH_3) were synthesized by condensation of indole with 3,5-dimethylbenzaldehyyde and 3-bromo-5-methoxybenzaldehyde (Sigma Aldrich, St. Louis, MO) respectively and 1,1-bis(3′-indolyl)-1-(p-hydroxyphenyl)methane (CDIM8) was synthesized by the condensation of indole and p-hydroxybenzaldehyde. The reaction conditions for synthesis of the CDIMs was performed as described (252). The purities of both compounds were > 98% and their nuclear magnetic resonance spectrum are included in the supplemental Figure 4. LC-MS was performed using a SHIMADZU 2010 EV using methanol as solvent.

siRNA interference assay:

Cells (3.0×10^5) were seeded in a medium supplemented with 10% FBS and were allowed to attach. After 24 hours, they were transfected with 100 nM of desired siRNAs using 50 umol/L of Lipofectamine-2000 in reduced serum medium. After 6 hours, the medium was removed and replaced with fresh medium supplemented with 10% FBS. The cells were then lysed after 48-72 hours with lysis buffer and the lysates were further used for western blot analysis.

Western blot analysis:

Cell lysates were obtained either from siRNA interference assay or by lysing the cells that have been treated with the desired compounds for 24 hours. The total protein in those lysates were quantified by Bradford assay. The protein content in all the lysates were then normalized and the equal amount of protein was loaded and was allowed to run on SDS polyacrylamide gel, connected to an electric source. The overall protein on the gel was then transferred to a PVDF membrane, which was then blocked using 5% skimmed milk for an hour. After that, it was incubated overnight with primary antibody that detects and binds the specific protein of interest. The membrane was then washed with TBST and then incubated with HRP-linked secondary antibody for 2 hours. After that, the membrane was once again washed with TBST. The chemiluminescent HRP-

substrate was then added to the blot and Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR) was used to detect the protein of interest in the membrane.

NR4A1-CDIMs binding assays:

The ligand binding domain (LBD) of NR4A1 was incubated with different concentrations of CDIM compounds and was used to obtain tryptophan fluorescence spectra with the excitation wavelength of 285 nm (slit width = 5 nm) and an emission wavelength of range 300-420 nm (slit width = 5 nm). The binding affinity (K_d) of CDIM8 analogs to NR4A1 was further determined by measuring NR4A1 tryptophan fluorescence intensity at emission wavelength of 330 nm. The binding affinity (K_d) and binding stoichiometry (B_{max}) of NR4A1/bisANS was determined as described (186). The ligand binding affinity (K_d) of CDIM8 analogs to NR4A1 was determined by measuring ligand-dependent decrease of NR4A1/ bisANS fluorescence intensity at emission wavelength of 500 nm. Ligand/bisANS fluorescence intensity at each ligand concentration was used to correct the NR4A1/bisANS/ligand fluorescence intensity as described (186, 253).

Polymerase Chain Reaction (PCR):

Cells (3.0×10^5) were seeded in a medium containing 10% FBS and were allowed to attach for 24 hours. The medium was then removed and replaced with fresh medium supplemented with 2.5% charcoal stripped FBS that also contained the desired compounds. After 24 hours, the cells were lysed and the RNA was extracted from them using Zymo Research Quick-RNA Miniprep kit (Irvine, CA) by following the manufacturer's protocol. The total RNA content was measured and then normalized. The high capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA) was then used to prepare cDNA from the isolated RNA, which was then used to quantify the total mRNA of the gene of interest by quantitative real-time PCR using amfiSure

qGreen Q-PCR master mix (genDEPOT, Katy, TX). The relative mRNA expression of the desired genes was determined by using human TATA binding protein mRNA as a control.

Chromatin immunoprecipitation (ChIP) assay:

The ChIP-IT express enzymatic kit (Active Motif, Carlsbad, CA) was used and the manufacturer's protocol was followed in order to perform this assay. Rh30 cells were treated with DMSO, CDIM8 or mithramycin for 24 hours and were then fixed with formaldehyde. Glycine was then used to stop the cross-linking reaction and the cells were scraped, collected and lysed to collect the nuclei which were then sonicated and sheared to get the chromatin fragments. Immunoprecipitation was then performed with the sheared chromatin fragments with protein specific antibodies (NR4A1, Sp1, IgG, PolII, or H3K9me2) in presence of protein G-conjugated magnetic beads for overnight. The beads were then washed with provided ChIP buffers, chromatin fragments were eluted, the protein-DNA cross-links were reversed and finally the DNA was obtained by protein K digestion. PCR was then performed with the designed primers for the promoters for specific genes (G9a or PTEN). The amplified fraction of the promoter was then resolved on 2% agarose gel in presence of ethidium bromide.

DNA-protein binding assay:

Rh30 cells (2.0×10^6) were seeded in a medium supplemented with 10% FBS and were allowed to attach for 24 hours. The Abcam (Cambridge, UK) nuclear extraction kit (ab113474) was then used and the manufacturer's protocol was followed in order to extract the nuclear protein from the cells. This nuclear protein was used with the Abcam DNA-protein binding assay kit (ab117139) and the manufacturer's protocol was followed to quantify the interaction of Sp1 protein with G9a promoter. The G9a oligonucleotide probes used were: WT, 5'-CCGGGGCGGC-3'; Mutant, 5'-CCGTGTCGGC-3'.

Animal studies:

Female athymic nude mice (3-4 weeks old) were purchased from Envigo Rms, LLC (Indianapolis, IN) and were housed at Lab Animal Care Center, Texas A&M University. The protocol for the animal studies was approved by Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. The mice were allowed to acclimate for a week and were fed standard chow diet. Rh30 cells (4.0×10^6) cultured in RPMI medium supplemented with 10% FBS were detached by trypsinization, washed with sterile PBS, and then resuspended in 100 µl of PBS and matrigel in 1:1 ratio. These cells were then injected into the mice subcutaneously. After the tumor size were palpable (~50 to 100 mm^3 in size), the mice were randomly divided into two groups – control and treatment groups. The mice in the control group were injected with 100 µl corn oil whereas the mice in the treatment group were injected with 100 µl of 12.5 mg/kg 3-Br-5-OCH_3 prepared in corn oil every other day intraperitoneally. The mice were weighed every week and the tumor volume in each mouse was calculated using a Vernier Calliper (V = L*W*W/2 mm^3). After three weeks, all the mice were sacrificed. The tumor from each mouse was then removed and weighed. A small piece of fresh tumor was homogenized in lysis buffer and was further used for western blot and PCR studies.

Cell survival (XTT) assay:

Cells (1×10^4) were seeded using 10% FBS containing medium and were allowed to attach for 24 hours. The medium was then replaced with a fresh medium containing 2.5% stripped charcoal serum supplied with the desired concentration of compounds for 24 hours. The XTT cell viability kit (Cell Signaling Technology, Danvers, MA) was then used and the manufacturer's protocol was followed to calculate the percentage of cell survival. Results for this are now illustrated in Supplemental Figure 5.

Statistical analysis:

Statistical significance of differences between the treatment groups was determined by Student t test. Each experiment was performed three times and the results were presented as means with error bars representing 95% confidence intervals. Data with a P value of less than 0.05 were considered statistically significant.

RESULTS

Previous studies showed that G9A was highly expressed in Rh30 and Rh41 ARMS cells (100) and examination of UALCAN and TCGA databases showed that in sarcoma patients, high expression of G9A was associated with decreased survival (Fig. 11A). In a limited data set, G9A is also expressed more in primary tumors than in non-tumor tissues (Fig. 11B). The major focus of this paper is to report our studies showing that the orphan nuclear receptor NR4A1 regulates G9a expression in ARMS cells. Knockdown of NR4A1 by RNA interference (RNAi) using multiple oligonucleotides in Rh30 and Rh41 cells decreased expression of NR4A1 and G9A (Fig. 11C) whereas knockdown of G9A by RNAi decreased expression of G9A but not NR4A1 proteins (Fig. 11D). These results indicate that NR4A1 regulates expression of G9A in ARMS cells whereas knockdown of G9A has minimal effects on NR4A1. 1,1-Bis(3'-indolyl)-1-(p-hydroxyphenyl)methane (CDIM8) is a prototypical NR4A1 antagonist in cancer cells including ARMS cells (77) and the 3,5-$(CH_3)_2$ and 3-Br-5-OCH_3 buttressed analogs of CDIM8 (Fig. 12A) bind NR4A1 and quench fluorescence of tryptophan in the ligand binding domain (Fig. 12B) as

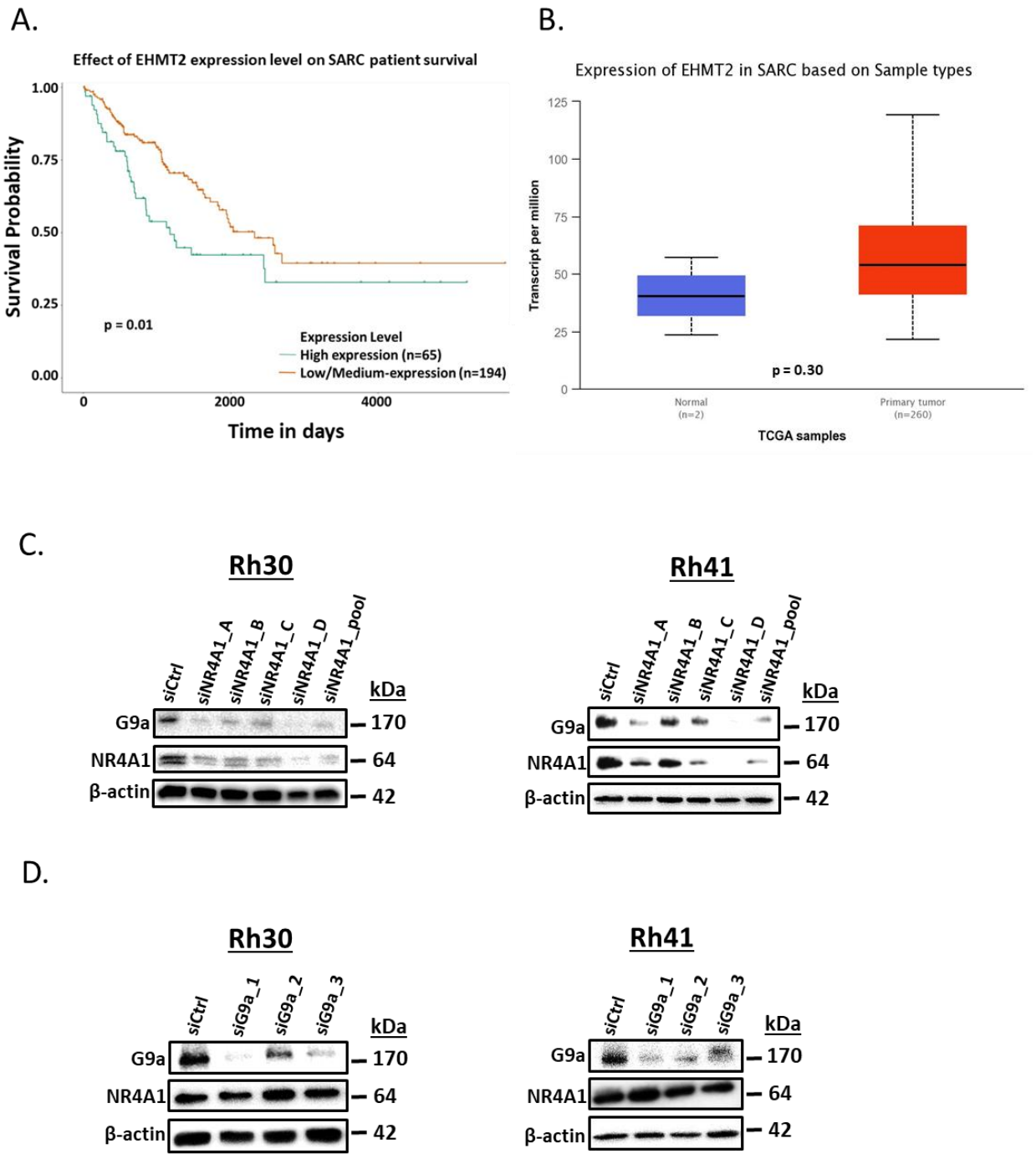

Figure 11. G9a (EHMT2) is an NR4A1-regulated gene in ARMS. High expression of EHMT2 is a negative prognostic factor for sarcoma patient survival (A) and is more highly expressed in tumors versus normal (B). Rh30 and Rh41 ARMS were transfected with oligonucleotides targeting NR4A1 (siNR4A1; C) and G9a (siG9a; D) and whole-cell lysates were analyzed by Western blots as outlined in Materials and Methods.

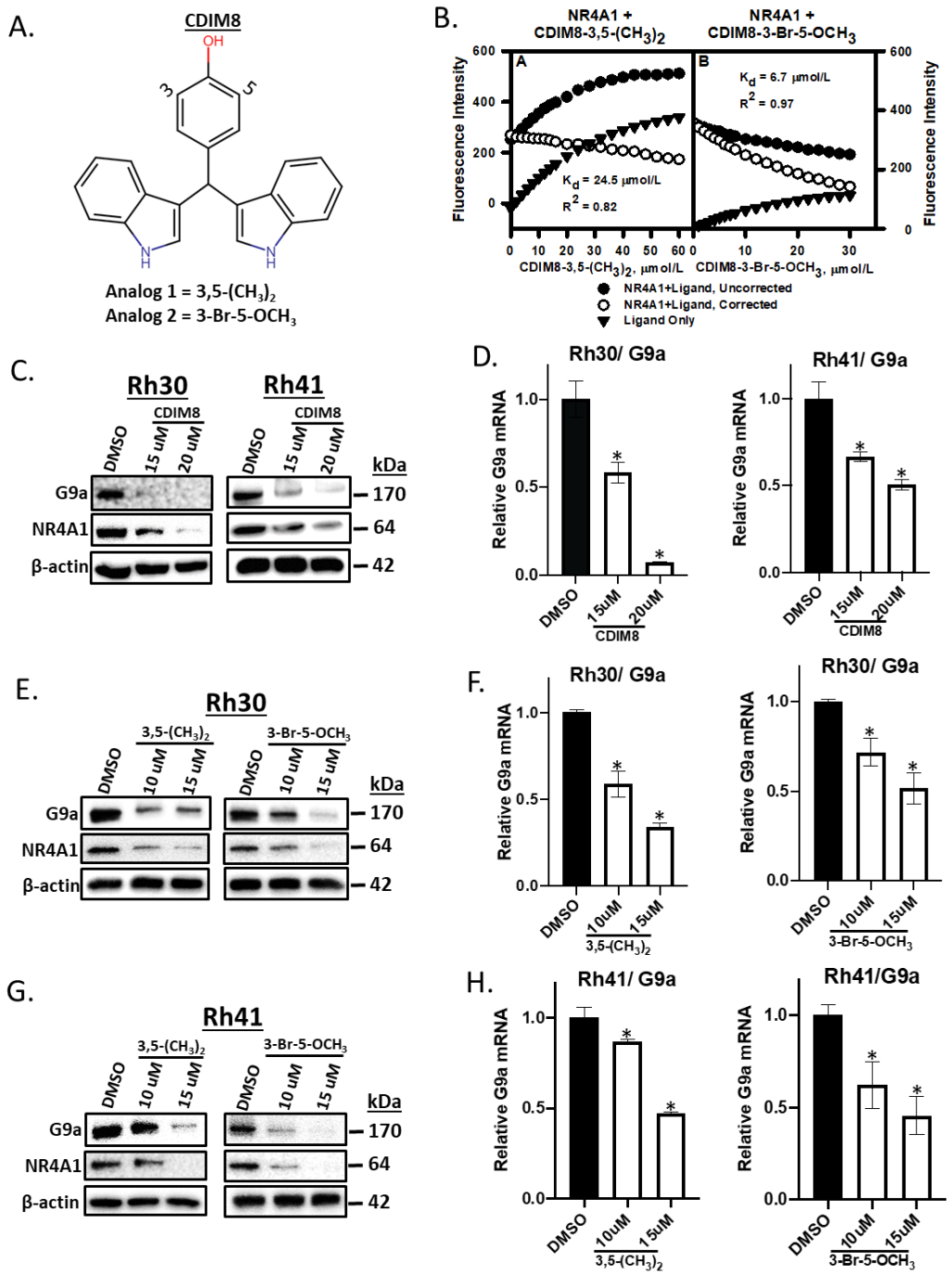

Figure 12. NR4A1 ligands act as antagonists and decrease G9a expression in ARMS cells. A. Structures of CDIM8 and buttressed analogs. B. The K_d values for 3,5-$(CH_3)_2$ and 3-Br-5-OCH_3 interactions with the LBD of NR4A1 were determined by fluorescent quenching of the tryptophan residue in the binding pocket as outlined in Materials and Methods. Rh30 and Rh41 cells were treated with CDIM8 (C and D), 3,5-$(CH_3)_2$ and 3-Br-5-OCH_3 (E–H) and effects on gene products and mRNA levels were determined by Western blots and real-time PCR, respectively, as outlined in Materials and Methods. Results (D, F, and H) are expressed as means ± SD for at least three replicated determinations for each treatment group and significantly ($P < 0.05$) decreased responses are indicated.

