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Rb-Raf-1 interaction as a therapeutic target for proliferative disorders

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Rb-Raf-1 Interaction as a Therapeutic Target for Proliferative Disorders

by

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DEDICATION

To my loving husband Christian Davis and my parents Barbara Kinkade and Gary Kinkade.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Rb	Retinoblastoma
SV40 T Ag	Simian virus 40 large tumor antigen
E1A	Early region 1A
DP	Dimerization partner
HDAC1	Histone deacetylase 1
HP1	Heterochromatin protein 1
DNMT1	DNA (cytosine-5-)-methyltransferase 1
SCLC	Small cell lung cancer
NSCLC	non-small cell lung cancer
Cdc2	Cell division cycle 2
Cdc25A	Cell division cycle 25 homolog A
HATs	histone acetyltransferase
TGF-β	transforming growth factor β
ChIP	Chromatin Immunoprecipitation
BRCA1	breast cancer 1 early onset
Bcl-2	B-cell leukemia/lymphoma 2
Ask1	apoptosis signal-regulating kinase 1
-/-	knockout
PcG	polycomb group

APAF1	apoptotic peptidase activating factor
Tg-GFAP	transgenic-human glial fibrillary acidic protein promoter
n	number of samples
MMP16	matrix metalloproteinase 16
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
FGFR	Fibroblast growth factor receptor
IL-8	interleukin-8
HIF1-α	hypoxia inducible factor 1 alpha
ATM	ataxia-telangiectasia mutated
ATR	Ataxia telangiectasia mutated and rad3-related protein
$p^{300/CREB}$	cAMP-response element-binding protein
G1	gap phase 1
G2	gap phase 2
EGFR	epidermal growth factor receptor
Erb2	avian erythroblastosis oncogene B
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
Grb2	growth factor receptor bound protein 2
SOS	son of sevenless
GDP	guanosine diphosphate
GTP	guanosine triphosphate
nAChRs	Nicotinic acetylcholine receptors

NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N′*-nitrosonornicotine
TNF-α	Tumor necrosis factor alpha
VSMC	vascular smooth muscle cells
HAEC	human aortic endothelial cells
HUVEC	Human Umbilical Vein Endothelial Cells
EC	endothelial cells
ECM	extra cellular matrix
Pak	p21 activated kinase
JAK	janus tyrosine kinase
PECAM1	platelet endothelial cell adhesion molecule
RKIP	Raf-1 kinase inhibitor protein
Mdr1	multi drug resistance gene 1
AML	acute myelogenous leukemia
kDa	kilodalton
nM	nanomolar
μM	micromolar

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ABSTRACT

The retinoblastoma tumor suppressor protein, Rb, is a key regulator of the mammalian cell cycle and its inactivation facilitates S-phase entry. Rb is inactivated through multiple waves of phosphorylation, mediated mainly by kinases associated with D and E type cyclins in the G1 phase of the cell cycle. Our earlier studies had shown that the signaling kinase Raf-1 (c-Raf) physically interacts with Rb upon growth factor stimulation and initiates the phosphorylation cascade. We had shown that an 8 amino acid peptide derived from Raf-1 could disrupt the Rb-Raf-1 interaction leading to an inhibition of Rb phosphorylation, cell proliferation and tumor growth in nude mice. Here, we describe a newly identified orally-active small molecule, RRD-251 (Rb – Raf-1 Disruptor 251), that disrupts potently and selectively the binding of Raf-1 to Rb; it had no effect on Rb-HDAC1, Rb-Prohibitin, Rb-Ask1, Rb-cyclin E, or Raf-1-Mek interactions. RRD-251 inhibited anchorage-dependent and –independent growth of human cancer cells; it could also potently inhibit angiogenesis both *in vitro* and *in vivo*. Oral or intra-peritoneal administration of RRD-251 resulted in a significant suppression of growth of tumors xenotransplanted into athymic nude mice; the tumor suppressive effects were restricted to tumors carrying a wild-type Rb gene.

Thus, selective targeting of Rb-Raf-1 interaction appears to be a promising approach for developing novel anti-cancer agents. In addition to mitogens, tobacco components like NNK and nicotine can induce cell proliferation and angiogenesis, contributing to lung cancer. Induction of cell proliferation by tobacco components required the binding of Raf-1 to Rb and RRD-251 could prevent nicotine induced cell proliferation. Our studies also show how nicotine not only promotes tumor growth *in vivo*, it also increases chance of tumor recurrence and metastasis. In addition to growth factors and tobacco components, cytokines like TNFα could induce Rb-Raf-1 interaction in vascular smooth muscle cells. Since TNFα-induced proliferation of vascular smooth muscle cells contributes to growth of atherosclerotic plaques, RRD-251 could be beneficial in controlling atherosclerosis as well. Thus, it appears that drugs that can disrupt the Rb-Raf-1 interaction might have beneficial effects in a wide spectrum of human diseases.

Chapter 1: Introduction

1. The retinoblastoma tumor suppressor gene and cell cycle

While the presence of tumor suppressor genes have been realized for many years, the retinoblastoma tumor suppressor gene (RB) was the first to be identified and cloned (1). Recognized as the first identified tumor suppressor gene, RB was identified based on studies on the inheritance pattern of retinoblastoma, which is a pediatric tumor of the eye. Retinoblastoma could be familial or sporadic; familial forms are bilateral and mutifocal while sporadic forms are unilateral (2). Based on these observations, Alfred Knudsen proposed that the retinoblastoma arises after two genetic events; this led to the “two-hit hypothesis” meaning that 2 independent “hits” (mutations) are required in the same cell to initiate cancer (2). Several laboratories cloned the RB tumor suppressor gene in the late 1980s through positional cloning of minimally deleted chromosomal regions (1,3,4). Rb protein was found to be a 928 amino acid nuclear phospho protein that has no catalytic activity and very weak DNA binding activity (3). It was found that viral oncoproteins such as SV40 large T antigen, adenovirus E1A and human papilloma virus E7 are capable of binding to Rb and disrupting its tumor suppressive function (5-7). Mutants of such viral oncoproteins that could not bind to Rb could not transform cells; further, mutant Rb proteins found in tumors could not bind to the viral oncoproteins (5,7). These findings led

to the hypothesis that binding of these viral oncoproteins caused an inactivation of the Rb protein equivalent to mutating its gene. This finding also revealed how viral oncoproteins can instigate tumor formation by inactivation of a tumor suppressor protein, Rb (5,7).

1.1 Regulation of cell cycle by Rb

Further studies showed that that there are many cellular proteins that bind to Rb and this allowed Rb to regulate a variety of cellular processes (8). Such proteins can be classified into two groups – those that are upstream of Rb and regulate or affect Rb function and those that are downstream of Rb, facilitating Rb functions. The major downstream targets of Rb are those involved in transcriptional control; these include the E2F family of transcription factors (9). E2F family members bind to DNA as heterodimers with DP proteins, DP1 or DP2 (Dimerization partner 1 or 2) (10). E2F-DP complexes bind to the canonical sequence TTTCGCGC or its derivatives present on many cellular promoters and regulate the expression of genes involved in DNA replication, cell cycle progression and DNA repair (11-15).

In quiescent cell (G_0) and early in G1, Rb remains hypo-phosphorylated and is in its active state; this is when Rb is most efficient at binding and repressing E2F regulated genes (16). Mutant Rb proteins that are unable to bind to E2F cannot inhibit transcription (17). In response to mitogenic stimulation, Rb becomes increasingly phosphorylated, causing Rb to become less efficient at

interacting with associated proteins such as E2F and co-repressor molecules (18). Therefore, phosphorylation of Rb weakens its ability to repress transcription. The phosphorylation of Rb is catalyzed by cyclin/ cyclin dependent kinase (CDK) complexes (19-21).