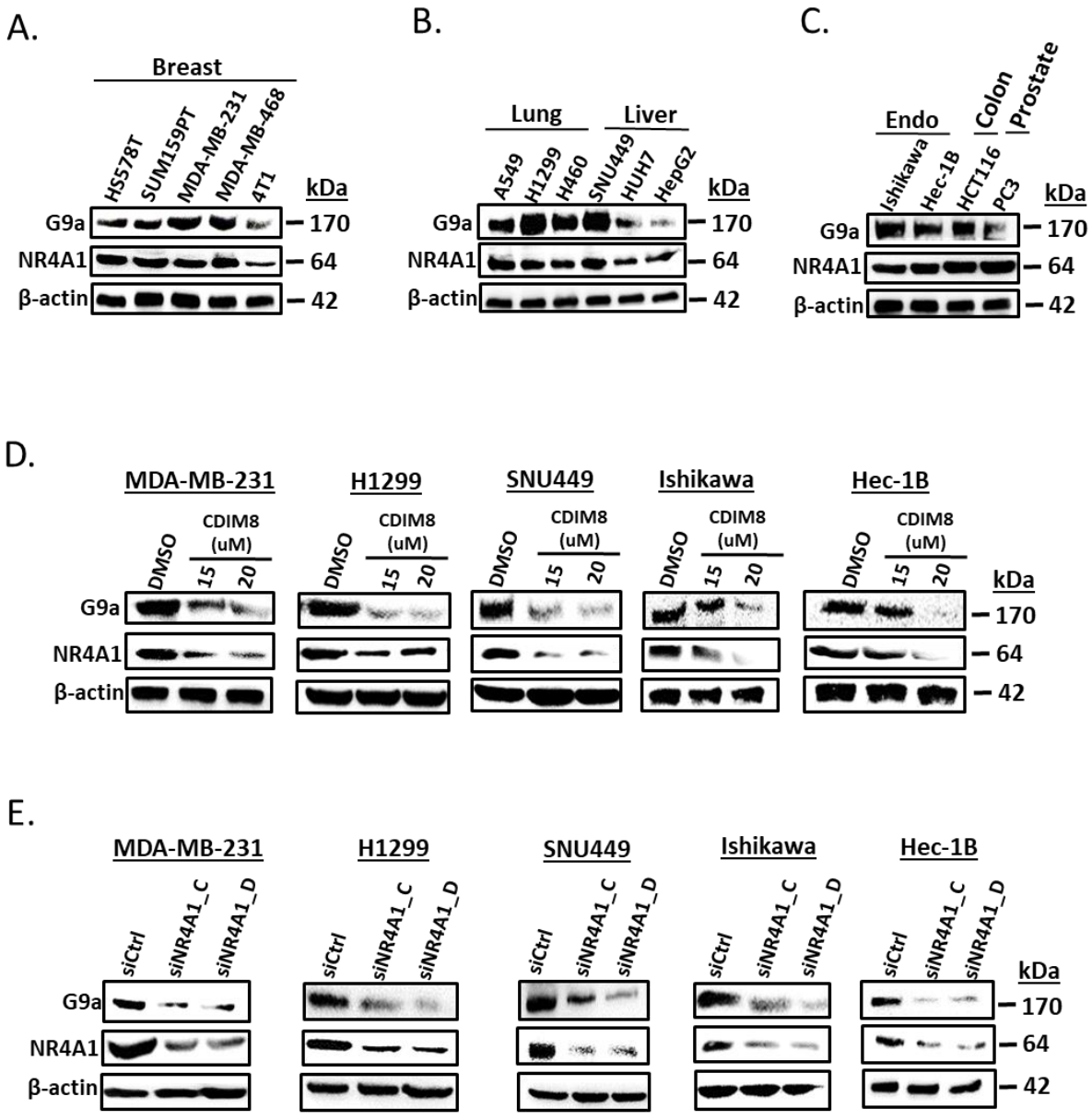

Figure 13. G9a is an NR4A1-regulated gene in multiple cancer cell lines. Lysates from several breast (A), lung and liver (B), endometrial, colon, and prostate (C) cancer cell lines were analyzed by Western blots as outlined in Materials and Methods. Selected breast (MDA-MB-231), lung (H1299), liver (SNU449), and endometrial (Ishikawa and Hec1B) cancer cell lines were treated with the NR4A1 antagonist CDIM8 (D) or transfected with siNR4A1 (2 oligonucleotides; E) and whole-cell lysates were analyzed by Western blot analysis as outlined in Materials and Methods.

described previously (186) and the growth inhibitory effects of these compounds are summarized in Supplemental Fig. 5; Supplemental Fig. 6A shows that the NR4A1 antagonists also decrease NR4A1/ bisANS fluorescence intensity as described previously (186). Treatment of Rh30 and Rh41 cells with CDIM8 decreased levels of G9A protein and this was also accompanied by decreased NR4A1 protein (Fig. 12C). CDIM8 also decreased expression of G9A mRNA levels in Rh30 and Rh41 cells (Fig. 12D). We also used buttressed CDIM8 analogs 3,5-$(CH_3)_2$ and 3-Br-5-OCH_3 (190, 207, 211) and investigated their effects on G9A expression in ARMS cells. Like CDIM8, both compounds inhibited growth of Rh30 and Rh41 cells (Supplemental Figure 5) and decreased G9A protein and mRNA levels in Rh30 (Figs 12E and 12F) and Rh41 (Figs. 12G and 12H) cells. These results suggest that NR4A1 regulates G9A expression in ARMS cells and this was further investigated in a panel of NR4A1-expressing cancer cell lines (Fig. 13). NR4A1 and G9A are also co-expressed in a panel of breast (Fig. 13A), lung and liver (Fig. 13B) and endometrial, colon and prostate (Fig. 13C) cancer cells. Moreover, in a subset of these cell lines (MDA-MB-231, H1299, SNU449, Ishikawa and Hec1B), treatment with C-DIM8 (Fig. 13D) or knockdown of NR4A1 by RNAi (Fig. 13E) decreased levels of G9A protein and these results were consistent with those observed in ARMS cells suggesting that NR4A1 regulates G9a in multiple cancer cell lines.

Previous studies in RMS and other cell lines show that NR4A1 regulates multiple genes containing GC-rich promoters by acting as a nuclear co-factor for DNA bound Sp1 or Sp4, (79, 194, 208, 254-256) and this was previously observed for NR4A1 regulation of PAX3-FOXO1 and β1-integrin in ARMS cells (79). Figure 14A illustrates that G9A contains a consensus GC-rich promoter site and knockdown of Sp1 (Fig. 14B) but not Sp4 (Fig. 14C) decreased expression of

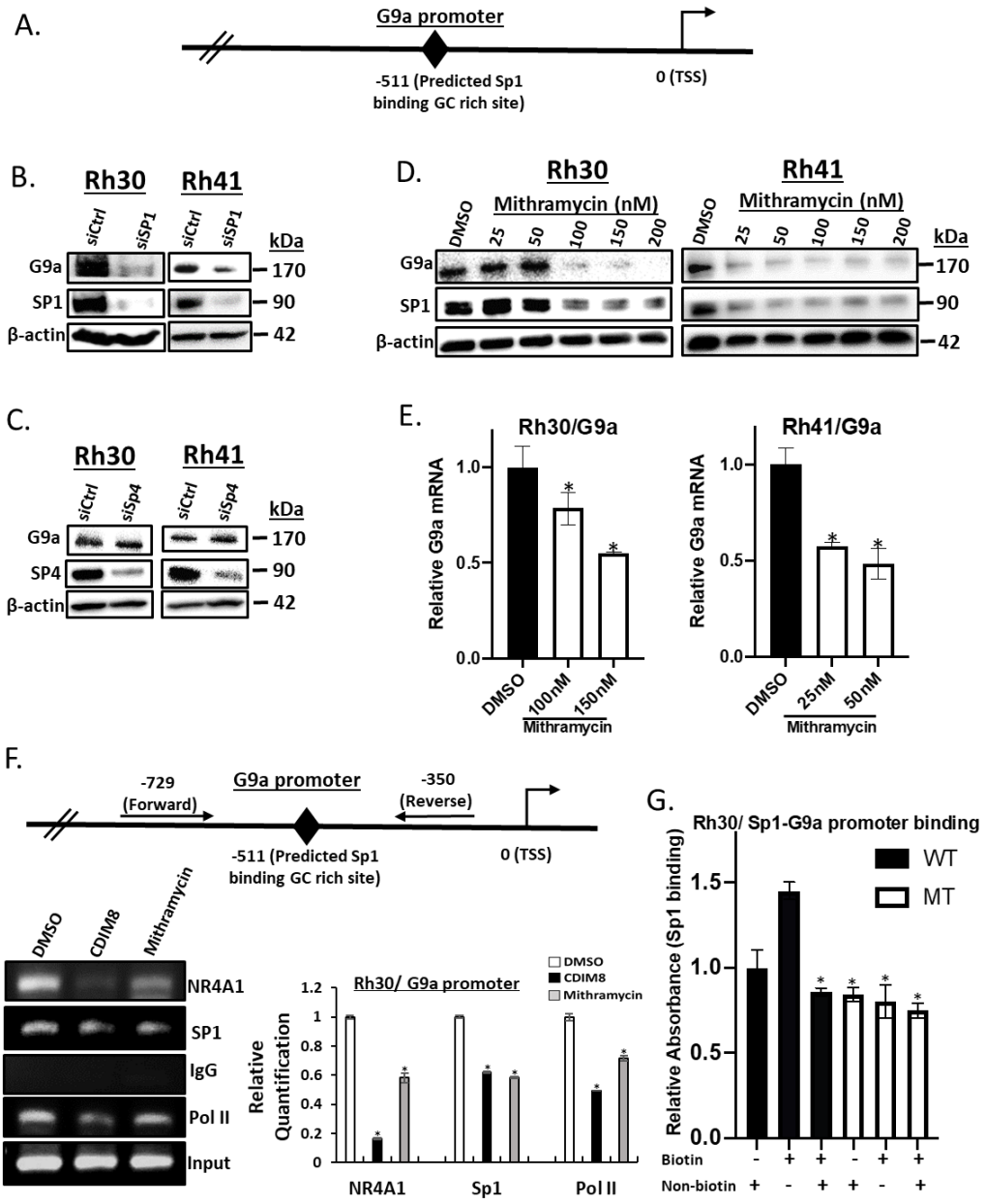

Figure 14. G9a is an NR4A1/Sp1-regulated gene in ARMS cells. A. G9a promoter and GC-rich Sp1-binding site. Rh30 and Rh41 cells were transfected with oligonucleotides targeting Sp1 (B) and Sp4 (C), and whole-cell lysates were analyzed by Western blots as outlined in the Materials and Methods section. Rh30 and Rh41 cells were treated with mithramycin and effects on G9a protein (D) and mRNA levels (E) were determined by Western blots and real-time PCR, respectively, as outlined in Materials and Methods. F. Rh30 cells were treated with DMSO, CDIM8 (20 mmol/L) or mithramycin (100 nmol/L) for 24 hours and analyzed for binding to the G9a promoter in a ChIP assay as outlined in Materials and Methods and the band intensities were quantitated. G. Binding of nuclear extracts from Rh30 cells to a GC-rich oligonucleotide (identical to the GC-rich/-511 G9a promoter) was determined as outlined in Materials and Methods. Results (E and G) are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) changes compared with controls are indicated (*).

G9A in Rh30 and Rh41 cells. The role of Sp1 in regulating G9A expression was further confirmed by showing that mithramycin, a drug that binds GC-rich sites to inhibit Sp-dependent gene expression (257), also decreased expression of G9A protein (Fig. 14D) and mRNA (Fig. 14E) in Rh30 and Rh41 cells. ChIP analysis shows that Sp1, pol II and NR4A1 bind to the GC-rich region of the G9A gene promoter and after treatment of Rh30 cells with CDIM8 or mithramycin for 24 hours, we observed decreased binding of Sp1, NR4A1 and pol II to the G9A promoter (Fig. 14F). These results are consistent with previous ChIP analysis of other NR4A1/Sp-regulated genes in RMS and other cell lines (79, 194, 208, 254-256). In addition, we show that Sp1 protein from nuclear extracts of Rh30 cells binds to a GC-rich oligonucleotide derived from wild type G9a promoter significantly higher in comparison to mutant (GC) oligonucleotide in a DNA protein binding assay (Fig. 14G).

The histone methyltransferase activity of G9A primarily enhances dimethylation of H3K9 and knockdown of G9A in Rh30 and Rh41 cells decreases overall H3K9me2 expression in Rh30 and Rh41 cells (Fig. 15A). Similar results were observed after knockdown of NR4A1 (Fig. 15B) or treatment of CDIM8 (Fig. 15C) with Rh30 and Rh41 cells. UNC0642 has previously been characterized as a substrate competitive inhibitor of G9A (152) and we observed that this compound also decreased levels of H3K9me2 in Rh30 and Rh41 cells (Fig. 15D). Thus, like UNC0642, knockdown of NR4A1 or treatment with CDIM8 not only decreases G9A, but also decreases G9A-dependent levels of H3K9me2.

Previous studies in ARMS cells reported that G9A silenced PTEN thereby activating Akt (100) and therefore we further investigated effects of NR4A1 antagonists on G9A, PTEN and phosphorylated Akt in ARMS cells. Treatment of Rh30 (Fig. 16A) and Rh41 (Fig. 16B) cells with

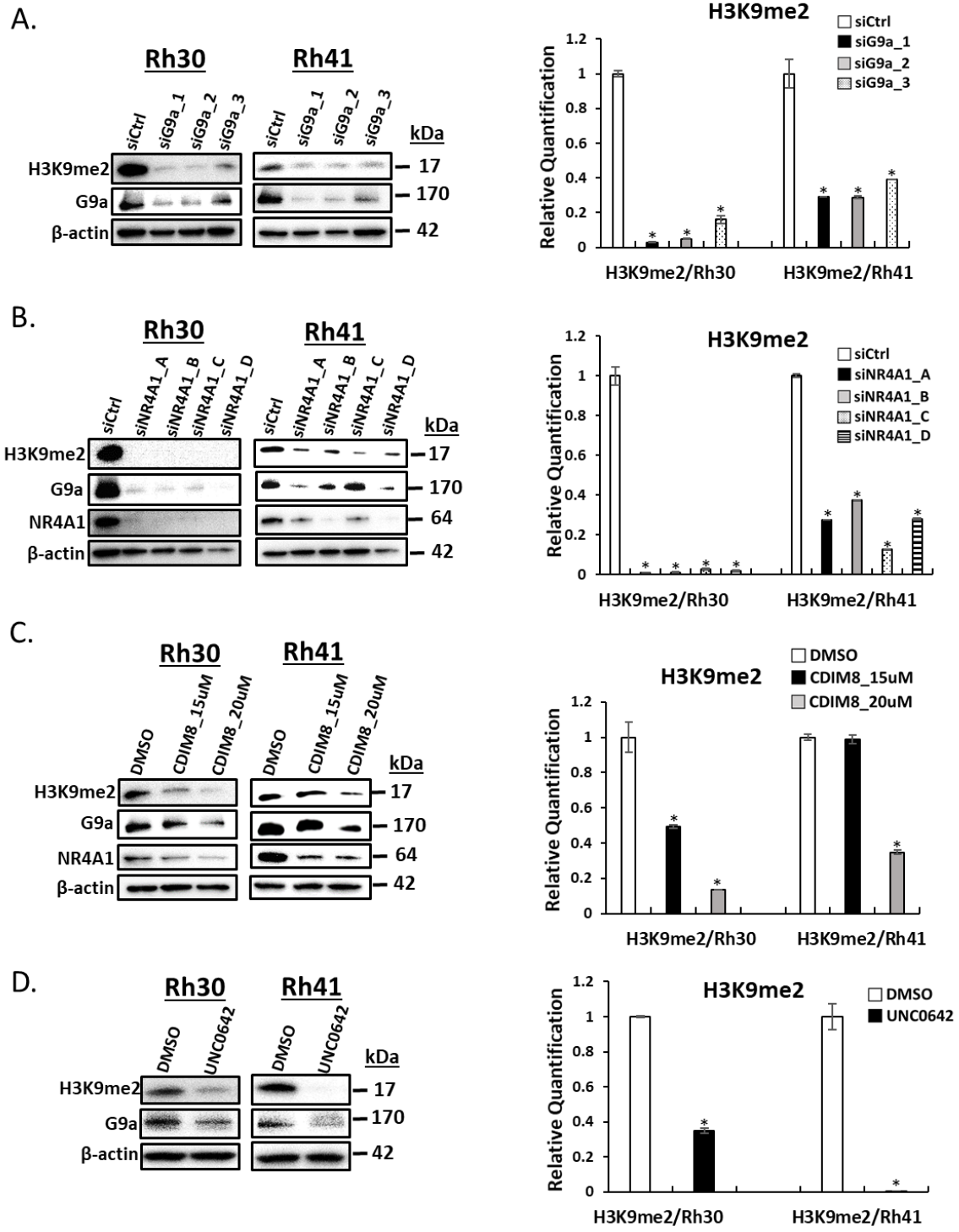

Figure 15. NR4A1/G9a regulate H3K9me2 levels in ARMS cells. The effects of knockdown of G9a (A) and NR4A1 (B), treatment with CDIM8 (C) or the G9a inhibitor UNC0642 (5 mmol/L; D) on G9a expression in Rh30 and Rh41 cells were determined by Western blot analysis of whole-cell lysates as outlined in Materials and Methods. Quantitation of each blot (relative to β-actin) was also determined for each blot.

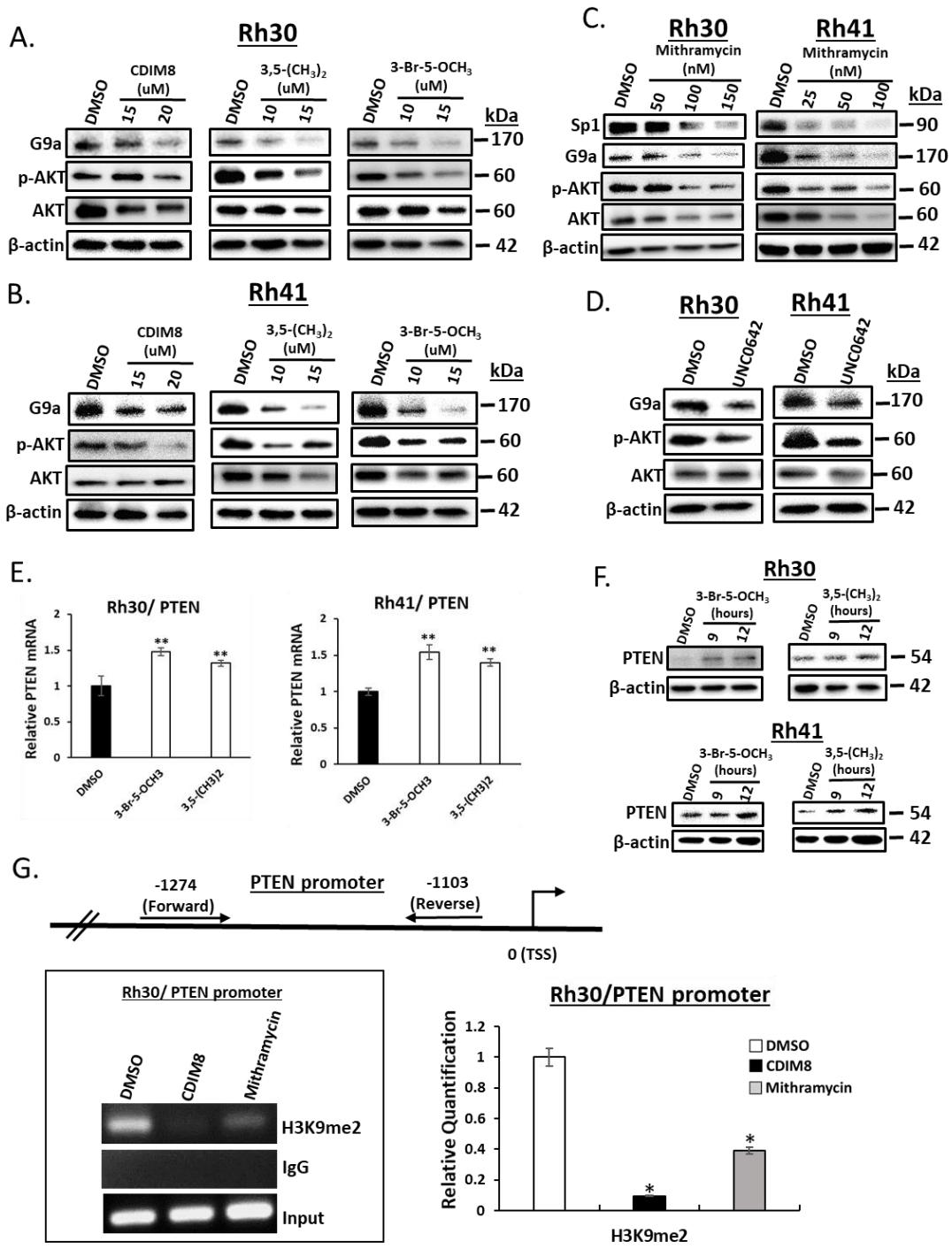

Figure 16. siNR4A1/NR4A1 antagonists, mithramycin, and UNC0642 inhibit phosphorylation of Akt. ARMS cells were treated with NR4A1 antagonists (A and B), mithramycin (C) and UNC0642 (5 mmol/L) (D) and whole-cell lysates were analyzed by Western blots as outlined in Materials and Methods and blots (A and B) were quantitated in Supplemental Fig. 6B and 6C. Cells were treated with 15 mmol/L 3-Br-5-OCH3 and 3,5-(CH3)2 and PTEN mRNA levels (E) and protein (F) were determined by real time PCR and Western blots, respectively. G. Cells were treated with DMSO, CDIM8 (20 mmol/L), or mithramycin (100 nm) for 24 hours and association of H3K9me2 with the PTEN promoter was determined (and quantitated) in a ChIP assay as outlined in Materials and Methods.