The cyclin/CDK complexes that phosphorylate Rb in G1 phase are cyclin D/CDK4/6 and cyclin E/cdk2. Mitogenic signaling (growth factor stimulation) leads to activation of cyclin/CDK complexes. Cyclin/CDK complexes in G1 efficiently phosphorylate Rb to completely inactivate its transcriptional repressor function, thus allowing expression of E2F target genes (22,23). E2F functions to activate genes that are essential for entry into S-phase (24,25). Rb remains inactive throughout the remainder of the cell cycle. Mitotic cyclins/CDK complexes CDK2/cyclin A and CDK1/cyclin B phosphorylate Rb and mediate the progression through the S/G2/M phases of the cell cycle (26). Rb phosphorylation is reversed by dephosphorylation, causing a transient reactivation. From anaphase to G1 protein phosphatase 1 (PP1) dephosphorylates Rb in response to growth inhibitory signals (27,28). Under normal conditions, it is assumed that PP1 complexes contain PP1 regulatory proteins termed PNUTs (29). During hypoxic conditions or in the presence of chemotherapeutics, PNUTS were found to dissociate from PP1 and this led to the activation of PP1 towards Rb, causing an early dephosphorylation of Rb (29). These findings support that inactive and active Rb serves as a critical controller of cell proliferation and growth suppression.

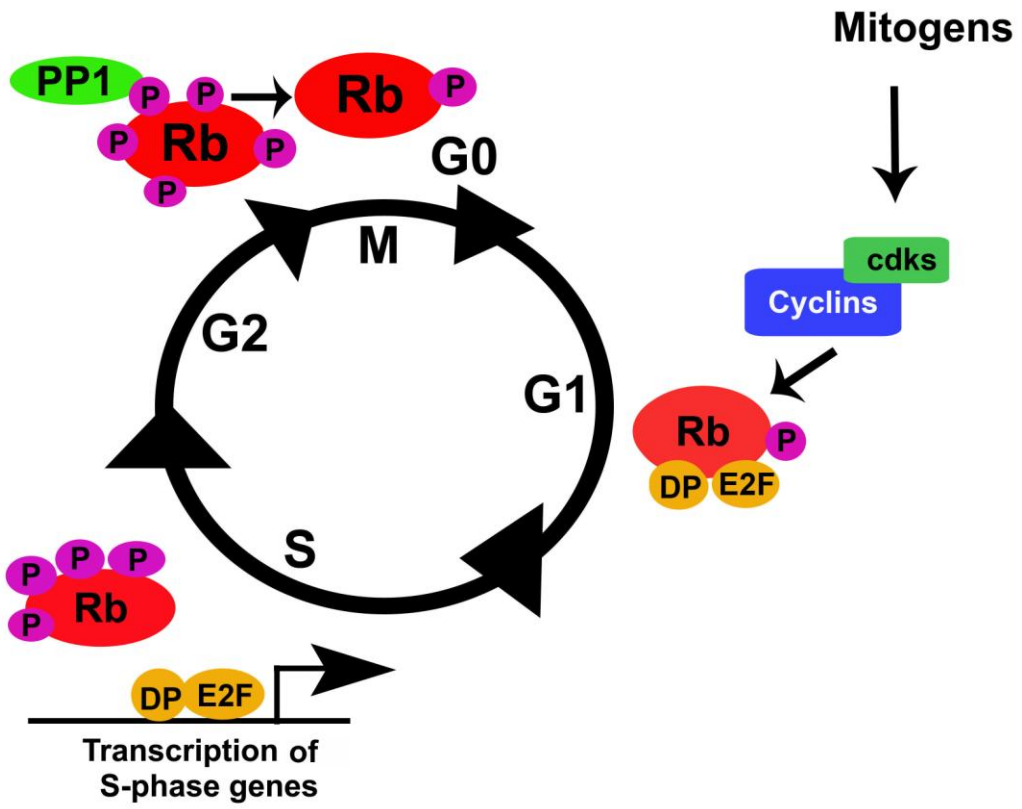


Figure 1. Cell cycle dependent regulation of Rb/E2F. Mitogenic signals stimulate the accumulation of cyclin dependent kinases and initiate phosphorylation of Rb in G1 phase of the cell cycle. Once Rb is inactivated though phosphorylation it releases E2F to induce S-phase genes. Late in mitosis, Rb is dephosphorylated by protein phosphatase 1 (PP1).

1.2 Rb family members

Rb belongs to a family of three proteins, generally referred to as the 'pocket proteins'; the family consists of Rb, p107 and p130 (30). They are termed pocket proteins because of a shared domain called the pocket through which these proteins bind viral oncoproteins, cellular proteins and transcription factors. RB and p130 are found on chromosomes 13q14 and 16p12.2, in which mutations are evident in cancer (31). The p107 gene has been mapped to chromosome 20q11.2, which is not frequently mutated in cancer (32). The pocket region of Rb family members contains two domains (A and B) separated by a spacer (S), which is different among the Rb proteins. Rb proteins also contain a C-terminal domain that has been referred to as the C-pocket and is involved in E2F binding (33) **(Figure 2)**. The majority of Rb binding proteins interact with the A and B pocket domains. Most proteins that bind to Rb share a LXCXE motif; this is present in the three viral oncoproteins mentioned earlier as well as the D-type cyclins and histone deacetylase (HDAC). Structurally and functionally, p107 and p130 are more related to each other than either is related to Rb. p107 and p130 proteins also bind to E2Fs and their phosphorylation by cyclins and cdks results in dissociation of E2F and genes that regulate S-phase (34). However, both p107 and p130 bind different E2Fs compared to Rb, and they regulate different E2F responsive genes (34). In addition, their expression pattern is unique from one another; Rb is expressed in both proliferating and non-proliferating cells, p107 is

predominantly expressed in proliferating cells and p130 is mainly expressed in arrested cells (34). Many years of research have suggested specific models for pocket protein-E2F networks. It is thought that in G0 and early G1, p107 and p130 form repressor complexes in conjunction with E2F4 or E2F5 at most of the E2F responsive promoters. At the same time, Rb is thought to bind E2F1-3 either at or sequestered away from E2F responsive promoters (30). Pocket proteins are inactivated by phosphorylation in late G1 leading to the dissociation of E2Fs; at this time, E2Fs 4 and 5 are thought to translocate to the cytoplasm, allowing E2Fs1-3 to occupy the proliferative promoters. This binding of E2Fs independent of the pocket proteins facilitates the expression of genes needed for DNA synthesis and cells enter S-phase. Thus the progression of cells from G1 to S-phase is a stringently regulated process that involves many vital components (30).

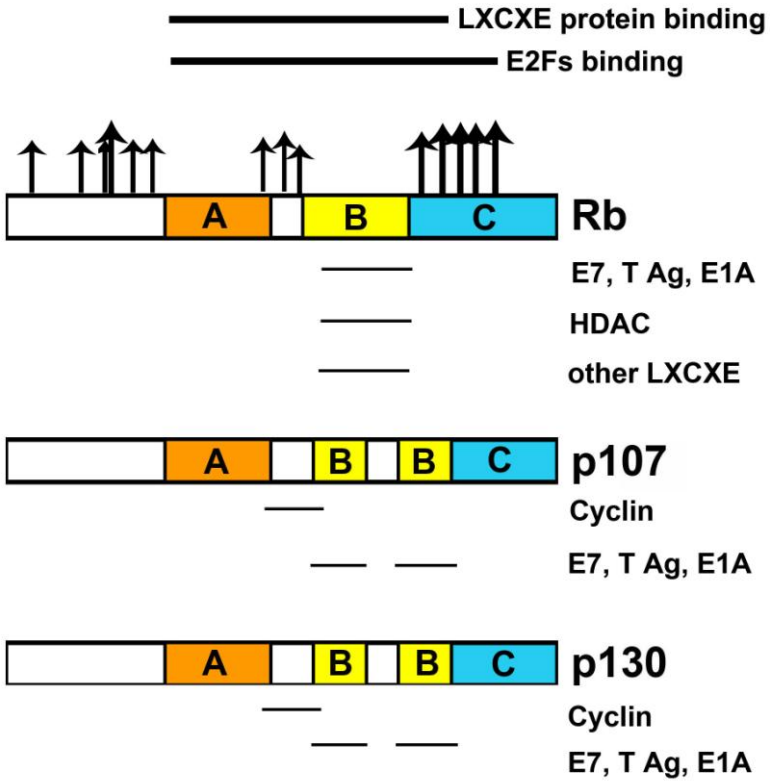


Figure 2. The pocket family of proteins consists of Rb, p107 and p130. Homology lies within the pocket domain made up of regions A and B separated by a spacer. All three proteins contain the C terminal domain, the region where E2Fs bind. Arrows indicate the many regulatory phosphorylation sites. Proteins containing the LXCXE mainly bind within the pocket region of these proteins.

1.3 Rb inactivation in cancer

As predicted by Knudson's hypothesis, both alleles of the Rb gene are inactivated by mutation in sporadic and inherited forms of retinoblastoma tumors (35). The most common mutations are point mutations, small deletions, or insertions in the gene, which results in frameshifts and premature termination of the protein product (35). It is rare for a gross deletion or rearrangement of the gene to occur (35). Inactivation of the Rb gene leads to oncogenesis, not only in eye tissue, but also in osteosarcoma, small cell lung cancer (SCLC), 20-30% of non-small cell lung cancers (NSCLC) (36), prostate cancer, and breast cancer (37,38). Rb inactivation is required for the tumors to overcome the Rb mediated restraint on cell cycle progression. The Rb 'pocket' is targeted by viral oncoproteins like adenovirus E1a (7), human papilloma virus E7 (5), and SV40 large T antigen (6), all of which disrupt Rb's function. Perturbations in the Rb pathway are present in almost all cancers, and several mechanisms have been identified for inactivating Rb. Over-expression of cyclin-D or CDK4 kinases from amplification, mutation, or chromosomal translocation can lead to enhanced Rb phosphorylation and poor prognosis (18,39-43). Also, loss or mutation in $p16^{INK4a}$ (cdk inhibitor) can induce excessive CDK4/cyclin D activity and will lead to increased Rb phosphorylation and inactivation. Since p16 is responsible for the control of cyclin D/cdk4 kinase activity, mutations or loss of p16 correlates with Rb activity and are often found in human cancers (37,44-47). Increased expression of cyclin E/cdk2 or reduced levels of the cdk inhibitor $p27^{Kip1}$ also give

a poor prognosis in many cancers since these too will lead to increased Rb inactivation (18). Another common method for Rb inactivation in cancers is through viral oncoprotein E7 (5,48). The tumor-promoting HPV contains at least two genes, E6 and E7, which encode for proteins that interfere with cell-cycle regulation. E7 disrupts the cell cycle via its direct binding to Rb and other members of the retinoblastoma family (p107 and p130). The human papilloma virus-16 is found associated with approximately 50 percent of cervical carcinomas (5).

2. Downstream effectors of Rb function

More than 100 proteins have been reported to bind to Rb. Rb interacts with kinases, phosphatases, transcriptional regulators, kinase regulators and various miscellaneous proteins (8). Although there are several Rb targets reported in the literature, most of the attention has been focused on the E2F family of transcription factors (8, 49, 50).

2.1 E2F Family of Transcription Factors and Cell Cycle Regulation

Each of the Rb family members bind to distinct members of the E2F family of transcription factors which regulate and drive cell cycle progression. A broad range of studies have revealed that Rb family members associate with a wide variety of transcription factors and chromatin remodeling enzymes to control gene expression, of which the E2F family of transcription factors are

predominantly studied. Our studies are focused on the regulation of E2F by Rb and mechanisms to prevent cell cycle progression via inhibiting Rb inactivation.

2.2 Discovery of E2F and its function

The mammalian cell cycle is stringently regulated by growth stimulatory and inhibitory signals from the environment. The transcriptional activity of the E2F family of proteins can respond appropriately to the wide array of signals the cell receives. E2F was originally discovered as a cellular activity that is required for the early region 1A (E1A) transforming protein of adenovirus to mediate transcription of the viral E2 promoter (51). Experiments later determined how E2F is regulated in normal cells, when Joe Nevins and colleagues determined that E2F is inhibited by its association with the retinoblastoma protein, Rb (51-53). E2F family members are the key downstream targets of Rb and regulators of S-phase entry (54). Rb can bind directly to the transactivation domain of E2Fs and block their ability to activate transcription (55,56) and can recruit chromatin remodeling enzymes to repress E2F activity (57-59). Transcriptional repression by Rb is mediated through its various co-repressors including HDAC1 (60,61) Brg1/Brm (62), HP1 (63), SuV39H (64), PcG proteins (65) and DNMT1 (66), but repression is not restricted to only these co-repressors. E2F1 also induces the expression of the cyclin-dependent kinase inhibitor p27, resulting in negative feedback regulation of E2F1 transcriptional activity through the inhibition of cyclin-dependent kinase activity and Rb hypophosphorylation (67).

In G0 and early G1, p107 and p130 bind E2F4 or E2F5 at most E2F-responsive promoters to form a repressor complex (54). At the same time, Rb is thought to bind E2F1-3 either at the promoters or sequestered away from the promoters (30). In late G1, pocket proteins are phosphorylated causing them to dissociate from E2Fs. E2F4 and E2F5 relocate to the cytoplasm, and the promoters are then occupied by E2Fs1-3. For most promoters the binding of E2F1-3 coincides with recruitment of histone acetyltransferases (HATs), leading to acetylation of histones at the promoters facilitating transcriptional activation (30,68). It is the activator E2Fs that interact with various HATs, whereas Rb recruits histone deacteylases (HDAC) and histone methyltransferases (69). Complexes of E2F family members with repressive pocket-proteins are high in G0 and early G1 phases of the cell cycle; these complexes are disrupted in late G1 (30). This allows E2Fs to induce the transcription of genes required for S-phase entry such as cyclin E (CCNE1), cyclin A, CDC2, CDC25A, p107, RB, c-Myc, N-Myc, B-Myb, E2F1 and E2F2 (70-72).

2.3 E2F family members

The E2F family of transcription factors is a large and continuously growing family of proteins, with the first member being cloned in 1992 (E2F1) till the recent identification of E2F8 in 2005, totaling 9 different proteins altogether (73) **(Figure 3)**. E2Fs heterodimerize with Dimerization Partner protein 1 and 2 (DP1, DP2) for optimal transcriptional activity and all possible combinations of E2F-DP

complexes can exist *in vivo* (74) (10). It is the specific identity of E2F and the proteins involved in the complex that determines the transcriptional response. The various E2F/DP complexes preferentially recognize the same nucleotide sequence – TTTCCCGC, or variants thereof (75). E2F activity is interconnected through complexes with any of the 9 E2Fs, 2 DP binding proteins (DP1 and DP2) and 3 pocket proteins (Rb, p130, p107) (76). E2Fs can be subdivided into three groups: the activating E2Fs, the passively repressing E2Fs and the actively repressing E2Fs. E2F1, E2F2 and E2F3a are the potent transcriptional activators that interact only with Rb and are expressed intermittently throughout the cell cycle (9). E2F4 and E2F5 are poor transcriptional activators and function as passive repressors by recruiting pocket proteins to the E2F regulated promoters (9). E2F4 interacts with all three pocket proteins yet E2F5 binds predominantly to p130 (30). The activator E2Fs are typically involved in promoting cell cycle progression and the repressor E2Fs function for cell cycle exit and differentiation (54). Unlike E2Fs 1-5, E2F6 and E2F7 lack transactivation and pocket protein binding domains; they actively repress transcription independent of pocket proteins (77). Both the E2F6 and E2F7 loci produce several alternatively spliced mRNAs, which encode distinct protein isoforms (78). E2F6 can repress transcription through binding to polycomb group (PcG) proteins (79); however, mechanisms underlying repression by E2F7 are still unclear. E2F7 associates with promoters during S and G2 phase of the cell cycle, suggesting that it may function to repress E2F targets once they are expressed and have executed their

functions (78). The newest identified member of the E2F family, E2F8, has transcriptional repressive functions similar to E2F7 (80).