C-DIM8, 3,5-$(CH_3)_2$ and 3-Br-5-OCH_3 decreased expression of phospho-Akt and these results are quantified in Supplemental Fig. 6B and 6C. Similar results were observed after treatment with mithramycin (Fig. 16C) and UNC0642 (Fig. 16D) in Rh30 and Rh41 cells demonstrating that inactivation of NR4A1 inhibits G9A-dependent phosphorylation of Akt. Mithramycin also decreases levels of Akt protein (Fig. 16C) and previous studies show that other drugs that downregulate Sp1 also decrease Akt levels in some cancer cell lines (258-260). Rh30/Rh41 cells were also treated with 3-Br-5-OCH_3 and 3,5-$(CH_3)_2$ for 12 hours and this results in significant induction of PTEN mRNA levels in both cell lines (Fig. 16E). Using a similar treatment protocol, we also observed induction of PTEN protein in Rh30 and Rh41 cells after treatment for 9 and 12 hours; the induction response was not observed after longer treatment times ($\geq$ 24 hours) (data not shown) (Fig. 16F). ChIP analysis of the PTEN promoter in Rh30 cells (Fig. 16G) showed that both CDIM8 and mithramycin decrease H3K9me2 associated with the PTEN promoter and this is consistent with their effects on decreasing G9A expression in these cells. We also investigated the effects of the NR4A1 antagonist 3-Br-5-OCH_3 as an inhibitor of tumor growth in athymic nude mice bearing Rh30 cells as xenografts. Tumor volumes in control (corn oil) mice were significantly increased compared to the 3-Br-5-OCH_3 treated mice (12.5 mg/kg every other day) over the 21 days duration of study (Fig. 17A). After sacrifice, the volumes (Fig. 17B) and the weights (Fig. 17C) of the excised tumors in control mice were also significantly larger/higher in comparison to the 3-Br-5-OCH_3 treated mice, however their body weights remained unchanged over the treatment period (Fig. 17D). Quantitative PCR and western blot analysis of the tumor extracts showed that the treatment with the NR4A1 antagonist also decreased expression of G9a mRNA (Fig. 17E) and protein (Fig. 17F). Results of both in vitro and in vivo studies were complementary and demonstrate for the first time that the histone methyltransferase G9a gene is regulated by

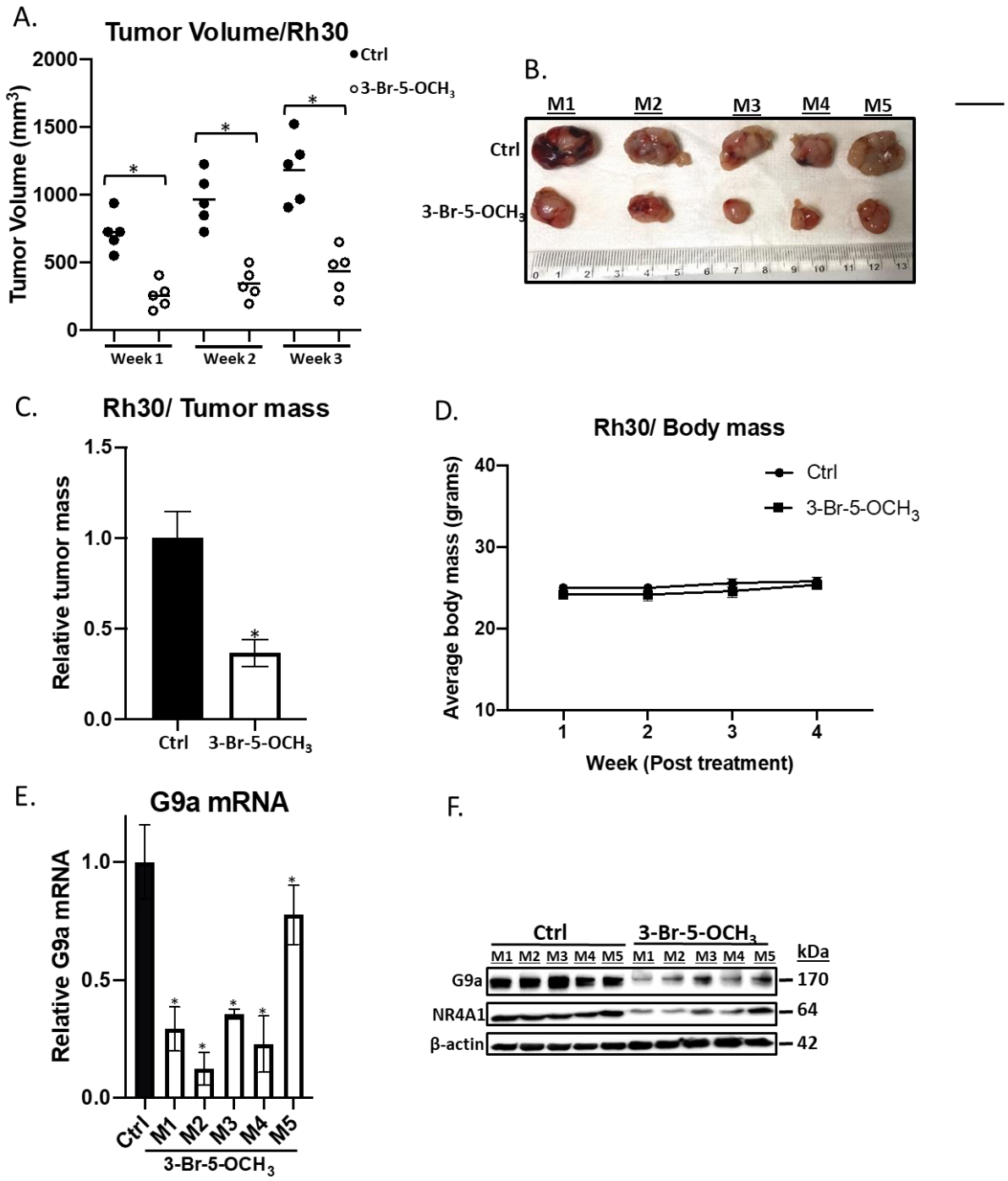

Figure 17. NR4A1 antagonists inhibit tumor growth and decrease G9a expression. Athymic nude mice bearing Rh30 cells as xenografts were treated with 3-Br-5-OCH3 (12.5 mg/kg/every other day) by intraperitoneal injection over a period of 3 weeks and tumor volumes (A) and weights (B and C) and changes in body mass (D) were determined as outlined in Materials and Methods. Real-time PCR and Western blot analysis were performed with the tumor extracts to obtain the expression of G9a mRNA (E) and protein (F).

NR4A1 in ARMS and the bis-indole derived NR4A1 antagonists target G9a and represent a novel class of G9a inhibitors.

DISCUSSION

The orphan nuclear receptors NR4A1, NR4A2 and NR4A3 are immediate early genes induced by multiple stressors, and the NR4A receptors play an important role in maintaining cellular homeostasis and disease. There is increasing evidence for the role of these receptors in metabolic, cardiovascular and neurological functions as well as in inflammation and inflammatory diseases and in immune functions and cancer (120, 152). NR4A1 is overexpressed in multiple solid tumors and in breast, colon and lung tumor patient's high expression of NR4A1 is a negative prognostic factor and predicts decreased survival (120, 178, 182, 215). The functional activity of NR4A1 in cancer has been extensively investigated in cancer cell lines by either knockdown or overexpression. In blood-derived cancers, the combined loss of NR4A1 and NR4A3 in mice results in rapid development of acute myeloid leukemia symptoms and both receptors exhibit tumor suppressor-like activity (164, 218). In contrast, NR4A1 is a pro-oncogenic factor in solid tumors and regulates one or more of cancer cell proliferation, survival, cell cycle progression, migration, and invasion in lung, melanoma, lymphoma, pancreatic, colon, cervical, ovarian, rhabdomyosarcoma and gastric cancer cell lines (77, 79, 178, 180, 186, 187, 190, 194, 207, 208, 215, 254). NR4A1 regulates many of the same pathways in RMS and most solid tumor-derived cancer cells, and this includes regulation of thioredoxin domain containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1) which maintains high reductant levels which indirectly affect mTOR signaling. Knockdown of NR4A1 or treatment with bis-indole derived NR4A1 antagonists decreases expression of TXNDC5 and IDH1 resulting in induction of ROS and ROS-dependent sestrin2 which in turn activates AMPK and inhibits mTOR signaling (77, 180, 190, 209-211, 253,

261). NR4A1 also acts as a cofactor for several pro-oncogenic Sp-regulated genes including Bcl-2/survivin, epidermal growth factor receptor (EGFR), several integrins and PAX3-FOXO1 in ARMS cells and knockdown of NR4A1 or NR4A1 antagonists decrease expression of these genes (79, 180, 186, 194, 207, 208, 254, 255).

Recent studies showed that NR4A1 regulates β1-integrin expression in breast cancer cells, and NR4A1 antagonists inhibit β1-integrin gene expression and β1-integrin-dependent cell migration/invasion (194). NR4A1 also plays an important role in TGFβ-induced breast and lung cancer invasion and DIM-C-pPhOH inhibits this response (178, 209, 210). The mechanism of regulation of several genes, including survivin, TXNDC5, and several integrins by NR4A1 involves interactions of the receptor with Sp1 or Sp4 transcription factors bound to GC-rich promoter regions of these genes. ChIP analysis shows that NR4A1, Sp1 and p300 bind to the GC-rich β1-integrin gene promoter and treatment with DIM-C-pPhOH (CDIM8) or its p-carbomethoxy analog decreases these interactions with the β1-integrin promoter and decreases expression of β1-integrin in MDA-MB-231 and SKBR3 breast cancer cells (207).

NR4A1 also plays an important pro-oncogenic role in RMS cells and regulates expression of genes associated with cell proliferation, survival and migration/invasion and this includes NR4A1/Sp4-dependent regulation of the PAX3-FOXO1 fusion oncogene and β1-integrin expressed in ARMS cells (79). A recent study also reported high expression of G9A in ARMS and like NR4A1, G9A also regulates ARMS cell growth and migration (100). This raised the possibility that pro-oncogenic functions of NR4A1 in ARMs cells and the potent anti-tumorigenic activity of bis-indole derived NR4A1 antagonists (77, 79) may also be linked to the regulation of G9A. Moreover, the G9A gene promoter contains GC-rich sequences that potentially bind Sp

transcription factors (Fig. 14A) and one mechanism of NR4A1 regulation of genes is due to the receptor acting as a co-factor of Sp1 or Sp4 (79, 180, 186, 194, 207, 208, 254, 255).

Results illustrated in Figures 11 and 12 demonstrate that knockdown of NR4A1 by RNAi decreased expression of G9A protein and NR4A1 antagonists decreased expression of G9A protein and mRNA suggesting that G9A is an NR4A1-regulated gene that can be targeted by NR4A1 antagonists. Moreover, this is supported by comparable results in multiple cancer cell lines suggesting that NR4A1 is an upstream regulator of G9A expression (Fig. 13). We also show by protein, gene and ChIP analysis that NR4A1 and Sp1 (but not Sp4) are important for regulation of G9A and both NR4A1 and Sp1 interact with the GC-rich region of the G9A gene in a ChIP assay (Fig. 14). Interestingly our previous studies showed that NR4A1/Sp4 regulates PAX3-FOXO1 gene expression in ARMS cells demonstrating that the transactivation functions of NR4A1/Sp1 and NR4A1/Sp4 are gene specific and this has previously been observed for regulation of integrins by NR4A1 (79, 194, 208, 254).

Previous studies in ARMS cells showed that G9A suppression/methylation of PTEN and subsequent activation of Akt was a critical factor in cell and tumor growth (100). We observed similar response after knockdown of G9A or treatment with UNC0642, a G9A substrate binding site inhibitor (Figs. 16A and 16B). However, this was also observed after treatment with NR4A1 antagonists demonstrating that G9A is regulated by NR4A1 and can be targeted by NR4A1 antagonists. Results of in vivo studies (Fig. 17) complemented data obtained in cell culture demonstrating that NR4A1 antagonists inhibit tumor growth and this is accompanied by downregulation of G9A. These observation in ARMS cells and tumors suggests that NR4A1 through regulation of G9A also enhances tumorigenesis via epigenetic pathways in ARMS and possibly other cancer cell lines and that bis-indole derived NR4A1 antagonists represent a new

class of G9A inhibitors that inhibit transcription of this gene and thereby act as modulators of epigenetic pathways in cancer cells.

CHAPTER IV

FLAVONOIDS KAEMPFEROL AND QUERCETIN ARE NUCLEAR RECEPTOR 4A1 (NR4A1, NUR77) LIGANDS AND INHIBIT RHABDOMYOSARCOMA CELL AND TUMOR GROWTH*

INTRODUCTION

Flavonoids are phytochemicals produced in fruits, nuts and vegetables that have been directly linked to the health promoting effects of diets enriched in flavonoid compounds. Consumption of total and individual flavonoids have been associated with increased lifetimes and protection from multiple adverse health effects including cardiovascular disease, diabetes and metabolic diseases, neurodegeneration, inflammatory diseases and cancer (262-270). For example, high dietary intakes of anthocyanins, flavonoids and flavonoid polymers by participants in the prospective Framingham Offspring cohort were correlated with lower risks of dementias including Alzheimer's disease (271, 272). Flavonoids exhibit multiple activities and the mechanisms of chemoprevention associated with high dietary intakes of flavonoids are difficult to establish. However, most dietary flavonoids exhibit antioxidant activities and they also enhance the immune system and these effects coupled with other individual flavonoid-dependent responses contribute to their chemoprevention of diseases (273-277).

There is also evidence that diets enriched in flavonoids also protect against development of cancer (263, 265, 277-280) and this is complemented by an extensive literature on the

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***** Reprinted from "Flavonoids Kaempferol and Quercetin are Nuclear Receptor 4A1 (NR4A1, Nur77) Ligands and inhibit Rhabdomyosarcoma Cell and Tumor Growth" by Shrestha R., Mohankumar K., Martin G., Hailemariam A., Lee SO., Jin, UH., Burghardt R., Safe S. Journal of Experimental & Clinical Cancer Research (In Review) 2021.

chemotherapeutic effects of individual flavonoids. In vitro and in vivo studies demonstrate that flavonoids inhibit cancer cell growth and migration, and modulate multiple pathways and genes associated with tumorigenesis. The studies on the chemotherapeutic mechanisms associated with flavonoids as anticancer agents primarily have focused on specific functions or genes that are affected. A recent report showed that the flavonoid cardamonin inhibited dextran sodium sulfate-induced inflammation in the gut and this anti-inflammatory response was linked to the AhR activity of this compound (281). Studies in this laboratory have been investigating the pro-oncogenic roles of the nuclear orphan receptor 4A1 (NR4A1, Nur77) in rhabdomyosarcoma (RMS) and other cancer cell lines and the anticancer activities of bis-indole derived (CDIMs) which are NR4A1 ligands that act as receptor antagonists (77, 79, 172, 282-285). The fusion oncogene PAX3-FOXO1 and G9a have been characterized as highly pro-oncogenic factors in RMS (100, 286) and NR4A1 regulated expression of both genes and also β1-integrin and treatment of RMS cells with CDIM/NR4A1 antagonists decreased expression of these genes (79, 282). A recent study reported that the flavonoid kaempferol decreased G9a expression in gastric cancer cells (287) and this was accompanied by growth inhibition, induction of markers of apoptosis and inhibition of mTOR signaling by induced phosphorylation of AMPK. This pattern of responses observed for kaempferol in gastric cancer cells has previously been observed for CDIM/NR4A1 antagonists or NR4A1 silencing in RMS and other cancer cell lines (21-27). Therefore, we hypothesized that kaempferol is an NR4A1 ligand and this study shows for the first time that both kaempferol and quercetin bind NR4A1 and act as NR4A1 antagonists in RMS cells. Both flavonoids inhibit expression of G9a, PAX3-FOXO1, and other pro-oncogenic NR4A1-regulated genes/pathways. Kaempferol and quercetin also inhibited tumor growth in an athymic nude mouse model in vivo suggesting that these nutraceuticals can be repurposed and used in a precision

medicine/nutrition approach for treating RMS patients and possibly patients with other cancers that express NR4A1.

MATERIALS AND METHODS

Cell lines, reagents and antibodies:

Rh30 (RMS) cell line was purchased from American Type Culture Collection (Manassas, VA) and was maintained in RPMI medium. The Rh41 (RMS) cell line was a generous gift from Mr. Jonas Nance, Texas Tech University Health Sciences Center- Children's Oncology Group (Lubbock, TX) and was maintained in IMDM medium and supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37^{o}C temperature in presence of 5% CO_2. The summary of the reagents/antibodies and oligonucleotides used are summarized in Supplemental Table 3 and Table 4 respectively. Both kaempferol and quercetin were dissolved in 100% DMSO. Rh30 and Rh41 cell lines were treated with the desired concentrations of flavonoids for 24 and 48 hours respectively. Knockdown studies by RNA interference (siNR4A1) were carried out essentially as described (282, 283).

NR4A1-CDIMs binding assay:

Quenching of NR4A1 Tryptophan Fluorescence by direct ligand binding tryptophan fluorescence spectra were obtained essentially as described (186); the ligand binding domain (LBD) of NR4A1 (0.5 μM) in buffer was incubated with different concentrations of ligands and the fluorescence was obtained using an excitation wavelength of 285 nm (excitation slit width = 5 nm) and an emission wavelength range of 300-420 nm (emission slit width = 5 nm). Ligand binding affinity (K_d) to NR4A1 was determined by measuring concentration-dependent NR4A1 tryptophan fluorescence intensity at emission wavelength of 330 nm (186).

Bis-ANS displacement assay:

Bis-ANS (Molecular Probes, Inc/ThermoFisher) is essentially non-fluorescent in aqueous solution, however, bisANS fluorescence increases significantly upon binding to protein such as NR4A1. The binding affinity (K_d) and binding stoichiometry (B_{max}) of NR4A1/bisANS was determined essentially as described (253). Ligand binding affinity (K_i) to NR4A1 was determined by measuring NR4A1/bisANS fluorescence intensity at emission wavelength of 500 nm as described (253). Ligand/bisANS fluorescence intensity at each ligand concentration was used to correct the NR4A1/bisANS/ligand fluorescence intensity.