These specific interactions between E2F transcription factors and pocket proteins have suggested several models of the pocket protein-E2F network. Once entry into S-phase has occurred, E2F1-3 continue to bind and activate some promoters whilst other promoters are bound by E2F1-3 only until G1-S transition, depending on the cellular function of the target gene (81). Certain studies have suggested a preferential role for E2F3a, the predominant form of E2F3, in regulating S-phase entry compared to other proliferative E2Fs. The activator E2Fs can also overcome growth arrest signals such as TGF-β or Cdk inhibitors (82). It should be mentioned that the activator E2Fs do not always promote S-phase entry (70). The ability of E2F to induce S-phase depends entirely on the cellular context and the nature of the signals.

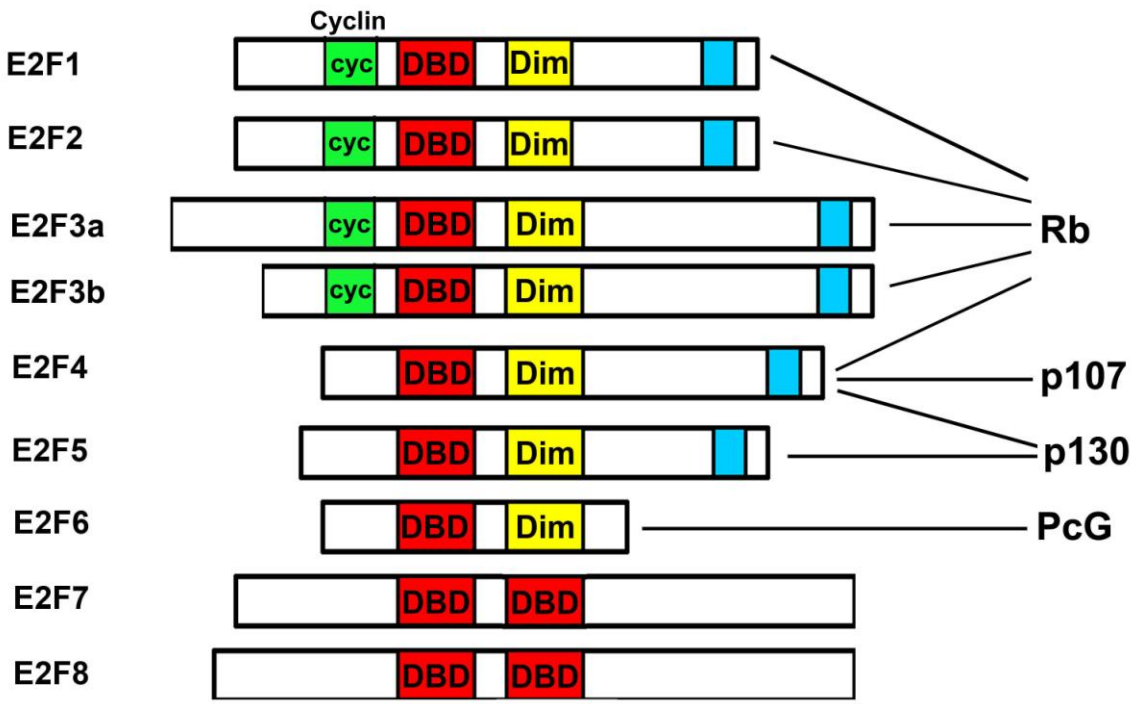


Figure 3. Domain structure of the E2F family. E2Fs 1-6 contain 1 DNA binding domain (DBD) and one DP dimerization domain (Dim). Transactivation domains (blue) and binding sequences for pocket proteins are found only in E2Fs1-5. E2F1-3 have cyclin binding domains in their N terminal region (green). E2F7 and E2F8 have two DNA binding domains.

2.4 E2F Target Genes

A diverse set of potential E2F target genes have been identified, shedding light on the many cellular functions that can be regulated by E2Fs. The list of E2F target genes has been growing from studies including microarray analysis and Chromatin Immunoprecipitation (ChIP) assays (13,14,83-93). The known target genes are no longer restricted to those involved in G1/S progression and DNA replication, although they remain the best studied.

2.4.1 Proliferative E2F target genes

E2F activity regulates a variety of genes required for entry into S-phase. In accordance with its ability to activate S-phase gene transcription, overexpression of the E2F1 product drove quiescent cells into S-phase (94). All three activating E2Fs are responsible for the expression of cyclin E and only E2F1 and E2F3 activate the expression of Cdc6 and p107 (95). Dihydrofolate reductase is involved in nucleotide synthesis and is preferentially targeted by E2F2, whereas E2F3 is the primary activator of cdk2, a G1/S cell cycle regulator (96). Both ribonucleotide reductase (RRN 1, 2) and cyclin A are activated by E2F1 and E2F2 (96). A set of G2 expressed genes regulated by E2F, namely, cyclin B1, cyclin A2, Cdc20, Bub1, and Importin α2 were discovered through DNA microarray analysis, along with several other targets involved in checkpoint regulation (86). Other groups have revealed new E2F target genes by microarray that are involved in DNA replication, such as DNA replication protein A2, MCM 2,

3, 4, 5, 6, 7, and DNA polymerase α (89). The repressive E2F4/p130 complex (97) regulates DNA repair enzymes such as BRCA1 and RAD51.

2.4.2 Apoptotic E2F target genes

Certain E2Fs are also equipped with pro-apoptotic functions to protect cells from undergoing aberrant oncogenic transformation. E2F1 appears to be the crucial family member facilitating E2F-dependent apoptosis (54). The role for E2F1 in apoptosis was confirmed by the observation that mice lacking E2F1 display a high incidence of tumors, implicating a role in tumor suppression probably by promoting apoptosis (98,99). While in certain tissues like skin and liver, E2F1 overexpression can result in tumor formation (100,101). A deficit in E2F1 can also impair the development of pituitary tumors in $Rb^{+/-}$ mice implying that E2F1 can play a tumor suppressive or oncogenic role depending on tissue type (102).

Different mechanisms have been identified by which E2F-1 induces apoptosis and the picture is becoming clearer with passing years. Interestingly, E2F1 can induce apoptosis in p53-dependent and independent pathways. Ectopic expression of E2F1 induces p53-dependent apoptosis both *in vitro* and *in vivo* (101,103-105) through the transactivation of $p19^{ARF}$ (106,107) and thus alleviation of MDM2-mediated degradation of p53 (106-109). In addition, E2F1 can induce the expression of p73 (110), Apaf-1 (111), caspases (112), and pro-

apoptotic BH3-only proteins of the Bcl-2 family (113) and thereby induces apoptosis through a p53-independent mechanism. Thus, pharmacologic activation of E2F1-mediated apoptosis in p53-deficient tumors can be explored to overcome the chemoresistance in these tumors.

In addition to p53 and p73, a variety of cellular proteins have been identified that can facilitate the induction of apoptosis by E2F1. E2F1 downregulates the expression of Mcl-1, an antiapoptotic member of the Bcl-2 family (114). E2F1 has also been shown to upregulate the expression of the pro-apoptotic BH3-only proteins PUMA, Noxa, Bim, and Hrk/DP5 through a direct transcriptional mechanism leading to apoptosis (88,112,113,115,116). Expression of the E7 protein of HPV16, which disrupts Rb/E2F complexes also upregulates the expression of these four BH3-only proteins, implicating endogenous E2F in this phenomenon. Furthermore, increased Noxa and PUMA levels have been shown to mediate E2F1-induced apoptosis (113). HDAC inhibitors such as SAHA and Trichostatin A can promote E2F1-mediated apoptosis through the induction of the pro-apoptotic Bcl2 family member Bim as well as ASK1, and this apoptosis does not require p53 or p73 (117); as a result cancer cells with deregulated E2F1 activity are sensitive to HDAC inhibitors. ASK1 induction contributes to SAHA-induced apoptosis through positive feedback regulation of E2F1 apoptotic activity (117).

These studies highlight that E2F1 regulates the apoptotic machinery by activating a number of pro-apoptotic genes. In addition to these studies it was found that depletion of E2F4 and not E2F1 could enhance apoptosis induced by chemotherapeutic drugs in human cancer cell lines, suggesting that E2F1 and E2F4 act in opposing manners in drug induced apoptosis (118). Such studies and many more reveal the complex contributions of E2F family members to the biology of the cell and a panorama of apoptotic target genes that may be less characterized than the well understood cell cycle progression genes.

2.5 E2Fs in Oncogenesis

The E2F family of transcription factors can execute opposing roles in activating or inhibiting cellular proliferation; this enables them to act as potential oncogenes or tumor suppressor genes. Elegant studies in the 1990's were the first to show that E2F is inhibited through its association with Rb (51-53). Nevins and colleagues also discovered that overexpression of E2F1 in cell culture could lead to proliferation (119); it was also found that E2F1 could transform cells in association with other oncogenes like Ras and c-myc (119,120). Unlike Rb, E2F itself is rarely found to be mutated in cancers, although recent findings have discovered deregulated levels or mutations of E2F family members in certain types of cancers (121). Gene disruption studies in mice lacking E2F family members clearly demonstrated the complexity and opposing roles of the E2F family.

2.5.1 Genetic Alterations of E2F in Cancer

Most reviews on E2F family members conclude that there are not many known mutations in this family of transcription factors in human cancer, and this holds true for the most part (122) (123). At the same time there are some reports indicating genetic alterations of E2F genes in cancer (124). Among the E2F family members, it has been the *E2F3* gene, located at chromosome 6p22, which is frequently amplified and overexpressed in certain types of cancers like retinoblastoma and transitional cell carcinomas of the urinary bladder (125,126). In bladder cancer especially, *E2F3* amplification is associated with a more malignant and invasive tumor (127,128). *E2F3* overexpression has been identified in prostate, ovarian, and non-small cell carcinoma and also correlates with poor survival rates (129-132). There is also evidence that other E2F genes are involved in some cancers. The *E2F5* gene is amplified in some breast cancers often along with c-myc and or c-mos amplification (133). It has also been reported that *E2F4* is also mutated in a number of stomach and colon cancers (134-137). In addition, E2F4 protein levels are often elevated in colon cancers and this is associated with low levels of apoptosis (134). Moreover, deregulated E2F1 (transgenic mice engineered to express E2F1 in Glial cells; tg-GFAP-E2F1) activity in the brain increases the onset of multilineage brain tumors in mice old and young; this demonstrates that *E2F1* functions specifically as an oncogene in mouse brain tissue (138).

The amplification of *E2F1* in cancer cell lines has been examined thoroughly, it has been demonstrated in esophageal, colorectal, ovarian, melanoma and lymph node metastasis of melanoma (139-144). High expression of *E2F1*, *E2F2* and *E2F8* were linked to ovarian cancer cell lines (n=77) and also correlated with histopathologic grade 3 ovarian tumors (145). The clinical relevance of E2F family members was assessed in ovarian cancers to predict if E2Fs provide resistance to chemotherapy with platinum based drugs. Low expression of activating E2F1 or E2F2 and high expression of inhibiting E2F4 or E2F7 was associated with favorable disease-free and overall survival of patients who had undergone surgery (145). Platinum resistant tumors were associated with lower E2F4 and E2F7 expression when compared to platinum sensitive tumors indicating that their downregulation could be contributing to mechanisms underlying platinum resistance (145). High levels of E2F1 in cancers of the lung, breast and pancreas correlate with poorer outcome (76). Conversely, it is reduced E2F1 expression in colon cancer, bladder cancer and diffuse large B-cell lymphoma that correlates with a more aggressive disease (76). Even though there are plenty of studies that link E2F expression with cancer, it is mainly alterations in the Rb-E2F pathway that are common in all types of cancers.

2.5.2 E2F knockout studies

Studies on E2F1 null mice provided novel insights into the opposing roles of E2F1 in oncogenesis and tumor suppression (99). $E2F1^{-/-}$ mice are viable and

fertile except, as they age these mice exhibit hyperplasia and neoplasia (98,99). As the mice age, a broad range of tumors was seen, including lymphomas, sarcomas of the reproductive tract and lung tumors (99). E2F2$^{-/-}$ mice die early due to autoimmune disease with splenomegaly, multiorgan inflammatory infiltrates, glomerulonephritis, and serum anti-DNA antibodies (146). The loss of E2F1 and E2F2 in mice results in tumor formation, primarily hematopoetic malignancies (147). Studies in E2F3 knockout mice revealed that although these mice die prematurely, the mice that do survive are significantly growth retarded. E2F3$^{-/-}$ mice have no obvious signs of tumor formation; instead they have the typical signs of congestive heart failure (148). A loss of both E2F1 and E2F3 did not increase the tumor incidence therefore demonstrating that it is E2F1 that has the tumor suppressive functions and not E2F3 (148). Mice lacking E2F4 surprisingly show no abnormalities in cell proliferation or cell cycle arrest; however, E2F4 was essential for normal development of the mouse. Mice lacking E2F4 have several developmental defects including a craniofacial abnormality (149,150). Knockout studies on E2F5 revealed that it was also not essential for proliferation; instead E2F5 was required in differentiated neural tissue, as these mice developed hydrocephalus at 7 weeks of age (151). Mice lacking E2F6 are viable and healthy, they only display skeletal transformations, these mice display posterior homeotic transformations of the axial skeleton, which is very similar to what is observed in mice lacking PcG proteins, which is consistent with the ability of E2F6 to associate with PcG proteins (79). The phenotype of knockout E2F7

and E2F8 have yet to be published. It can be concluded that mice lacking repressive E2Fs have developmental defects but are not predisposed to developing tumors. Oddly enough, tumor suppressive activity is associated with the E2Fs that are activators of transcription and promoters of proliferation.

2.6 E2Fs regulate Angiogenesis

While a role for E2Fs in cell proliferation and apoptosis is well established, their role in angiogenesis is less clear. Recent studies raise the possibility that E2Fs might be contributing to the growth and progression of tumors by affecting angiogenesis. Overexpression of E2F1 is associated with increased tumor cell invasiveness and metastatic progression (152,153). Many E2F1 target genes include genes involved in angiogenesis such as bFGF, metalloproteinase 16 (MMP16) and VEGF-B through a direct or indirect transcriptional regulation of the promoters (154). Several genes whose expression is regulated by VEGF contain E2F binding sites in their promoter. Human metallothionein 1G (hMT1G) is involved in metal metabolism and regulation of angiogenesis. Stimulation of cells with VEGF led to a dissociation of Rb, p130 and p107 and an increase in activator E2Fs on this promoter (155). In other studies, E2F1, E2F2 and E2F3 can activate the fibroblast growth factor receptor 2 (FGFR-2) promoter that is involved in angiogenesis (156). These findings raise the possibility that even though E2F genes by themselves are seldom mutated in cancers, E2Fs can promote the growth of solid tumors by promoting angiogenesis.