Luciferase assay:

Cells (8×10^4) were seeded in a medium supplemented with 10% FBS and were allowed to attach. After 24 hours, Lipofectamine-2000 reagent (50 umol/L) in reduced serum medium was used to co-transfect those cells with upstream activation sequence a) 400 ng $(UAS)_{x5}$-Luc and 40 ng Gal4-NR4A1 or b) 200 ng $NBRE_{x3}$-Luc and 20 ng Flag-NR4A1. The medium was removed after 6 hours and replaced with 2.5% charcoal-stripped FBS supplemented medium containing either DMSO or flavonoids. After 24 hours, the cells were lysed and the cell extract was processed for chemiluminescence quantification of luciferase activity. The Lowry protein assay was used to determine the protein concentration in the cell extract which was used to normalize the luciferase activity as described in (172, 186). Both Gal4-NR4A1 and Flag-NR4A1 that are used for this study contained full length NR4A1 coding sequence. The plasmids used for this study are constructed as described previously (172, 186).

Cell survival (XTT) assay:

Cells (1×10^4) were seeded in 10% FBS containing medium and were allowed to attach. After 24 hours, the medium was replaced with a fresh medium containing 2.5% stripped charcoal

serum supplied with either DMSO or flavonoids. The XTT cell viability kit (Cell Signaling Technology, Danvers, MA) was then used and the manufacturer's protocol was followed to calculate the percentage of cell survival.

Western blot analysis:

Cells treated with DMSO or flavonoids were lysed and the protein concentrations in cell extracts were quantified using the Lowry protein assay. After normalization, an equal amount of protein was loaded and allowed to run on an SDS polyacrylamide gel. The proteins from the gel were transferred to a PVDF membrane, blocked, and incubated with the primary antibodies (overnight) followed by secondary antibodies (two hours). The HRP-substrate was then added to the membrane and the expression of the protein of interest was detected using Kodak 4000 MM Pro image station (Molecular Bioimaging, Bend, OR).

Annexin V staining:

Cells were seeded and allowed to attach overnight. They were then treated with either DMSO or the desired concentration of flavonoids. Cells were then trypsinized, washed with PBS, and were then suspended in annexin binding buffer. The Annexin V and propidium iodide provided with the Alexa fluor® 488 annexin V/dead cell apoptosis kit (Invitrogen, Carlsbad, CA) were added to the cells. The manufacturer's protocol was then followed to analyze the stained cells by flow cytometry.

Migration (Scratch) assay:

Cells ($3 \text{ X } 10^5$) were seeded and were allowed to attach. After 24 hours, the medium was removed and a scratch was made on the surface using a sterile 200 µl pipette tip. The dead cells were then removed by washing the cells with PBS (2x). The medium supplemented with 2.5% charcoal stripped FBS that contained either DMSO or the desired concentration of flavonoids were

then added to the cells. After 48 hours, the medium was removed, replaced with PBS and the pictures of migrated cells were taken using an Evos digital inverted microscope.

Boyden chamber invasion assay:

Cells (2 X 10^5) were seeded and were allowed to attach. After 24 hours, the medium was removed and replaced with the fresh medium supplemented with 2.5% charcoal stripped FBS that contained either DMSO or the desired concentration of flavonoids. After 48 hours, cells were trypsinized, counted and 75,000 cells were allowed to invade through the Boyden chamber. After 24 hours, the invaded cells trapped in the chamber were fixed, stained and counted. At least 3 replicates were performed for each treatment group.

Spheroid invasion assay:

Rh41 cells (3 X 10^3) were seeded in 200 µl 10% FBS supplemented medium in a low attachment round bottom 96 well plate. After 24 hours, when the spheroid had formed, medium (100 µL) was gently removed and the plate was allowed to chill on ice. Matrigel solution (100 µL) was then added to each well without disturbing the spheroid while the plate was still on the ice. The cells were then incubated at 37ºC for an hour and flavonoids (3X the desired final concentration) were added to each well. The cells were then incubated at 37ºC for 48 hours. After flagella like invading structures were developed from the spheroids, medium (100 µL) was removed and replaced by 100 µL of 3.6% formaldehyde for fixation. Pictures of the invasion were then taken using an Evos digital inverted microscope.

PCR:

Cells (3 X 10^5) were seeded in a 10% FBS containing medium and were allowed to attach. After 24 hours, the medium was removed and replaced with 2.5% charcoal stripped FBS supplemented medium that contained either DMSO or flavonoids. The manufacturer's protocol on

Zymo Research Quick-RNA Miniprep kit (Irvine, CA) was then followed to lyse the cells and extract RNA from them. The RNA concentration in the extract was then determined, normalized and the high capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA) was used to prepare cDNA from the isolated RNA. The amfiSure qGreen Q-PCR master mix (genDEPOT, Katy, TX) was then used to quantify the expression of mRNA of the gene of interest by quantitative real-time PCR. The human TATA binding protein mRNA was used as a control.

Chromatin immunoprecipitation assay:

The manufacturer's protocol on the ChIP-IT express enzymatic kit (Active Motif, Carlsbad, CA) was followed to perform this assay. Rh30 cells were seeded and allowed to attach for 24 hours, then treated with DMSO or flavonoids for 24 hours and fixed using formaldehyde. The cross-linking reaction was stopped with glycine and the cells were lysed and nuclei were collected, sonicated, and sheared to collect chromatin fragments. These chromatin fragments were immunoprecipitated with protein specific antibodies in presence of protein G-conjugated magnetic beads. The chromatin fragments were then eluted, the protein-DNA crosslinks were reversed and digestion with protein K was performed to obtain ChIP DNA. The primers designed for specific genes were then used to perform PCR with the ChIP DNA and the amplified promoter fraction was resolved on 2% agarose gel in presence of ethidium bromide (Denville Scientific, Metuchen, NJ).

Immunohistochemistry (IHC):

Tumor tissues were fixed in formaldehyde, embedded in paraffin, sectioned at 4 µM and then mounted on charged slides. These slides were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was then performed and the slides were washed with Tris buffer. The IHC procedure was then performed on an automated platform (intelliPATH FLX,

Biocare Medical, Pacheco, CA). All incubations were carried out at room temperature. Endogenous peroxidase activity was blocked by incubating the slides with 3% hydrogen peroxide for 10 minutes. A non-serum blocking reagent (Background Punisher, Biocare Medical) was then used to block non-specific protein binding. The Ki-67 antibody (Biocare Medical) was diluted 1:200 and incubated for 50 minutes and then a polymer detection reagent (Mach 2 HRP Polymer, Biocare Medical) was applied for 25 minutes. The sites of antigen-antibody interaction were visualized by incubating slides with a DAB chromogen (ImmPACT DAB substrate kit, peroxidase, Vector Laboratories, Burlingame, CA) for 5 minutes. Mayer's hematoxylin was used to counterstain the sections. The slides were then dehydrated in 100% alcohol and cleared with xylene. The sections were coverslipped with a permanent mounting medium (Permount Mounting Medium, Electron Microscopy Sciences, Hatfield, PA). IHC images for Ki-67 staining were captured on a Zeiss Axio Imager.M2 motorized microscope using a 20x/0.8 NA PlanApo objective lens (Carl Zeiss Microscopy, LLC, Thornwood, NY).

Live Cell Imaging:

For imaging of live RMS cells following treatment, cells were grown on 2-well Nunc™ Lab-Tek™ II Chambered Coverglass slides with a No. 1.5 borosilicate coverglass and imaged using a motorized Zeiss Axiovert 200 MOT with a 20X 0.8 NA objective lens and DIC optics, a Roper Scientific Photometrics CoolSnap HQ Microscope Camera and incubator providing temperature and CO_2 control.

Animal studies:

All the protocols for the animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Three to four weeks old female athymic nude mice (3 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA)

and were housed in the Lab Animal Care Center, Texas A&M University. Mice were allowed to acclimatize for a week and fed the standard chow diet. Two million Rh30 cells suspended in 100 µl of 1:1 matrigel and PBS solution were injected in each flank of the mouse subcutaneously. When the tumor size was palpable (~50 to 100 mm^3 in size), the mice were randomly divided into control and treatment groups. Each mouse in the control group was administered 100 µl of DMSO: corn oil (1:4) solution by intraperitoneal injection every day. Each mouse in the treatment group was injected with 100 µl of 50 mg/kg flavonoid prepared in DMSO: corn oil (1:4) solution by intraperitoneal injection every day. The mice were weighed and a Vernier Caliper was used to calculate their tumor volume (V = L*W*W/2 mm^3) every week. After the third week of drug administration, mice were sacrificed and tumors were removed and weighed. A small piece of tumor was homogenized in the lysis buffer and its extract was used for western blot analysis.

Statistical analysis:

The statistical significance of differences between the treatment groups was determined by Student's t-test. Each assay was performed in triplicate and the results were presented as means with error bars representing 95% confidence intervals. Data with a *P* value of less than 0.05 were considered statistically significant.

RESULTS

NR4A1 binding and transactivation induced by flavonoids

The histone methyltransferase (EHMT2) G9a is an NR4A1-regulated gene in RMS (288) and the observation that kaempferol decreased expression of G9a in gastric cancer cells (287) suggested that kaempferol may be an NR4A1 ligand acting as an antagonist. In this study we used kaempferol and the flavonoid quercetin (Figure 18A) and examined their activity as NR4A1

ligands in Rh30 and Rh41 RMS cells. Incubation of kaempferol and structurally related quercetin with the ligand binding domain (LBD) of NR4A1 resulted a concentration-dependent quenching of the fluorescence of Trp (186) in the LBD of NR4A1 with K_D values of 3.1 and 0.93 μM respectively (Fig. 18B). Kaempferol and quercetin also displaced the fluorescent probe bis-ANS in a competitive binding assay (289) with K_i values of 0.77 and 0.23 μM respectively (Fig. 18C). Kaempferol and quercetin also decreased transactivation in Rh30 and Rh41 cells transfected with the Gal4-NR4A1 chimera and a reporter gene (UAS-luc) containing 5 tandem yeast Gal4 response elements linked to a luciferase reporter gene (Fig. 18D). In addition, kaempferol and quercetin decreased transactivation in Rh30 and Rh41 cells transfected with an NBRE-luc reporter plasmid containing 3 tandem NBRE sites that bind NR4A1 monomer (Fig. 18E). Thus, like the CDIM/NR4A1 antagonists both kaempferol and quercetin directly bound NR4A1 and antagonized NR4A1-dependent transactivation in Rh30 cells.

Inhibition of RMS cell growth, survival, migration and invasion by flavonoids

Previous studies show that NR4A1 regulates ARMS cells growth, survival and invasion, and related genes including the PAX3-FOXO1 fusion oncogene and G9a (79, 282) and we therefore further investigated kaempferol and quercetin as inhibitors of these NR4A1-dependent pathways/genes. Treatment of Rh30 cells with 10-100 μM kaempferol and quercetin decreased growth (Fig. 19A) and similar effects were observed in Rh41 ARMS cells (Fig. 19B). Treatment of Rh30 cells with 25 or 50 μM kaempferol and quercetin significantly induced markers of apoptosis including cleavage of PARP and caspase 3 in Rh30 (Fig. 19C) and Rh41 (Fig. 19D) cells; these compounds also significantly induced Annexin V staining in Rh30 (Fig. 19E) and Rh41

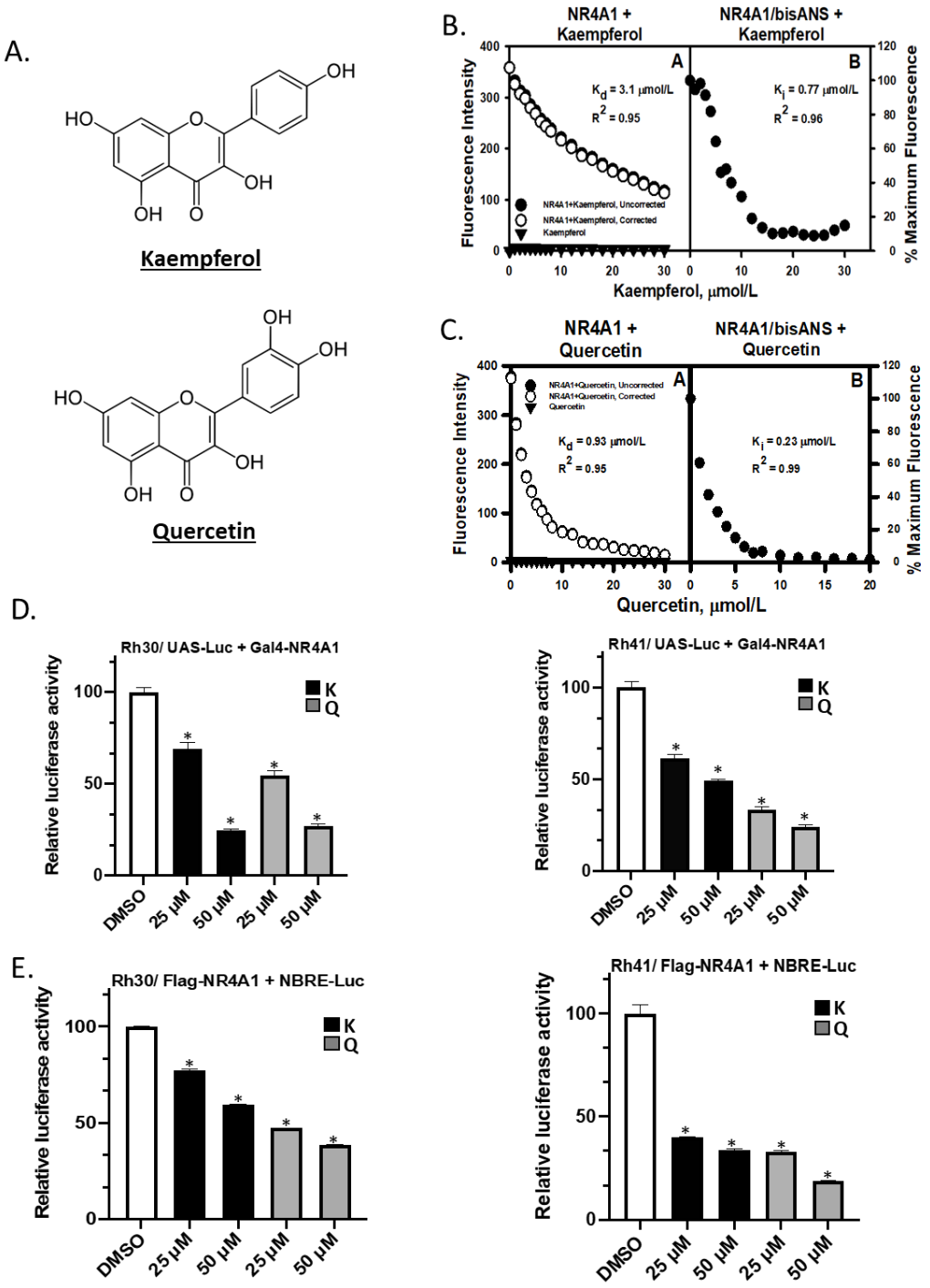

Figure 18. Kaempferol and quercetin bind NR4A1 and inhibit NR4A1-dependent transactivation. A. Structures of kaempferol and quercetin. Different concentrations of kaempferol (B) and quercetin (C) were incubated with NR4A1 (LBD) and binding was determined in fluorescent quenching direct binding or a competitive displacement (of bis-ANS) assay as outlined in the Methods. Rh30 and Rh41 cells were transfected with (D) UAS-luc/Gal4-NR4A1 or (E) an NBRE-luc/flag-NR4A1 constructs and after treatment with kaempferol (K) or quercetin (Q) luciferase activity was determined as outlined in the Methods. Results are expressed as means ± SD for at least 3 replicated determinations and significant ($p<0.05$) inhibition is indicated (*).

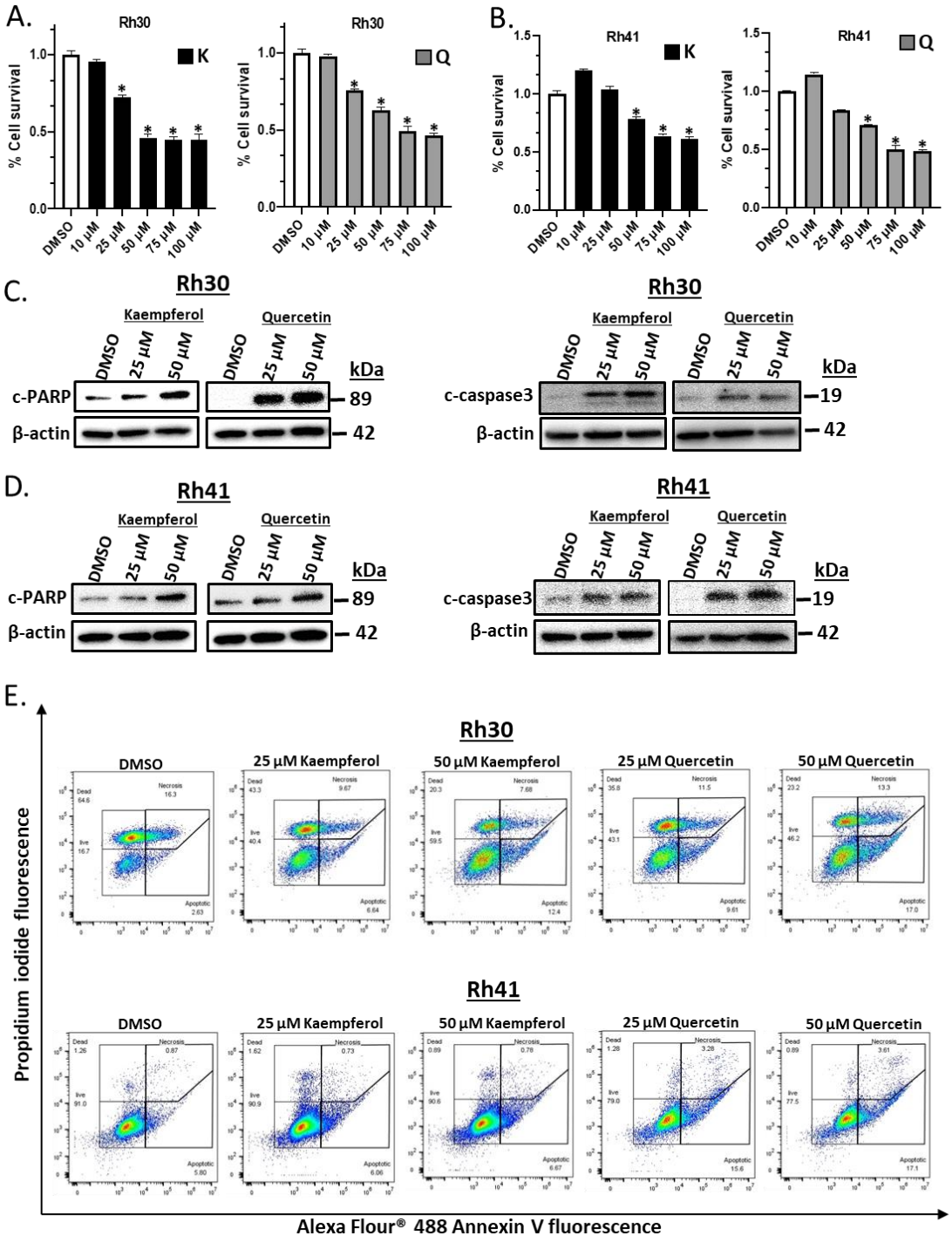

Figure 19. Kaempferol and quercetin inhibit growth and survival of RMS cells. Rh30 (A) and Rh41 (B) cells were treated with different concentrations of kaempferol (K) and quercetin (Q) and cell survival was determined as outlined in the Methods. Rh30 (C) and Rh41 (D) cells were treated with kaempferol and quercetin and whole cell lysates were analyzed by western blot analysis as outlined in the Methods. E. Cells were treated with kaempferol or quercetin and Annexin V staining was determined as outlined in the Methods. Results (A, B) are means ± SD for at least 3 determinations and significantly ($p<0.05$) decreased growth is indicated (*).

(Fig. 19F) cells. NR4A1 knockdown or treatment with NR4A1 antagonists also inhibits RMS cell migration and invasion (79, 282) and kaempferol and quercetin inhibit migration of Rh30 (Fig. 20A) and Rh41 (Fig. 20B) cells in a scratch assay (quantification in Supplemental Fig. 7A). Both flavonoid compounds also inhibited invasion of Rh30 (Fig. 20C) and Rh41 (Fig. 20D) cells in a Boyden Chamber assay and using Rh41 cells as a model 25 and 50 μM kaempferol and quercetin inhibited invasion in a 3-D spheroid invasion model (Fig. 20E). Rh30 cells do not form 3D spheroids. Thus, like CDIM/NR4A1 antagonists, kaempferol and quercetin inhibited RMS cell growth, survival, migration and invasion.

Inhibition of NR4A1-regulated genes by flavonoids

The histone methyltransferase G9a (EHMT2) and the PAX3-FOXO1 fusion oncogene are regulated by NR4A1 in ARMS cells (79, 288) and treatment of Rh30 cells with 20 and 50 μM kaempferol and quercetin decreased expression of G9a and PAX3-FOXO1 gene products (Fig. 21A). Similar results were observed in Rh41 cells (Fig. 21B). Kaempferol and quercetin also decreased expression of G9a and PAX3-FOXO1 mRNA levels in Rh30 (Fig. 21C) and Rh41 (Fig. 21D) cells demonstrating that both flavonoids antagonized NR4A1-dependent gene expression in RMS cells. Both the G9a and PAX3-FOXO1 promoters contain GC-rich Sp binding sites and are regulated by NR4A1/Sp where NR4A1 acts as a cofactor (79, 282). Results in Figures 21E and 21F demonstrate association of NR4A1 and Sp1 with the G9a and NR4A1 and Sp4 with the PAX3-FOXO1 promoters and treatment with either kaempferol or quercetin did not significantly increase or decrease NR4A1 or Sp association with the G9a and PAX3-FOXO1 promoters.

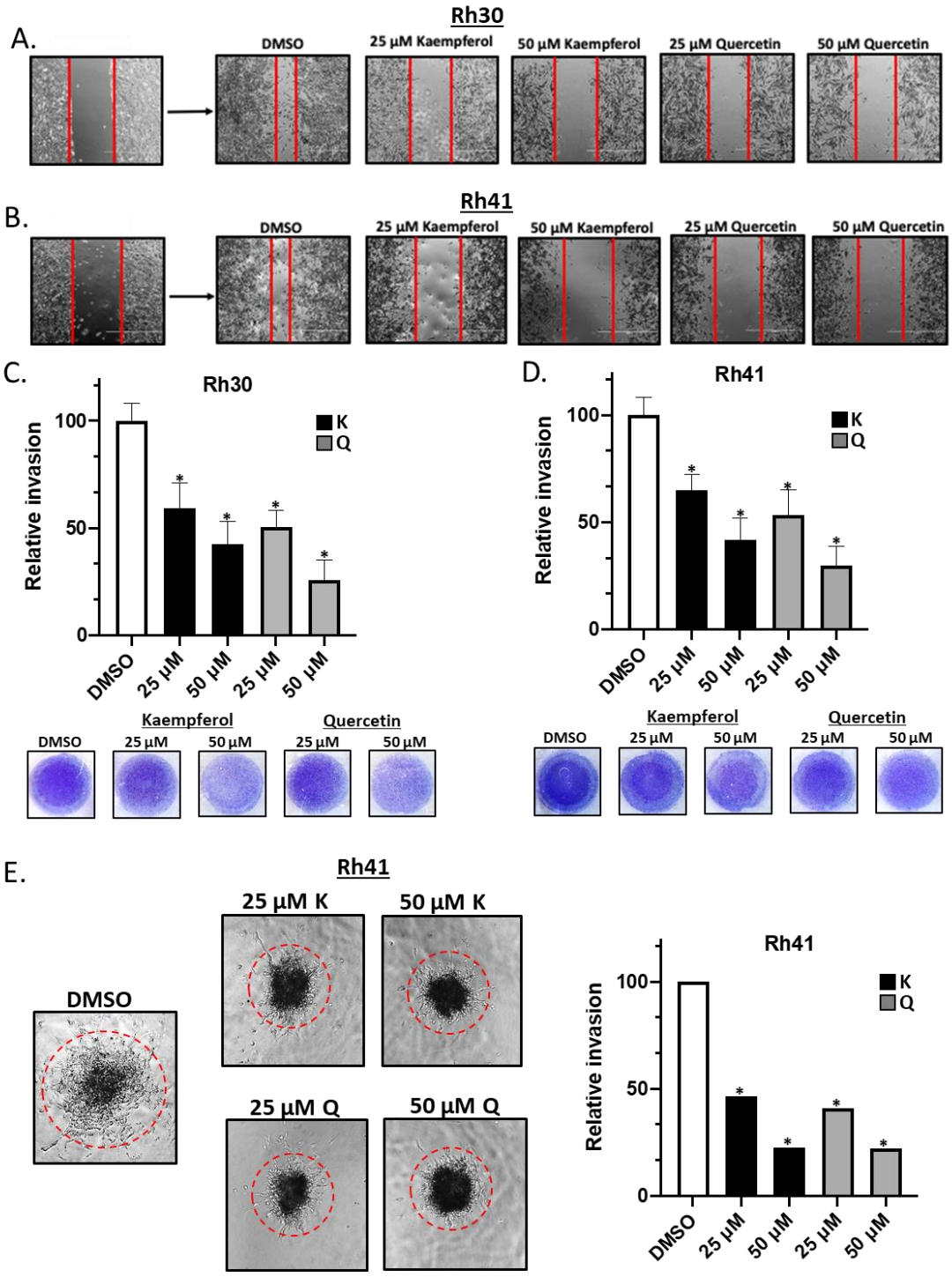

Figure 20. Kaempferol and quercetin inhibit RMS cell migration and invasion. Rh30 (A) and Rh41 (B) cells were treated with DMSO, kaempferol (K) or quercetin (Q) and effects on cell migration were determined in a scratch assay as outlined in the Methods. C. Invasion of Rh30 (C) and Rh41 (D) cells and effects of kaempferol and quercetin were determined in a Boyden chamber invasion assay and results were quantified. E. Rh41 cells were grown as spheroids and effects of kaempferol and quercetin on spheroid cell invasion were determined as outlined in the Methods. Results are expressed as means ± SD for at least 3 determinations and significant ($p<0.05$) inhibition is indicated (*).

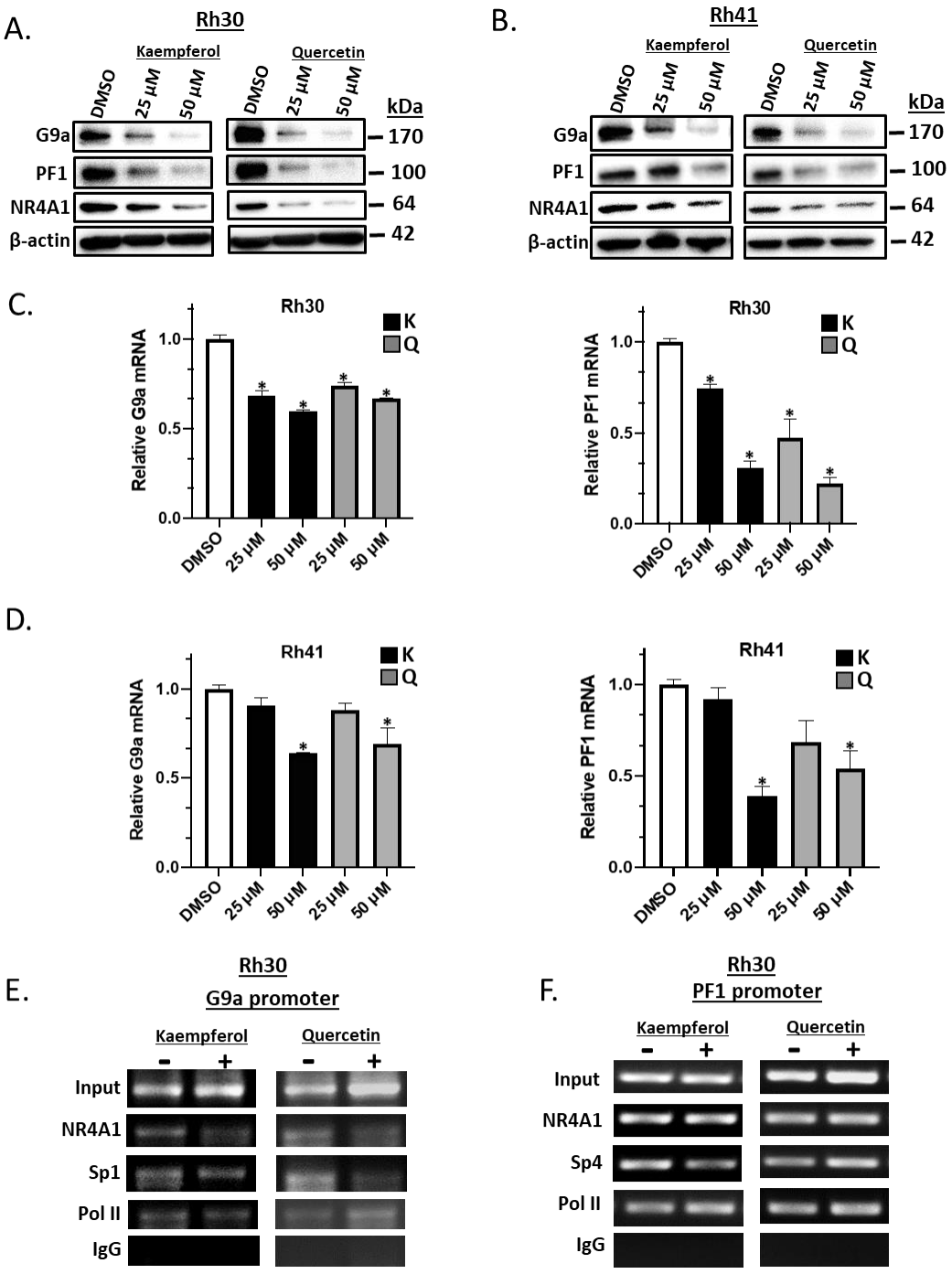

Figure 21. Kaempferol and quercetin downregulate G9a and PAX3-FOXO1 in RMS cells. Rh30 (A) and Rh41 (B) cells were treated with kaempferol or quercetin and whole cell lysates were analyzed by western blots as outlined in the Methods. Rh30 (C) and Rh41 (D) cells were treated with kaempferol or quercetin and G9a and PAX3-FOXO1 mRNA levels were determined by real time PCR as outlined in the Methods. E. Rh30 cells were treated with 50 μM kaempferol and quercetin for 24 hours and analyzed in a ChIP assay and the PAX3-FOXO1 gene (F) was also normalized to IgG using the appropriate primers and RT-qPCR as outlined in the Methods. Results (C and D) are means ± SD for at least 3 determinations and significant ($p<0.05$) inhibition is indicated (*).

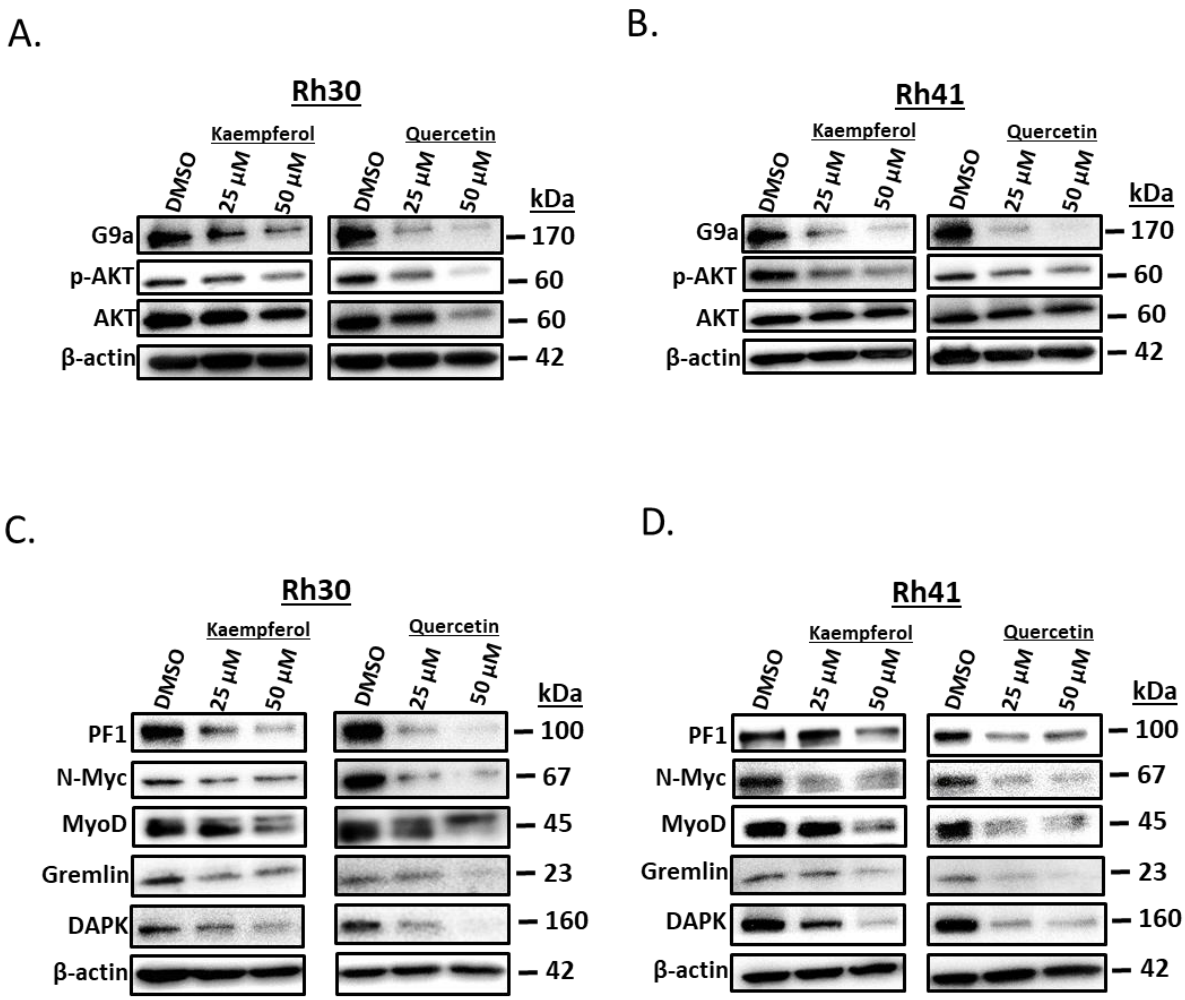

Figure 22. Kaempferol and quercetin inhibit expression of G9a- and PAX3-FOXO1-regulated gene products. Rh30 (A) and Rh41 (B) cells were treated with kaempferol or quercetin and whole cell lysates were analyzed by western blot analysis as outlined in the Methods. A similar protocol was used to determine expression of PAX3-FOXO1 downstream gene products in Rh30 (C) and Rh41 (D) cells treated with kaempferol or quercetin.

The histone methyltransferase gene regulates Akt phosphorylation in RMS cells (100) and NR4A1 knockdown or treatment with NR4A1 antagonists decreased G9a expression and this resulted in decreased Akt phosphorylation (pAkt). Results illustrated in Figures 22A and 22B show that similar effects are observed for kaempferol and quercetin, in Rh30 and Rh41 cells respectively. Kaempferol and quercetin also downregulate PAX3-FOXO1 and PAX3-FOXO1 regulated gene products (N-MYc, MyoD, Gremlin and DAPK) in Rh30 (Fig. 22C) and Rh41 (Fig. 22D) cells, and

these responses were also previously observed after NR4A1 knockdown or inhibition (79) demonstrating the activity of kaempferol and both kaempferol and quercetin as NR4A1 antagonists. NR4A1 also regulates mTOR signaling in RMS and other cancer cell lines (285) and NR4A1 knockdown or antagonists inhibit mTOR through reactive oxygen species-dependent activation of AMPK (i.e.: pAMPK) (160, 172, 190, 261) and, both kaempferol and quercetin induced pAMPK in Rh30 (Fig. 23A) and Rh41 (Fig. 23B) cells and this was accompanied by decreased phosphorylated mTOR and the downstream kinase p70S6K. NR4A1 also regulates gene products associated with attachment and migration (77, 79, 282-284) and treatment of Rh30 (Fig. 23C) or Rh41 (Fig. 23D) cells with kaempferol or quercetin inhibits expression of these gene products. Moreover, image analysis of Rh30 and Rh41 cells after treatment with kaempferol, quercetin or after knockdown of NR4A1 (siRNA) resulted in some changes in cell morphology and decreased cell attachment (Fig. 23E).