2.7 Targeting E2F biology for cancer therapy

In certain circumstances, either an increase or decrease in E2F1 activity can inhibit tumor development; although this is entirely context dependent there is great potential for the design of cancer therapies targeting E2F1. Recombinant adenovirus E2F1 has been shown to kill human tumor cells *in vitro* and in nude mouse models (157-162). Since deregulated E2F activity appears to be a common event in various malignancies, these treatments would have the potential to reach a broad range of cancers. It is important to note that E2F activity has been shown to influence chemotherapeutic response (163). It can be assumed that when E2F upregulates p73 and APAF-1, this could sensitize cells to other pro-apoptotic signals from DNA-damaging agents (110). A key target for therapy could be the downstream targets in E2F1 induced apoptosis. It can be imagined that chemotherapeutic agents that are most active in S-phase could benefit from E2F activity to induce cell death. Agents such as 5-flurouracil and hydroxyurea that target E2F responsive genes thymidylate synthase and ribonucleotide reductase respectively, would benefit from forced expression of E2F (164,165). Experiments with E2F1 transient transfection have been shown to enhance the efficacy of etoposide, camptothecin, and adriamycin (166). Induction of DNA damage leads to E2F1 protein stability through several mechanisms, including phosphorylation by the ataxia-telangiectasia mutated (ATM) kinase, the ATM and RAD3-related (ATR) and the Chk2 kinase. E2F1

stability is also mediated by acetylation through p300/CREB-binding protein factor (P/CAF). The common deregulation of Rb/E2F pathway in human cancers in combination with E2F's apoptotic potential and stabilization after damage suggest that E2F1 plays an important role in tumor cells sensitivity to DNA damage induced cell death. In the same context, loss of E2F1 is protective and anti-apoptotic.

The important question is how E2Fs can be targeted to induce apoptosis in cancer cells without inducing cell proliferation and tumor growth. Since the absence of all three activating E2Fs leads to abrogation in cellular proliferation and mouse development (167), it appears that targeting any one of the activating E2Fs or all of them would be a viable mechanism to shut down tumor cell proliferation. Studies from the Harlow lab demonstrated how dominant negative mutants of DP1 that can prevent DNA binding and transcriptional regulation by E2F leading to cell cycle arrest in G1 (168). These studies and others collectively support the idea that inhibiting E2F with small molecules would inhibit cellular proliferation. Introduction of peptides into human cells that functionally antagonize E2F DNA binding activity resulted in rapid onset of apoptosis (169). In another setting, peptides that bound to the DNA binding domain of E2F and blocked its association with DP1 resulted in a G1 block in mammalian fibroblasts (170). Although these experiments clearly indicate that blocking E2F DNA-binding activity could inhibit cell proliferation and sometimes cause apoptosis, it

is not clear how inhibiting this interaction would selectively target cancer cells in comparison to normal proliferating cells.

The latest findings of new family members, new biological activities and a plethora of novel target genes have gained significant attention towards the E2F transcription factor. It is almost impossible to tie E2F to one unified model of transcriptional regulation. It is clear however, that this family has diverse and sometimes opposing activities (oncogene or tumor suppressor), the signaling pathways involved vary depending on the setting. The E2F field has matured and new discoveries will possibly conclude the specific roles of E2F in normal development and tumorigenesis. The possibility of using E2F effector pathways for enhancement of chemotherapeutic drugs may provide useful tools for drug development of E2F activators or repressors. Since E2F can both stimulate proliferation as well as induce apoptosis, developing both E2F antagonists (to block proliferation) and E2F agonists (to promote apoptosis) seems a daunting task. It is difficult to determine whether there will be a positive or negative effect from these therapies. E2F family of proteins are involved in regulating an abundance of genes; determining which genes to shut off or turn on by E2F may result in complicated and insufficient therapies. A more simplistic approach to inhibiting proliferation would incorporate targeting upstream of E2F, i.e. Rb protein.

3. Upstream regulators of Rb function in proliferation

It is well established that the Rb protein is inactivated by kinases associated with D and E type cyclins and this facilitates inactivation of Rb and S-phase entry (22). Cyclin dependent Rb regulation is well studied; it is the non-cyclin dependent regulation of Rb that is less understood. This section discusses the upstream regulators of Rb in proliferation by various stimuli including growth factors and nicotine.

3.1 Regulation of Rb by growth factor stimulation

Since inactivation of the Rb protein is widespread in many forms of disease it is vital to understand the mechanisms involved. The Ras/Raf/MEK/MAPK pathway signaling pathway functions in a growth factor dependent manner to upregulate cyclin D1 dependent kinase activity and this in turn regulates Rb phosphorylation and its cell cycle functions (171). It has been shown that components of the MAP kinase cascade, including ERK kinases and Raf-1 kinase can phosphorylate Rb in response to proliferative signals (172,173). One study revealed that Rb is rapidly phosphorylated on Serine 795 upon treatment of vascular smooth muscle cells with angiotensin II or 5-hydroxy-tryptamine and this phosphorylation could be inhibited by blocking MEK activity (172). In other studies, the role for MAPK cascade in inactivating Rb has been shown using wild type mouse embryonic fibroblasts (MEFs). MEFs containing wild type Rb required the activation of the MAPK cascade to enter the cell cycle and MEFs lacking Rb did not (65,174).

It has been suggested that the MAP kinase cascade can phosphorylate Rb in response to proliferative signals. This cascade is initiated by ligand bound cell surface tyrosine kinase receptors such as epidermal growth factor receptor (EGFR), HER-2, vascular EGFR (VEGFR) and platelet derived growth factor receptor (PDGFR) leading to the activation of Ras (175,176). The tyrosine kinase receptor becomes phosphorylated upon ligand binding and recruits the adaptor protein Grb2 and SOS the guanine nucleotide exchange factor to activate Ras by exchanging GDP for GTP. Active Ras-GTP recruits Raf from the cytosol to the plasma membrane for activation by itself and other kinases, such as PAK and Src (177-179). Active Raf binds and phosphorylates MEK on two serine residues (217, 221) in the kinase domain (175). MEK then binds and phosphorylates ERK1 and ERK2 on Thr202/Tyr204 and Thr185/Tyr187 respectively (175). Activated ERK acts on several downstream substrates involved in the induction of numerous transcription factors and genes such as myc, c-fos, elk1, p90rsk (175,180-182) **(Figure 4)**. These genes are known to be involved in promoting cellular proliferation, differentiation, cytoskeletal changes, cellular motility and extracellular matrix remodeling among many others (183-185). This pathway is hyperactivated in 30% of all human tumors. Although the proteins of the Ras/Raf/MEK/ERK pathway are mutated in many cancers, direct mutations of Raf-1 leading to tumorigenesis have not been identified (186,187). It is the downstream effector of Raf-1, ERK that impinges on all stages of malignant transformation (188). The potential for Raf-1 to play a huge role in tumorigenesis

is evidenced by its broad activation by many kinases independent of Ras, like PKCα, Src, JAK, and Pak (177-179). Raf-1 has also been implicated in promoting expression of the multi-drug resistance gene *MDR1* (189). There are several clinical trials currently underway that target Raf-1 (190,191).

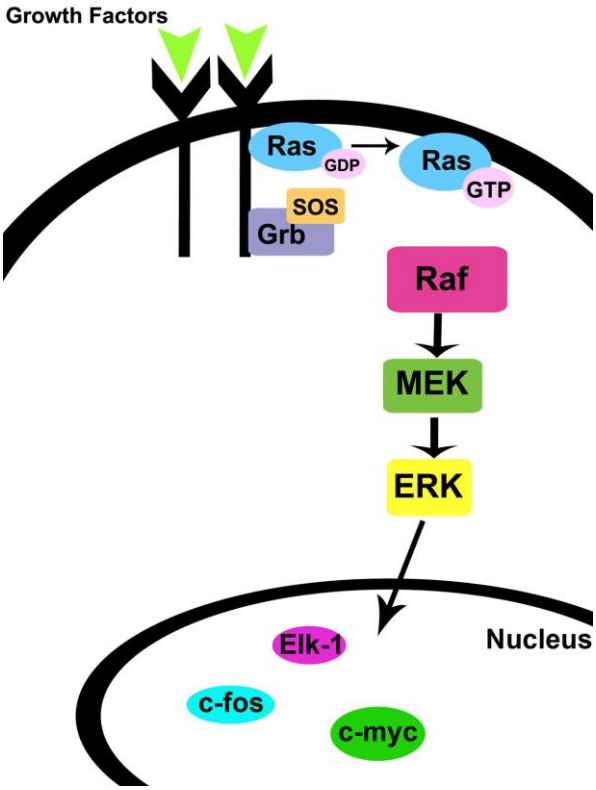


Figure 4. Binding of growth factors to the cell surface receptor tyrosine kinases (RTKs) signals through adaptor proteins such as growth factor receptor bound-2 (Grb2) and guanine nucleotide exchange factors like son-of-sevenless (SOS) activate Ras by exchanging GDP for GTP on Ras. Active Ras initiates membrane recruitment and activation of Raf, which leads to activation of dual specificity mitogen activated protein kinase (MAPK or MEK) and subsequently extracellular signal regulated kinase (ERK). Activated ERK acts on several nuclear transcription factors such as c-myc, c-fos and Elk-1.

3.1.1 Raf-1 Kinase

C-Raf, in particular (referred to as Raf-1), was identified in humans as the cellular homologue of the *v-Raf* oncogene (192). Shortly after, A-Raf was identified and had 85% sequence homology to Raf-1 in its central 100 amino acids (193). Identification of B-Raf, another member of the Raf family, linked all three *Raf* genes together **(Figure 5)**; approximately 75% of the kinase domain was conserved on all Raf genes (194). Raf-1 is the most studied of the isoforms, yet it remains poorly understood. Raf-1 ranges in size from 72-74 kDa and can be localized in the membrane, cytoplasm as well as the mitochondria; its presence in the mitochondria has been correlated with a role in apoptosis (176,195). Raf-1 has also been shown to translocate to the nucleus upon mitogenic stimulation (173). Expression of Raf-1 is ubiquitous in adult tissues and has the highest expression in muscle, cerebellum, and fetal brain (196). Raf-$1^{-/-}$ mice show a recessive lethal phenotype, are growth retarded and die midgestation. The fetal livers of these mice contain a high number of apoptotic cells. Raf-$1^{-/-}$ embryos stained with platelet endothelial cell adhesion molecule 1 (PECAM-1) revealed a reduction in the number of vessels and showed abnormal vascular network formation in the head region. ERK activation was not affected in Raf-1 knockout mice indicating that the phenotypes seen are due to lack of signaling through Raf-1 effector proteins independent of the ERK pathway (197). It has been shown that B-Raf can compensate for Raf-1 by activating MEK kinase raising the possibility that it is compensating for Raf-1 function in the MAP

kinase cascade in Raf-1 null mice. Mice containing a ‘knockin’ mutation of endogenous 340 and 341 tyrosines to phenylalanine (*raf-1*$^{FF/FF}$) resulting in an inactive form of Raf, survive to adulthood and ERK activation was not compromised despite the nonfunctional Raf-1 (197). MEK-independent functions of Raf-1 have garnered a significant amount of attention. The Bcl-2 anti-apoptotic protein can target Raf-1 to the mitochondria and together they cooperate in suppressing apoptosis (195). Bcl-2 binding protein Bag1 can activate Raf-1 *in vitro* and *in vivo* further increasing Raf-1’s antiapoptotic activities (198). Pak1 phosphorylates Raf-1 on S338 and S339, this activation by Pak is thought to direct Raf-1 to the mitochondria where it interacts with Bcl-2 by phosphorylating BAD and displacing BAD from Bcl-2 (199). Just as the role of Raf-1 in cell proliferation has provided therapeutic avenues for development of treatments, the role of Raf-1 and Bcl-2 in resistance to apoptosis may provide a target for inducing apoptosis in cancers with a constitutive activation of Raf-1 kinase. The cAMP-dependent protein kinase (PKA) is activated by second messenger cAMP. PKA then phosphorylates serine 43 on Raf-1 which is inhibitory for Raf-1 kinase activation (185).

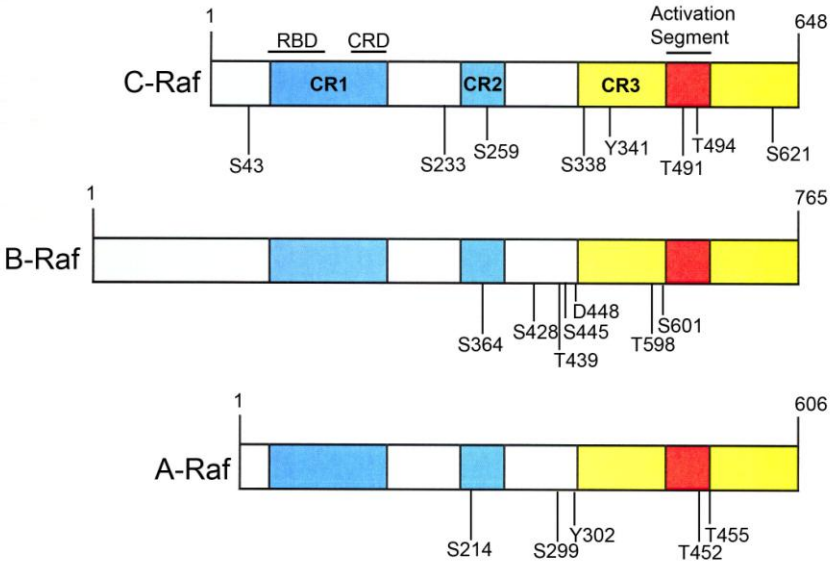


Figure 5. Domain structures of the Raf kinase family. There are three conserved regions on Raf kinases and remain conserved across isoforms and species. The N terminal CR1 domain contains a Ras binding domain (RBD and CRD). The CR2 domain is a serine/threonine rich domain. CR3 is the catalytic kinase domain. S43 and S259 in C-Raf (Raf-1), S364, S428 and T439 in B-Raf are inhibitory phosphorylation sites.

3.1.2 Raf-1 in Cancer

Gene rearrangements, point mutations, and truncations leading to constitutive activation of Raf-1 have been identified in several cancers, most notably in small cell lung cancer (SCLC) (200). Raf-1 overexpression has been linked with colon cancer and lung cancer cell lines, but not in human cancer tissues (175,183). One group from the Cancer Research UK Centre has linked Raf-1 expression with ovarian cancer cell lines. It was found that Raf-1 was the predominant Raf isoform accountable for regulating cell growth and apoptosis in ovarian cancer cell lines (201). Although the studies of Raf-1 expression have only been linked to cancer cell lines, it does confirm the role for Raf-1 in cancer development. Another study implicating Raf-1 in cancer involves the Raf-1 kinase inhibitor protein (RKIP). RKIP inhibits the phosphorylation of MEK by Raf-1 (202). In 103 human breast cancer specimens and lymph node metastasis examined, it was observed that RKIP expression was significantly reduced or completely lost in the lymph node metastasis compared to the normal levels of RKIP in the primary tumor (203). This suggests that the loss of an endogenous Raf-1 inhibitor might contribute to breast cancer metastasis. Although Raf-1 is not mutated extensively in cancers like Ras or p53 genes, it might contribute to tumorigenesis independent of Ras as well. This contention is supported by the fact that Raf-1 can be activated by Bcl-2 protein binding protein Bag1, protein kinase C-alpha (PKC-α) and has been linked to expression of the multidrug resistance gene mdr1 (176,195,198,204,205). Thus Raf-1 appears to be ideally placed to affect

the proliferation as well as apoptosis of cells in Ras-dependent and independent fashions, depending on the signaling event. Alterations in the signaling events or components can potentially lead to oncogenesis, via mediation of Raf-1.