Kaempferol and quercetin inhibit RMS tumor growth in vivo

The in vivo anticancer activity of the flavonoids quercetin and kaempferol was investigated in athymic nude mice bearing Rh30 cells as xenografts where cells were injected into the flanking region of mice. At a dose of 50 mg/kg/d, both flavonoids inhibited tumor growth (Fig. 24A) but did not affect body weights (Fig. 24B) over the 3-week treatment period. At sacrifice, tumor weights were decreased (Fig. 24C) and analysis of tumor lysates showed the expression of PAX3-FOXO1 and G9a proteins were decreased (Fig. 24D) and Ki67 staining was also decreased in tumors from mice treated with quercetin and kaempferol (Fig. 24E). The complementary in vitro and in vivo studies indicate that kaempferol and quercetin are NR4A1 antagonists that are highly effective against NR4A1-dependent pro-oncogenic pathways/genes in RMS. These results suggest

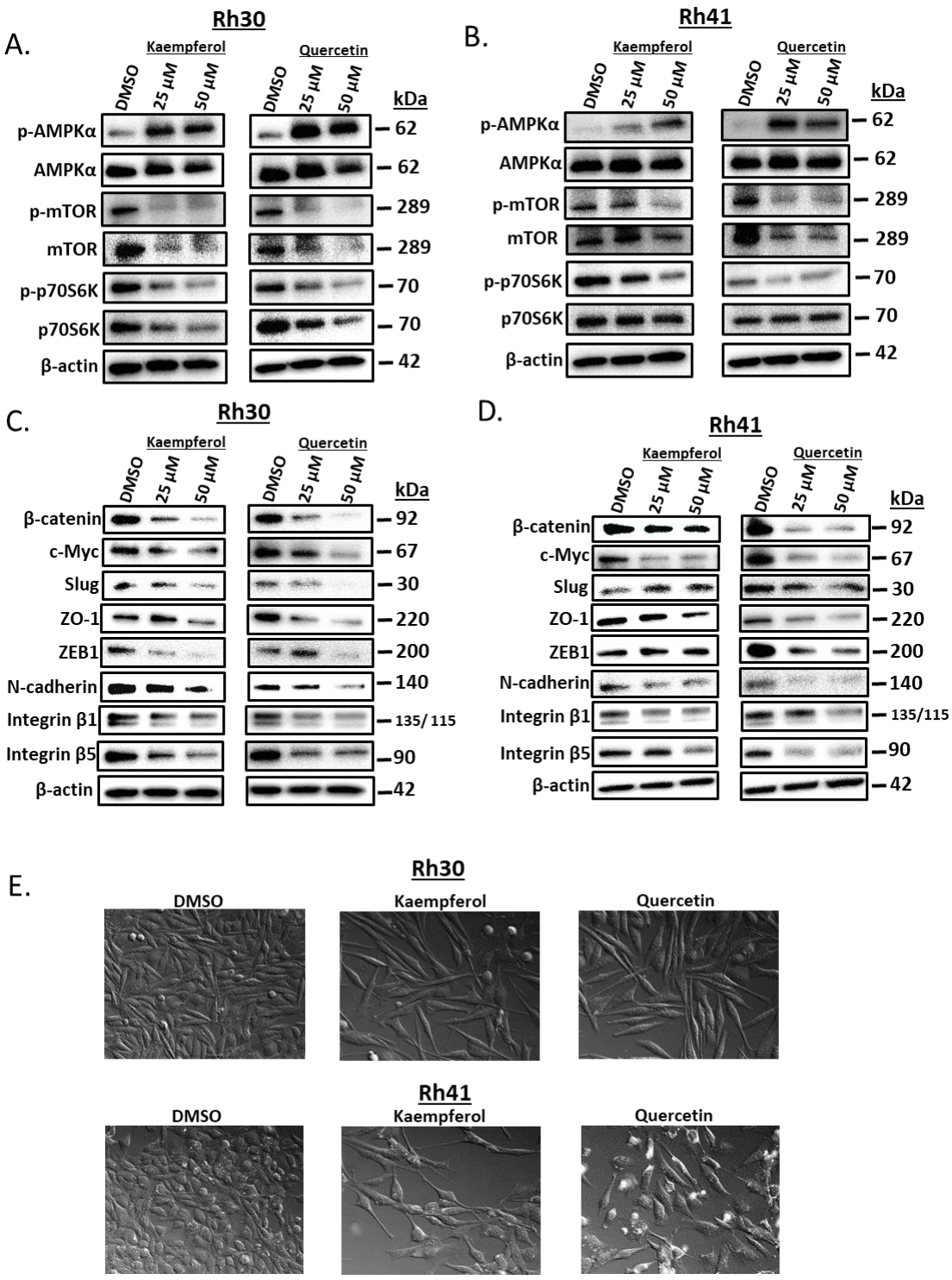

Figure 23. Kaempferol and quercetin act as mTOR inhibitors and induce cell detachment in RMS cells. Rh30 (A) and Rh41 (B) cells were treated with kaempferol or quercetin for 24 hours and whole cell lysates were analyzed by western blots. A comparable protocol was used to determine effects of kaempferol and quercetin on EMT marker gene products in Rh30 (C) and Rh41 (D) cells. E. Cells were treated with kaempferol and quercetin and also transfected with and examined by differential interference contrast imaging as outlined in the Methods.

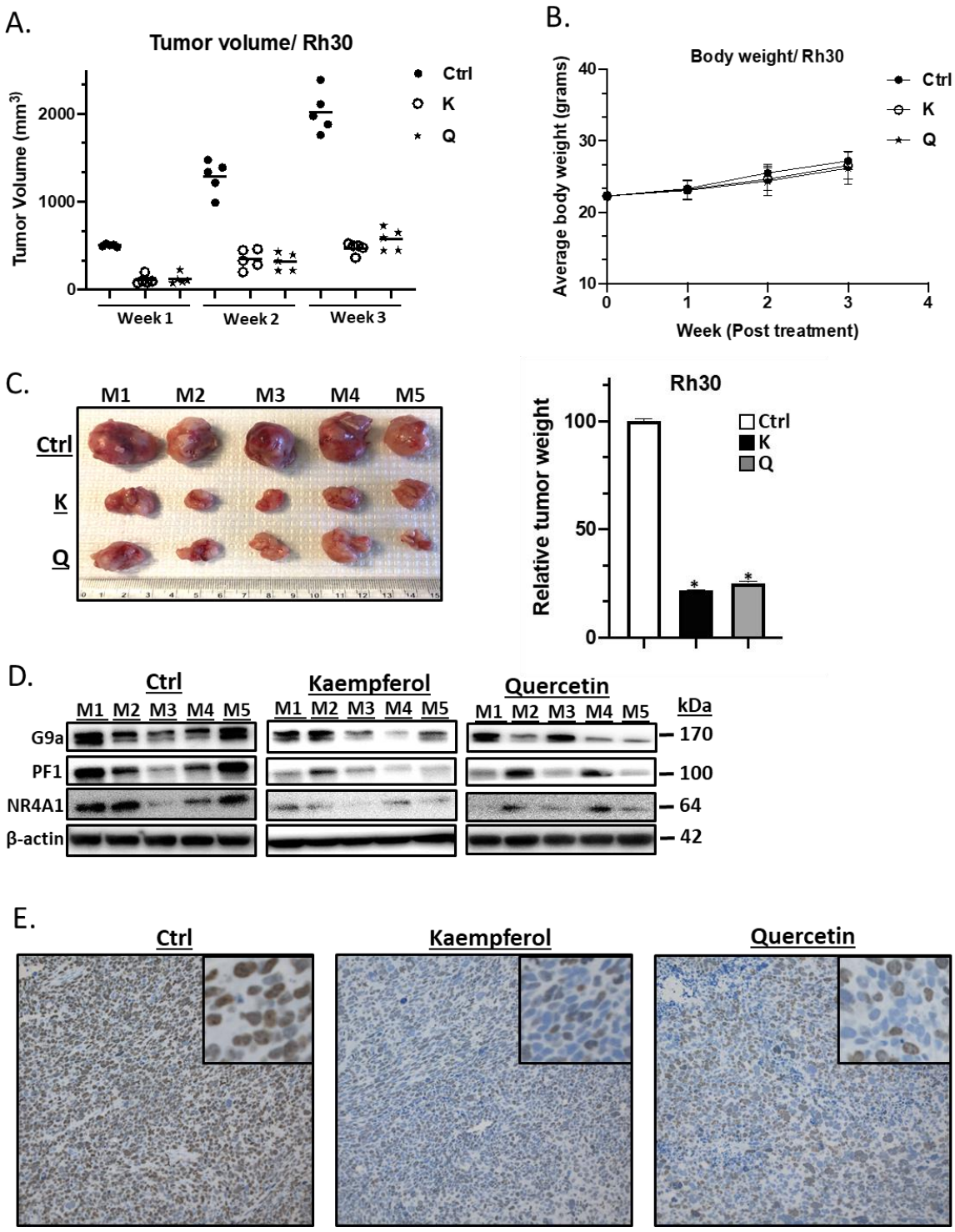

Figure 24. Kaempferol and quercetin inhibit RMS tumor growth. Rh30 cells were injected into flanks of Balb/c athymic nude mice that were treated with kaempferol or quercetin (50 mg/kg/d) by intraperitoneal injection and effects on (A) cell growth, (B) body weight changes and (C) tumor weights were determined. D. Tumor lysates were analyzed by western blots for changes in gene expression (relative to the solvent control). E. Ki67 staining in control and treated tumor sections was determined as outlined in the Methods. Significant ($p<0.05$) flavonoid-induced effects are indicated (*).

that NR4A1-active flavonoids can be repurposed from their broad nutraceutical applications for use as targeted agents for clinical treatment of RMS patients with tumors expressing NR4A1.

DISCUSSION

NR4A1 is a nuclear orphan receptor with no known endogenous ligands and there is increasing evidence that this receptor and other members of this family (NR4A2 and NR4A3) play an important role in maintaining cellular homeostasis and in pathophysiology (124, 152, 285). NR4A are typically induced by cellular stressors and in many diseases, including solid tumors where NR4A1 or other NR4A members are elevated and are potential drug targets. The role of NR4A in cancer is somewhat paradoxical (159); in many blood-derived tumors NR4A is a tumor suppressor and levels are low. Therefore, agents that induce NR4A1 and its nuclear export are potential therapeutics since the extranuclear receptor can form a proapoptotic NR4A1-Bcl-2 complex. In contrast, nuclear NR4A1 is pro-oncogenic in solid tumors and regulates cell growth, survival, migration/invasion and related genes (77, 79, 160, 172, 186, 190, 261, 282-285). Studies on the NR4A1 antagonist activities of CDIMs demonstrate that treatment of colon, lung, breast, pancreatic, kidney, RMS, endometrial cancer cells with CDIM/NR4A1 antagonist inhibited the pro-oncogenic NR4A1-regulated functional responses (rev in (159)). Moreover, the effects observed after treatment with CDIMs were comparable to those observed after NR4A1 knockdown.

RMS is a cancer primarily diagnosed in adolescents and accounts for 5% of all pediatric cancers and 50% of soft tissue sarcomas in children with an overall incident rate of 4.5×10^6 (55, 290, 291). Embryonal RMS (ERMS) and alveolar RMS (ARMS) are the two major classes of RMS in children and adolescents and differ with respect to their histology, genetics, treatment, and

prognosis (292, 293). ERMS accounts for over 60% of RMS patients and is associated with loss of heterozygosity at the 11p15 locus (292). ERMS patients have a favorable initial prognosis; however, the overall survival of patients with metastatic ERMS is only 40% (293). ARMS occur in approximately 20% of RMS patients and is associated with translocations from the fusion of *PAX3* or *PAX7* with the Forkhead gene *FOXO1* resulting in formation of pro-oncogenic gene products (294, 295). ARMS patients have a poor prognosis and patient survival is low for metastatic ARMS (296). Treatments include radiotherapy, surgery, and chemotherapy with cytotoxic drugs and/or drug combinations; RMS patients that survive current cytotoxic drug therapies have a > 95% increased risk for several diseases as adults ≥ 45 years of age (58). Thus, there is a critical need for development of new therapeutic regimens for treating childhood RMS and for developing innovative therapies for treating ARMS patients since the current cytotoxic drug therapies have limited effectiveness. Our previous research has identified NR4A1 as a new drug target for treating RMS. NR4A1 is overexpressed in RMS and correlates with expression of PAX3-FOXO1 in ARMS patients and treatment with synthetic CDIMs that are NR4A1 antagonists are highly effective in both cell culture and in vivo studies. The efficacy of NR4A1 antagonists is due, in part to their suppression of NR4A1-regulated mTOR signaling, PAX3-FOXO1, β1-integrin and downstream gene products and the histone methyltransferase G9a (79, 288). The origins of this study were based on a recent report showing that the flavonoid kaempferol downregulated G9a in gastric cancer cells (287) and we hypothesized that kaempferol and possible other flavonoids may be NR4A1 ligands that act as receptor antagonists.

Results in Figure 18 confirm that kaempferol and quercetin directly bind NR4A1 and competitively displace a fluorescent bound ligand (bis-ANS) and they also inhibit NR4A1-dependent transactivation. These results coupled with the effects of kaempferol and quercetin on

cell growth, survival, migration and invasion (Figs. 18-20) are also observed in RMS cells after NR4A1 knockdown or treatment with CDIM/NR4A1 antagonists (77, 79, 172, 282-285).

PAX3-FOXO1 and G9a are genes that play pro-oncogenic roles in RMS (100, 286) and these genes are regulated by NR4A1 which acts as a co-factor to enhance Sp1- or Sp4- mediated gene expression through NR4A1/Sp1/4 binding GC-rich promoter elements (77, 79, 282-284). This mechanism of NR4A1/Sp gene regulation is not uncommon and is observed for many other nuclear receptors (297). Both kaempferol and quercetin decrease expression of PAX3-FOXO1 and G9a mRNA and proteins and downstream gene products (Fig. 21-22). Similar results were observed for activation of pAMPK and inhibition of mTOR signaling and for inhibition of genes associated with cell attachment/migration and accompanying morphological changes (Figure 23). We also observed that at doses of 50 mg/kg/d quercetin and kaempferol were potent inhibitors of RMS tumor growth in athymic nude mice bearing Rh30 cells injected into their flanking regions (Fig. 24). The complementary results of cell culture and in vivo studies demonstrate for the first time that kaempferol and quercetin are NR4A1 ligands that act as antagonists in RMS cells and mimic the effects of NR4A1 knockdown by RNA interference (77, 79, 282-284). These results suggest that NR4A1-active flavonoids can be repurposed for clinical applications in the treatment of RMS and possibly other cancers where NR4A1 is a potential drug target. This type of precision medicine/nutrition approach for using flavonoids would specifically target patients that overexpress NR4A1 and could be used clinically for increasing the efficacy and decreasing the dose of currently used cytotoxic therapies.

CHAPTER V

SUMMARY, CONCLUSIONS AND FUTURE STUDIES

NR4A1, NR4A2, and NR4A3 are members of orphan nuclear receptor 4A group of immediate early genes induced by several stressors and stimuli. NR4A receptors are important in maintaining cellular homeostasis and are involved in multiple diseases such as cardiovascular, neurological, inflammation and inflammatory diseases, immune-related diseases, and cancer. In cancer, the role of NR4As is paradoxical. The expression levels of NR4As in blood-derived cancers such as leukemia and lymphoma are low, where NR4As exhibit tumor suppressor like activities. In contrast, NR4A1 is pro-oncogenic in solid tumors and is overexpressed in multiple solid tumors such as breast, lung, colon, pancreatic, cervical cancers, melanomas, and RMS. NR4A1 regulates cancer cell growth, survival, migration and invasion and related downstream genes. Consequently, overexpression of NR4A1 is associated with poor prognosis and decreased survival of lung, colon, breast and ovarian cancer patients. Previous studies have shown that NR4A1 regulates many crucial pro-oncogenic pathways in cancer cells and this includes regulation of pro-reductant genes such as TXNDC5 and IDH1, which maintain high reductant levels in cancer cells resulting in low levels of ROS-induced stress. NR4A1 also binds and inactivates p53 and further blocks activation of sestrin-2 and AMPKα, and this results in activation of mTOR signaling, facilitating cancer cell proliferation. NR4A1 is a transcription factor that binds NBRE and NuRE sequences on target gene promoters and regulates their transcription. In addition, NR4A1 also binds DNA-bound Sp proteins as a transcription co-factor and this NR4A1/Sp complex regulates expression of several important genes such as PAX3-FOXO1, integrins, and Bcl-2 in cancer cells. Although endogenous ligands for NR4A1 have not been identified, several compounds that bind NR4A1 as ligands have been discovered. Studies in this laboratory have

identified several bis-indole derived CDIM molecules that bind NR4A1-LBD and these CDIM molecules have been characterized as NR4A1 antagonists in cancer cells. Studies in this laboratory have also shown that CDIM molecules inhibited NR4A1-regulated pro-oncogenic functional responses in multiple cancer cell lines and mimic effects of NR4A1 knockdown by RNAi.

RMS is the most common sarcoma in children that accounts for 50% of all sarcomas in children and 5% of all pediatric cancers. Based on genetics, histology, prognosis, and treatment, RMS is divided into two major subtypes; ERMS and ARMS. ERMS occurs in 60% of RMS patients and ERMS patients have favorable initial prognosis, however, the survival rate for patients with metastatic ERMS is only 40%. In contrast, ARMS accounts for 20% of RMS patients and is more aggressive. In ARMS, chromosomal translocation results in the fusion of PAX3 or PAX7 gene with FOXO1 that forms fusion oncogenes PAX3-FOXO1 or PAX7-FOXO1. PAX3-FOXO1 is the major driver of aggressiveness in ARMS patients. Treatment of RMS tumor includes combination of surgery, radiation and chemotherapy however, drugs used for RMS treatment are cytotoxic with significant long term adverse health effects and therefore it is important to develop new agents for RMS treatment.

Previous studies in this laboratory have identified NR4A1 as a novel drug target in RMS treatment. Recently, it was shown that NR4A1 plays an important role in TGFβ-induced invasion in lung and breast cancer cells. TGFβ induced phosphorylation of NR4A1 and triggered NR4A1-nuclear export. This receptor then formed a complex with Axin2, Arkadia and RNF12, which induced proteasome-dependent degradation of inhibitory SMAD7. NR4A1 antagonist CDIM8 inhibited TGFβ-induced lung and breast cancer cell invasion by inhibiting nuclear export of NR4A1, thereby inhibiting the proteasome-dependent degradation of SMAD7. Our results in RMS cells showed that TGFβ also induced invasion in ERMS but not ARMS cells and the NR4A1

antagonist CDIM8 inhibited basal as well as TGFβ-induced ERMS cell invasion. However, the mechanism that was observed in ERMS was different compared to lung and breast cancer cells since NR4A1 was primarily cytosolic in RD and SMS-CTR ERMS cells and CDIM8 did not change the intracellular localization of NR4A1 in ERMS cells. In addition, CDIM8 did not inhibit SMAD7 degradation and had no effects on TGFβ-induced activation of regulatory-SMADs (SMAD3 or SMAD3) indicating that other pathways are involved in TGFβ-induced ERMS cell invasion. Our results showed that TGFβ-induced invasion in ERMS cells was β-catenin dependent and β-catenin was regulated by NR4A1. The NR4A1 antagonist CDIM8 inhibited NR4A1 and also inhibited β-catenin and suppressed TGFβ-induced ERMS cell invasion. Our study showed a novel mechanism of CDIM8-mediated inhibition of basal and TGFβ-induced ERMS cell invasion which was due to activation of the NR4A1-Bcl2 complex, mitochondrial disruption, induction of the tumor suppressor-like cytokine interleukin-24 (IL-24) which in turn downregulated β-catenin expression. Thus, the NR4A1 antagonist inhibited TGFβ-induced invasion of ERMS cells through initial targeting of cytosolic NR4A1. The mechanisms of cross talk between NR4A1 and growth factors/cytokines and effects of CDIM8 in ERMS cells are not well defined and will be investigated in future studies.

Previous studies in this lab have shown that NR4A1 regulates the fusion oncogene PAX3-FOXO1 in ARMS. NR4A1 binds DNA bound Sp4 protein and regulates PAX3-FOXO1 transcription. In addition, NR4A1 also regulates expression of PAX3-FOXO1-regulated downstream genes and their functions. PAX3-FOXO1 is an important oncogenic factor in ARMS and is also the primary reason for metastasis and recurrence in ARMS patients. Recently, a study also reported that G9a is overexpressed in PAX3-FOXO1 positive ARMS patients and displays pro-oncogenic activities in ARMS patients. Since NR4A1 and G9a regulate comparable pro-

oncogenic responses in ARMS, we hypothesized that NR4A1 may regulate G9a in ARMS. Our results showed that knockdown of NR4A1 or treatment of Rh30 and Rh41 ARMS cell lines with NR4A1 antagonists decreased G9a protein and mRNA expression. The prototypical NR4A1 antagonist CDIM8 and two buttressed 3,5-disubstituted phenyl analogs of CDIM8 (3,5-$(CH_3)_2$ and 3-Br-5-OCH_3) were used for this study. Treatment with NR4A1 antagonists or NR4A1 knockdown decreased G9a protein expression in a panel of NR4A1 expressing cell lines that included breast, lung, liver, endometrial, colon, and prostate cancers. Chromatin immunoprecipitation (ChIP) assay showed that NR4A1 and Sp1 are associated with a GC rich sequence on G9a promoter and regulates G9a transcription, which is decreased by both CDIM8 and the Sp1 inhibitor mithramycin. A study reported that in ARMS cells, G9a silences the tumor suppressor PTEN by catalyzing dimethylation of lysine 9 residue on histone 3 (H3K9me2) on PTEN promoter. G9a-mediated PTEN silencing induces Akt phosphorylation and activates PI3K pathway in ARMS. NR4A1 antagonists and mithramycin decreased Akt phosphorylation in Rh30 and Rh41 ARMS cells. In addition, ChIP assays showed that CDIM8 and mithramycin also decreased G9a-mediated H3K9me2 marks on PTEN promoter. G9a also induces ARMS tumor growth in vivo. Therefore, we investigated the effects of the NR4A1 antagonist 3-Br-5-OCH_3 as an inhibitor of G9a-mediated tumor growth in athymic nude mice bearing Rh30 cells as xenografts. Our results showed that the 3-Br-5-OCH_3 analog of CDIM8 inhibited ARMS tumor growth. In addition, the immunoblot and RT-PCR analysis showed decreased NR4A1 and G9a protein and mRNA expressions in tumors lysates extracted from mice treated with NR4A1 antagonist 3-Br-5-OCH_3. Therefore, our study showed that NR4A1 regulates G9a in ARMS cells. Furthermore, we have also characterized NR4A1 antagonist CDIM molecules as novel class of G9a inhibitors in this study.

A recent study reported that the flavonoid kaempferol induced apoptosis and inhibited G9a-mediated growth, proliferation, and mTOR activation in gastric cancer cells. The reported pattern of G9a inhibition by kaempferol was similar to what has been previously observed for NR4A1/G9a inhibition by NR4A1 antagonists CDIMs in RMS cells. Therefore, we hypothesized that kaempferol and quercetin (another natural flavonoid structurally related to kaempferol) are NR4A1 ligands. We further hypothesized that these flavonoids will inhibit NR4A1-regulated ARMS cell and tumor growth by inhibiting NR4A1 and NR4A1-regulated G9a and PAX3-FOXO1. Fluorescence quenching assays showed that kaempferol and quercetin bind NR4A1-LBD with K_d values of 3.1 µM and 0.93 µM respectively. Moreover, a luciferase reporter assay showed that kaempferol and quercetin decreased NR4A1-dependent transactivation in Rh30 and Rh41 ARMS cells, similar to what has been previously observed for NR4A1 antagonists. In addition, kaempferol and quercetin inhibited ARMS cell growth, survival, migration and invasion. Treatment of ARMS cells with kaempferol and quercetin also triggered cleavage of PARP and caspase 3 indicating that these flavonoids induce apoptosis in Rh30 and Rh41 ARMS cells. Furthermore, immunoblot and RT-PCR analysis showed that kaempferol and quercetin decreased expression of NR4A1-regulated G9a and PAX3-FOXO1 protein and mRNA in ARMS cells. Moreover, these flavonoids also inhibited ARMS tumor growth in athymic nude mice bearing Rh30 cells as xenografts. Our study showed that kaempferol and quercetin are NR4A1 ligands that inhibit NR4A1-regulated pro-oncogenic functions in ARMS. In addition, our results also demonstrate the clinical potential for repurposing these flavonoids for clinical applications as precision medicine for treating RMS patients that express NR4A1 in order to increase the efficacy and decrease dosages of currently used cytotoxic drugs. For future studies, experiments such as isothermal calorimetry, surface plasmon resonance, and circular dichroism will be performed to

further confirm the interaction of kaempferol and quercetin with NR4A1-LBD. Experiments such as ChIP-seq will also be performed to determine where flavonoids bind on NR4A1 promoter. Finally, this study also serves as a model to investigate if other flavonoids and natural compounds are NR4A1 ligands.

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APPENDIX A

SUPPLEMENTAL FIGURE 1

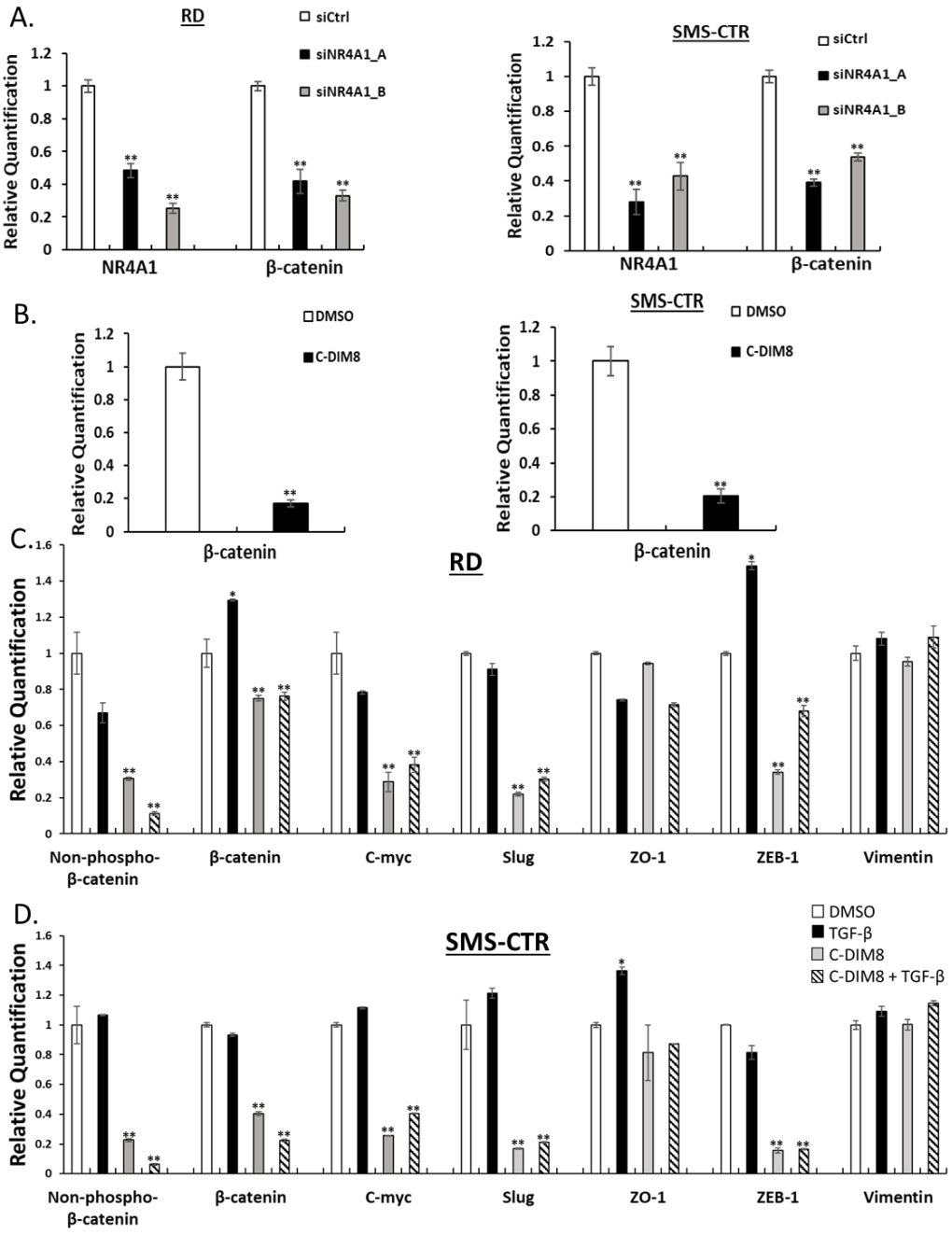


Figure S1. Quantification of protein levels in Figures 7A, 7B, 8A and 8B. Levels of (A) NR4A1 and β-catenin (From Figure 7A), (B) β-catenin (From Figure 7B) and (C, D) β-catenin and EMT markers (From Figure 8A and 8B) in RD and SMS-CTR cells after different treatment conditions as indicated were quantitated relative to control and normalized to β-actin. Results are means ± SD of at least three separate determinations and significant ($p<0.05$) induction (*) or inhibition (**) of an induced response are indicated.

SUPPLEMENTAL FIGURE 2

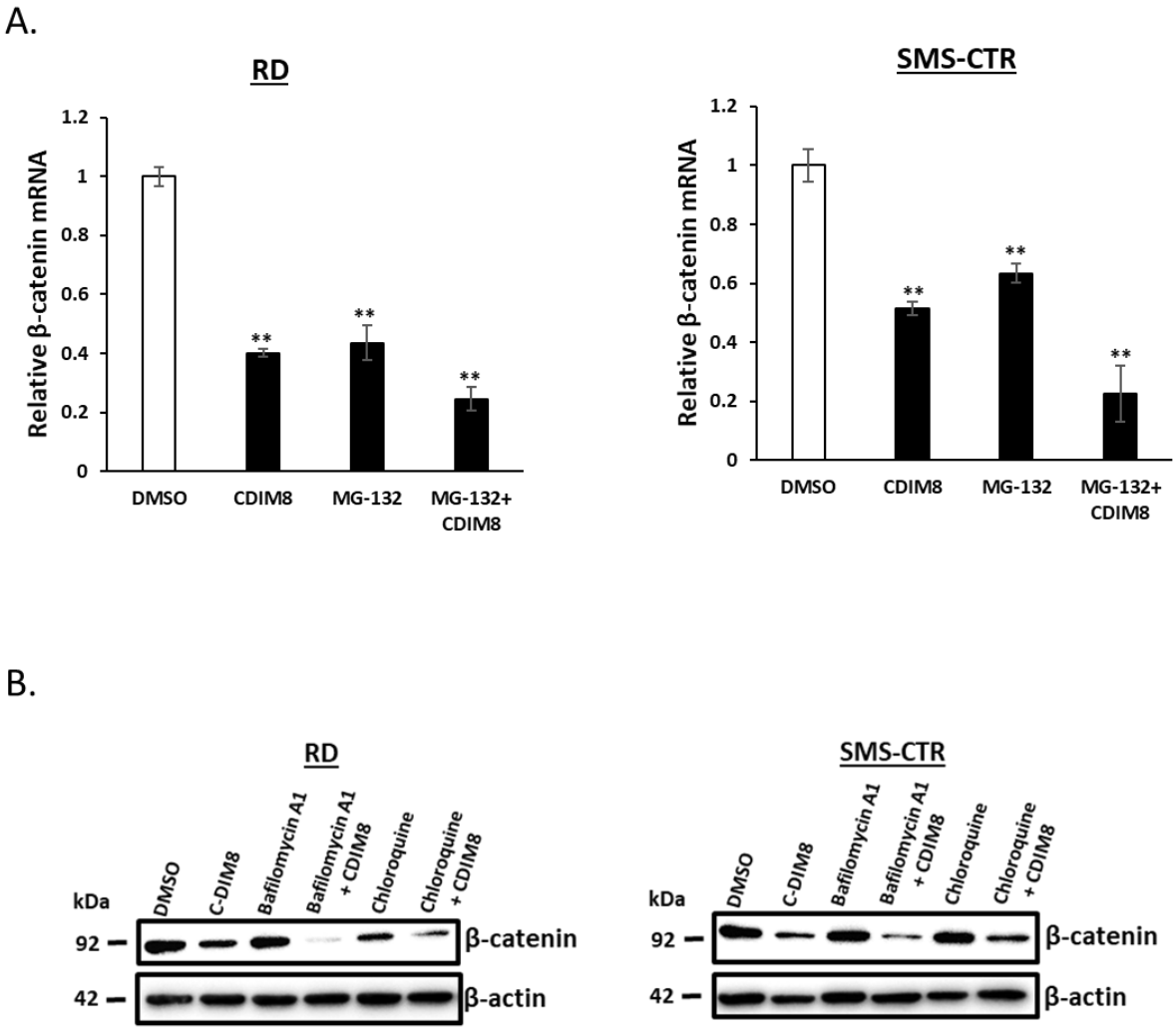


Figure S2. β-catenin downregulation by CDIM8 is proteasome and lysosome independent. A. RD and SMS-CTR were treated with DMSO, 20 μM CDIM8, 5 μM MG132 (3 hours) or their combination for 24 hours and the effect on β-catenin mRNA level was determined by real time PCR as outlined in the Methods. B. RD and SMS-CTR cells were treated with DMSO, 20 μM CDIM8, 1 μM Bafilomycin A1, 50 μM Chloroquine, or their combination for 24 hours and the effect on β-catenin protein level was determined by western blot as outlined in Methods. Results are expressed as means ± SD for at least 3 replicated determinations for each treatment group and significant ($p < 0.05$) decreased (**) response are indicated.

SUPPLEMENTAL FIGURE 3

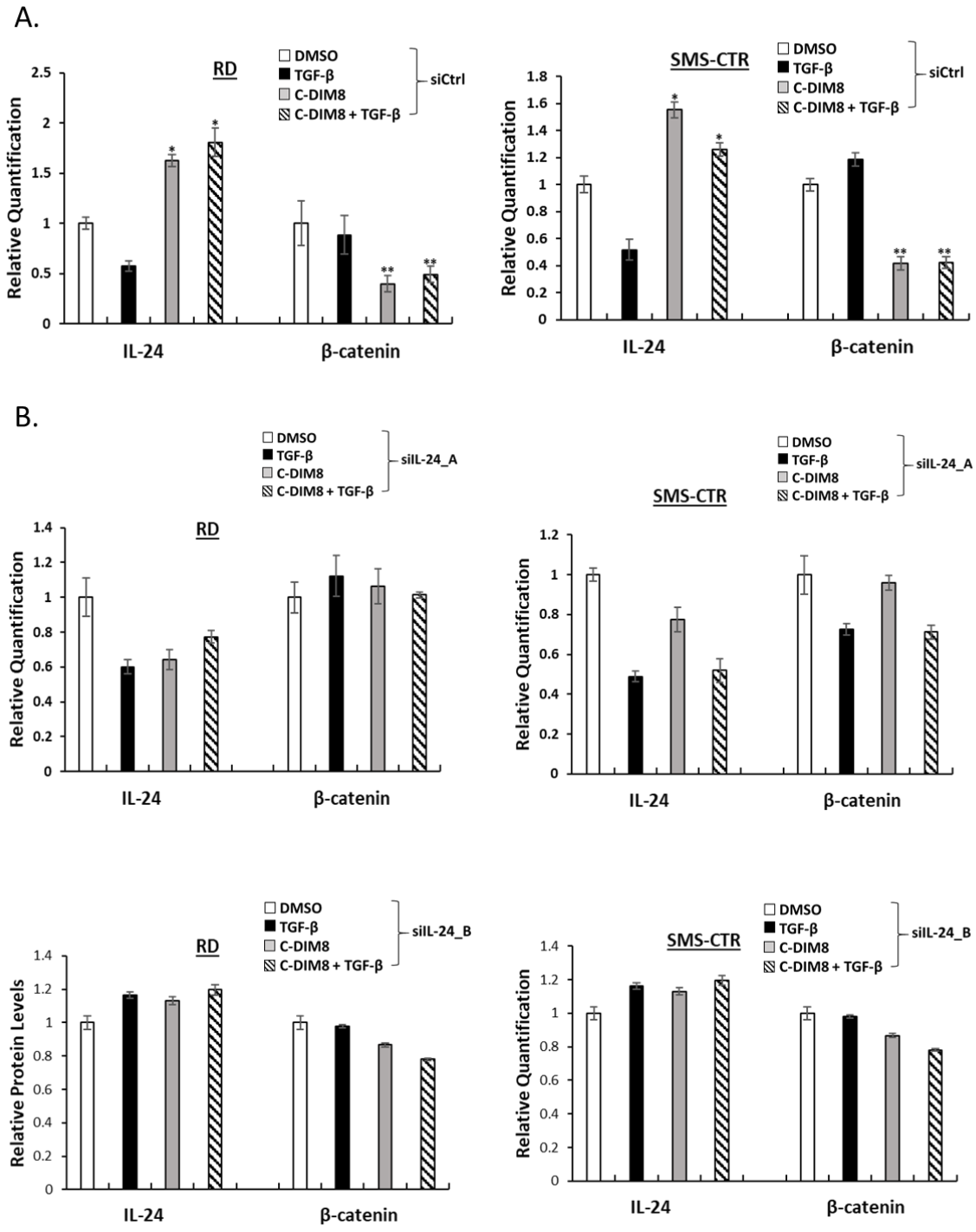


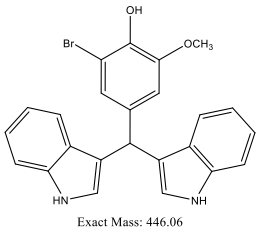
Figure S3. Quantification of protein levels in Figures 9C and 9D. Levels of IL-24 and β-catenin in RD and SMS-CTR cells transfected with (A) siCtrl (From Figure 9C) or (B) siIL-24 (From Figure 9D) were quantitated relative to control and normalized to β-actin. Results are means ± SD of at least three separate determinations and significant ($p<0.05$) induction (*) or inhibition (**) of the induced response are indicated.

SUPPLEMENTAL FIGURE 4

NMR RESULTS

^{1}H NMR spectra were measured on Bruker Advance spectrometer (400 MHz) spectrometers and are reported in ppm (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad; integration; coupling constant(s) in Hz), using TMS as an internal standard (TMS at 0.00 ppm) in $CDCl_3$. Liquid chromatogram mass spectra (LCMS) were obtained on SHIMADZU2010 EV using methanol as solvent.

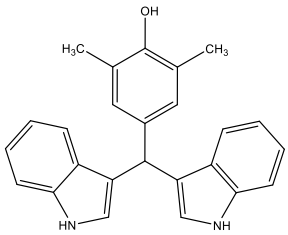
Sample 1: 3-Br-5-OCH_3



^{1}H NMR (400 MHz, DMSO): δ 10.78 (s, 2H), 9.14 (s, 1H), 7.35 (d, J = 8.1 Hz, 2H), 7.30 (d, J = 7.9 Hz, 2H), 7.04 (t, J = 7.7 Hz, 2H), 7.02 (d, J = 1.7 Hz, 1H), 6.96 (d, J = 1.5 Hz, 1H), 6.88 (t, J = 7.5 Hz, 2H), 6.84 (d, J = 1.9 Hz, 2H), 5.76 (s, 1H), 3.72 (s, 3H).

MS (LCMS): m/z 446.93 (M)+

Sample 2: 3, 5-$(CH_3)_2$



^{1}H NMR (400 MHz, DMSO): δ 10.71 (s, 2H), 7.89 (s, 1H), 7.37 (s, 2H), 7.33 (d, J = 8.1 Hz, 2H), 7.28 (d, J = 7.9 Hz, 2H), 7.02 (t, J = 7.2 Hz, 2H), 6.89 (s, 2H), 6.85 (t, J = 7.4 Hz, 2H), 6.77 (d, J = 1.8 Hz, 2H), 2.09 (s, 6H).

SUPPLEMENTAL FIGURE 5

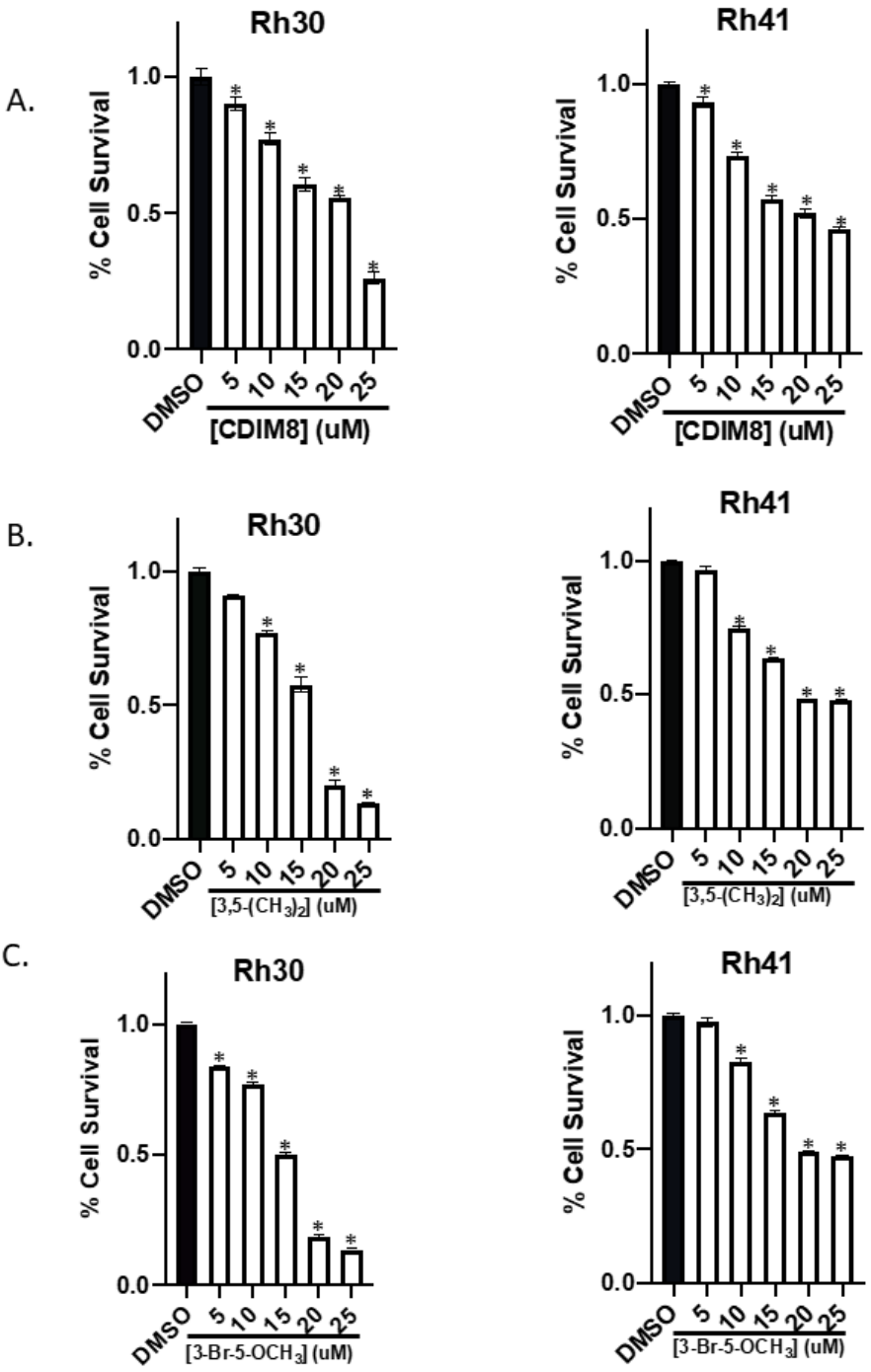


Figure S5. NR4A1 antagonists inhibit Rh30 and Rh41 cell growth. Cells were treated with CDIM8 (A), 3,5-$(CH_3)_2$ (B) and 3-Br-5-OCH_3 (C) and percent cell survival were determined as outlined in the Methods. Results are expressed as means ± SD for at least 3 replicates for each dose and significant ($p<0.05$) inhibition is indicated (*).

SUPPLEMENTAL FIGURE 6

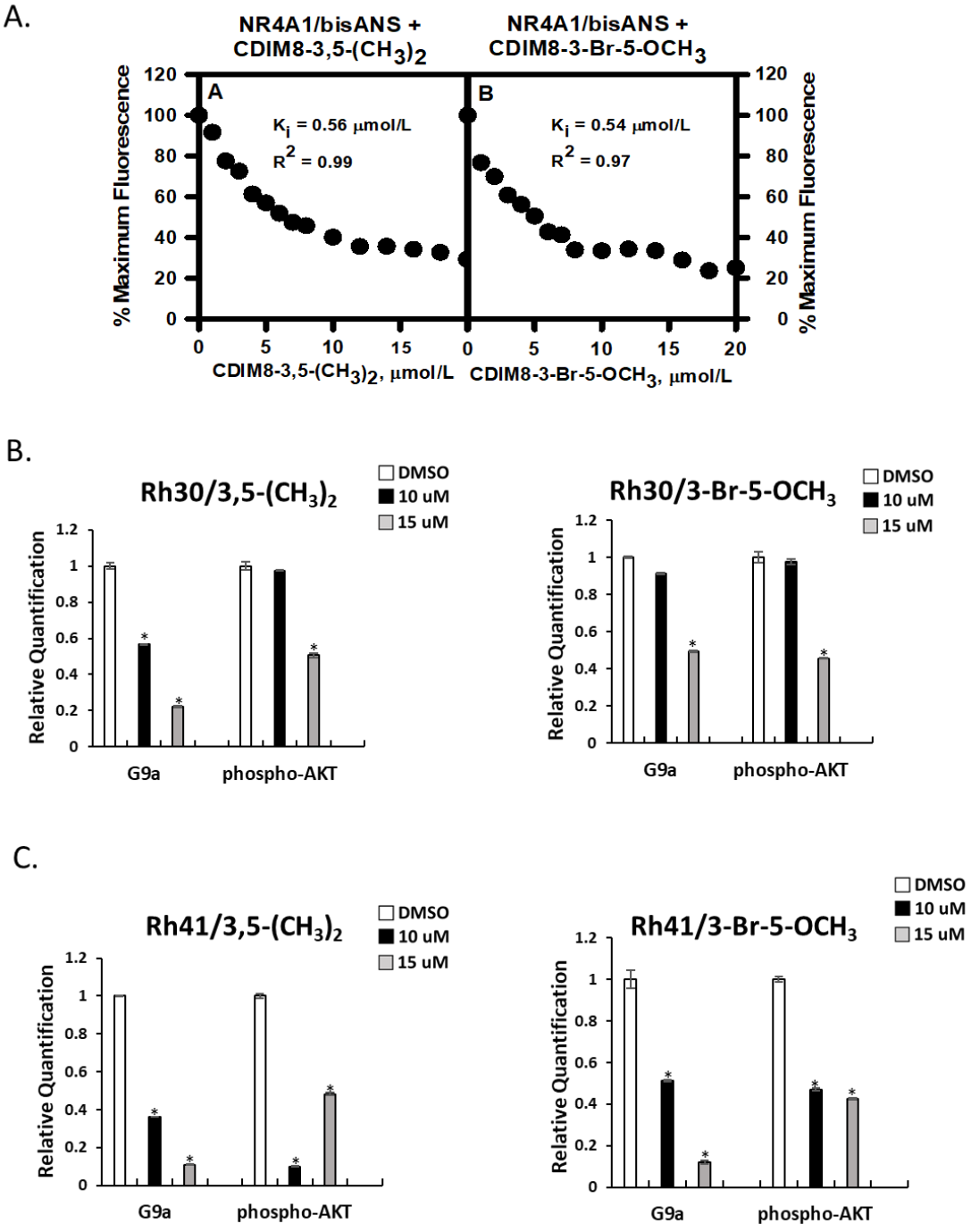


Figure S6. NR4A1 binding quantitation assays. A. Binding of 3,5-$(CH_3)_2$ and 3-Br-5-OCH_3 were determined by displacement of bisANS from NR4A1 (ligand binding domain) as outlined in the Methods. Results in Figures 16A and 16B were quantitated (B, C) and standardized relative to β-actin as outlined in the Methods.

SUPPLEMENTAL FIGURE 7

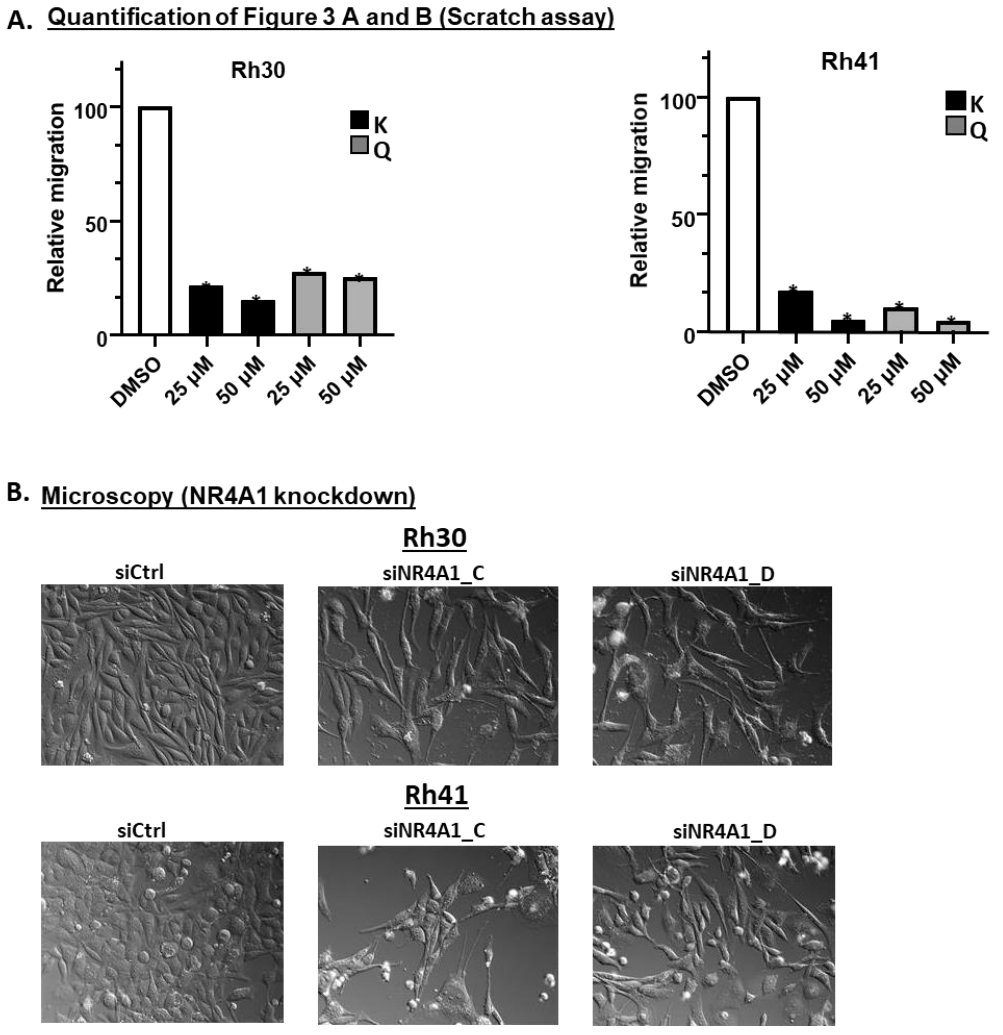


Figure S7. Quantification of the effects of quercetin (Q) and kaempferol (K) on cell migration (A) as illustrated in Figure 20A and 20B respectively. C. Cell morphological changes after knockdown of NR4A1 in Rh30 and Rh41 cells.

APPENDIX B

SUPPLEMENTAL TABLE 1

	Reagents/Antibodies	Purchased From:
1.	FBS (26140079), Trypsin (25200056), RPMI (11875093), DMEM (11995065), IMDM (12440053), Bis-ANS molecular probes (B153)	Thermofisher Scientific (Waltham, MA)
2.	CDIM8, CDIM8-3,5-$(CH_3)_2$, CDIM8-3-Br-5-OCH_3	Synthesized in the lab
3.	G9a (68851), H3K9me2 (4658), PTEN (9188), p-AKT (4060), AKT (4685), and HRP-linked secondary antibodies (7074, 7076)	Cell Signaling Technology (Danvers, MA)
4.	NR4A1 and Sp1 (ab13370) antibodies	Abcam (Cambridge, UK)
5.	Sp4 antibody (sc-390124)	Santa Cruz Biotechnology (Dallas, TX)
6.	β-actin antibody (A5316), oligonucleotides for siRNA interference assay	Sigma-Aldrich (St. Louis, MO)
7.	Lipofectamine-2000 (11668019)	Invitrogen (Carlsbad, CA)
8.	Mithramycin (11434)	Cayman Chemical (Ann Arbor, MI)
9.	PCR/ChIP primers and double stranded DNA probes	Integrated DNA Technologies (Coralville, IA)
10.	UNC0642 (HY-13980)	MedChemExpress LLC (Monmouth Junction, NJ)
11.	Ethidium bromide (BP130210)	Denville Scientific Inc. (Metuchen, NJ)
12.	Matrigel (08774122)	Corning Inc. (Corning, NY)
13.	Chemiluminescent Immobilon western HRP-substrate (WBKLS0500)	MilliporeSigma (Burlington, MA)

SUPPLEMENTAL TABLE 2

	Oligonucleotides	**Sequence**
1.	siNR4A1_A	5’-GGACAGAGCAGCUGCCCAA-3’
2.	siNR4A1_B	5’-CAGUCCAGCCAUGCUCCUC-3’
3.	siNR4A1_C	5’-CAGUGGCUCUGACUACUAU-3’
4.	siNR4A1_D	5’-GAGAGCUAUUCCAUGCCUA-3’
5.	siNR4A1_pool	Combination of siNR4A1_A, siNR4A1_B, siNR4A1_C, siNR4A1_D
6.	siG9a_1	5’-CCAUGAACAUCGAUCGCAA-3’
7.	siG9a_2	5’-UCACACAUUCCUGACCAGA-3’
8.	siG9a_3	5’-CCAACUGGUUCCUUUUGUU-3’
9.	siSp1	SASI_Hs01_00070994
10.	siSp4	SASI_Hs01_00114421
11.	G9a primer (human) (PCR)	F: 5'- TGGGCCATGCCACAAAGTC-3' R: 5'-CAGATGGAGGTGATTTTCCCG-3'
12.	G9a promoter (human) (ChIP assay)	F: 5’- CAGATGGGGACAGAGACGC -3’ R: 5’- CCCGGAGCATTGCACG-3’
13.	PTEN promoter (human) (ChIP assay)	F: 5′-GCAGGAAGGGTTGGGGTTCC-3′ R: 5′-GGATACACGGGCCACAGTCG-3’
14	PTEN primer (human) (PCR)	F: 5'-TTTGAAGACCATAACCCACCAC-3' R: 5'-ATTACACCAGTTCGTCCCTTTC-3'

SUPPLEMENTAL TABLE 3

	Reagents/Antibodies	Purchased From:
1.	FBS (26140079), Trypsin (25200056), RPMI (11875093), IMDM (12440053), Bis-ANS molecular probes (B153)	Thermofisher Scientific (Waltham, MA)
2.	Kaempferol (HY-14590)	MedChemExpress LLC (Monmouth Junction, NJ)
3.	Quercetin (Q00110)	Pfaltz & Bauer Inc. (Waterbury, CT)
4.	C-parp (5625), c-caspase3 (9661), G9a (68851), PAX3-FOXO1 (2880), IgG (2729), p-AKT (4060), AKT (4685), p-AMPKα (50081), AMPKα (5832), p-mTOR (2971), mTOR (2972), p-p70S6K (9205), p70S6K (9202), Slug (9585), ZO-1 (8193), ZEB1 (3396), N-cadherin (13116), β-catenin (8480), Integrin β1, Integrin β5 and HRP-linked secondary antibodies (7074, 7076)	Cell Signaling Technology (Danvers, MA)
5.	NR4A1 antibody	Abcam (Cambridge, UK)
6.	Sp1 (17824), Pol II (47701), N-myc (53993), MyoD (32758), Gremlin (515877), DAPK (136286), c-Myc (sc-40) antibodies	Santa Cruz Biotechnology (Dallas, TX)
7.	β-actin antibody (A5316), oligonucleotides for siRNA interference assay	Sigma-Aldrich (St. Louis, MO)
8.	Lipofectamine-2000 (11668019), Alexa Flour® 488 annexin V/Dead cell apoptosis kit (V13245)	Invitrogen (Carlsbad, CA)
9.	Reporter lysis buffer and luciferase reagent (E1483)	Promega Corporation (Fitchburg, WI)
10.	PCR/ChIP primers	Integrated DNA Technologies (Coralville, IA)
11.	Ethidium bromide (BP130210)	Denville Scientific Inc. (Metuchen, NJ)
12.	Invasion chambers (08774122), Matrigel (08774122)	Corning Inc. (Corning, NY)
13.	Chemiluminescent Immobilon western HRP-substrate (WBKLS0500)	MilliporeSigma (Burlington, MA)

SUPPLEMENTAL TABLE 4

	Oligonucleotides	**Sequence**
1.	G9a primer (human) (PCR)	F: 5'-TGGGCCATGCCACAAAGTC-3' R: 5'-CAGATGGAGGTGATTTTCCCG-3'
2.	PAX3-FOXO1 primer (human) (PCR)	F: 5'-CCCACTGCCATGCCGACCTTG-3' R: 5'-ACGAATTGAATTCTGAGGTGAGAG-3'
3.	G9a promoter (human) (ChIP)	F: 5'-CAGATGGGGACAGAGACGC -3' R: 5'-CCCGGAGCATTGCACG-3'
4.	PAX3-FOXO1 promoter (human) (ChIP)	F: 5'-TGCCTGTGCTTCACATTAGC-3' R: 5'-AATTCCAATAAGAAGGCATCTG-3'
5.	siNR4A1_C	5'-CAGUGGCUCUGACUACUAU-3'
6.	siNR4A1_D	5'-GAGAGCUAUUCCAUGCCUA-3'