Several advances have been made toward understanding the potential of improper activation of ERK signaling. Alterations in *Ras* genes are the most frequently detected mutations in cancer. Ras alterations are associated with 90% of pancreatic cancer, 50% of thyroid cancer, 50% of colon cancer, 30% of lung cancer, and 30% of acute myeloid leukemia (AML) (206).

3.1.3 Regulation of Rb by Raf-1

Experiments in yeast two-hybrid assays and in vitro binding assays revealed that Raf-1 could bind to Rb and p130, not p107 (173). Raf-1 was also found to bind Rb and p130 in Immunoprecipitation-Western blot experiments (173). The Rb-Raf-1 interaction was time course dependent; Rb-Raf-1 interaction is not detected in quiescent cells. Cells that were subsequently stimulated with serum showed Rb-Raf-1 interaction from 30 minutes to 2 hours of stimulation. After 2 hours of serum stimulation, Raf-1-Rb interaction was no longer detected (173). Rb is a nuclear protein and Raf-1 is predominantly cytoplasmic with activation occurring at the plasma membrane. A portion of Raf-1 was found to translocate to the nucleus upon serum stimulation where it bound to Rb (173) **(Figure 6)**. Raf-1 could efficiently phosphorylate Rb *in vitro*. Raf-1 could inactivate Rb and reverse Rb mediated repression of E2F1 in transcriptional activity assays as well as S-phase entry assays.

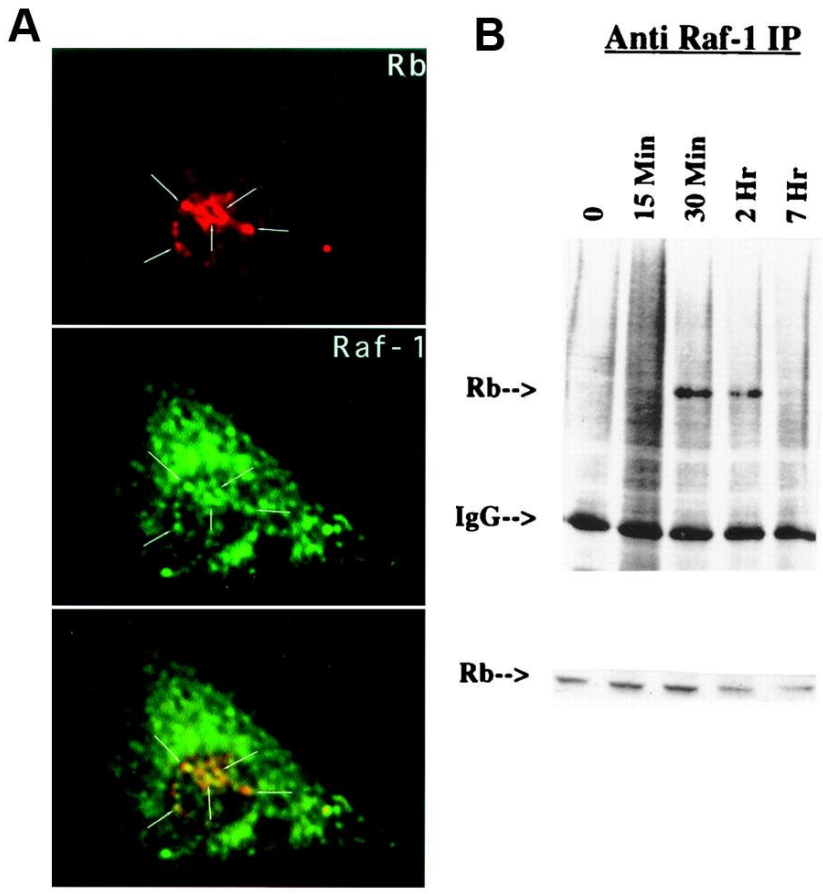


Figure 6. (A) Colocalization of Raf-1 and Rb in the nucleus of HSF8 cells. Colocalization can be observed in yellow (bottom panel). (B) The Rb-Raf-1 interaction is induced by serum. Extracts from HSF8 fibroblast cells stimulated with serum for the indicated time points were immunoprecipitated with monoclonal Raf-1 antibody. The presence of Rb was detected by western blot analysis. The Rb-Raf-1 interaction occurs from 30minutes to 2 hours of serum stimulation. Adapted with permission from Wang et al (173).

3.1.4 Raf-1 as a target for cancer therapy

Mutated Ras and Raf-1 are constitutively active and have transforming potential *in vitro.* It is apparent that mutations leading to Raf activation are the force behind many different types of malignancies and there is solid proof of principle for B-Raf and Raf-1 to serve as targets in cancer therapy (207). Although antisense oligonucleotide (ASO) therapy has been attempted and not been efficacious in clinical trials, this underscores the need to optimize this therapy in a patient specific manner (208). Poor results with ASO therapy does not mean that Raf-1 does not serve as an outstanding potential target, essentially, this therapy needs better regimens for inhibiting Raf-1 mRNA. Recent candidate drugs such as nanoparticles conjugated to a mutant form of Raf-1, B-Raf inhibitors and Rb-Raf-1 protein-protein inhibitors (discussed in this thesis) will provide valuable insights into the molecular biology of Raf signaling in cancer (209-211).

The BAY-43-9006 compound, termed sorafenib, was originally identified as a small molecule inhibitor of Raf-1. Further characterization of the bi-aryl urea compound demonstrated inhibition of wild type B-Raf and mutant B-Raf kinase, VEGFR-2, mVEGFR-3, mPDGFR-β, Flt-3, c-KIT, and FGFR-1 (212). Sorafenib inhibits Raf-1 and mVEGFR2 activity with an IC_{50} of 6nM; it's IC_{50}s for B-Raf mut, B-Raft wt, VEGFR2, mVEGFR3, Flt-3, c-kit, p38α, and mPDGFR-β ranges from 12-68nM. Sorafenib is highly selective for Raf-1 and B-Raf showing no activity

against downstream MEK and ERK (212). The FDA has approved Sorafenib for the treatment of advanced RCC since previous phase II and phase III results showed significant responses specifically in RCC patients. A phase II placebo controlled randomized discontinuation trial of sorafenib for patients with metastatic RCC resulted in 50% of patients being progression free at 24-weeks. This result showed significant disease stabilizing activity with the tolerability of daily therapy in comparison to the standard of care (cytokine therapy, IL-2) for RCC (213). RCC commonly has mutations in VHL (Von Hippel-Lindau) gene leading to increased production of VEGF, which makes these tumors largely dependent on VEGF-mediated angiogenesis (214). Sorafenib is likely functioning in RCC because of its ability to inhibit VEGFR and the kinases involved in signaling production of VEGF.

3.1.5 Role of Rb-Raf-1 Interaction in Cancer

Given the fact that both Rb and Raf-1 play important roles in cancer cell signaling pathways; the Rb-Raf-1 interaction was examined in cancer. Whole-cell lysates were prepared from ten non-small cell lung carcinomas (NSCLC) as well as the adjacent normal tissue that were resected from patients. The Rb-Raf-1 interaction was examined by IP-WB. In eight out of 10 matched pairs the Rb-Raf-1 interaction was elevated in the tumor tissue compared to the normal adjacent tissue (215). ChIP assays also revealed a similar result in NSCLC tumor tissues; more Raf-1 was found on the proliferative promoters *cdc6* and *cdc25A* in tumor

tissue compared to the normal tissue **(Figure 7)** (215). This suggests that the Rb-Raf-1 interaction might have contributed to the oncogenesis of the tumors.

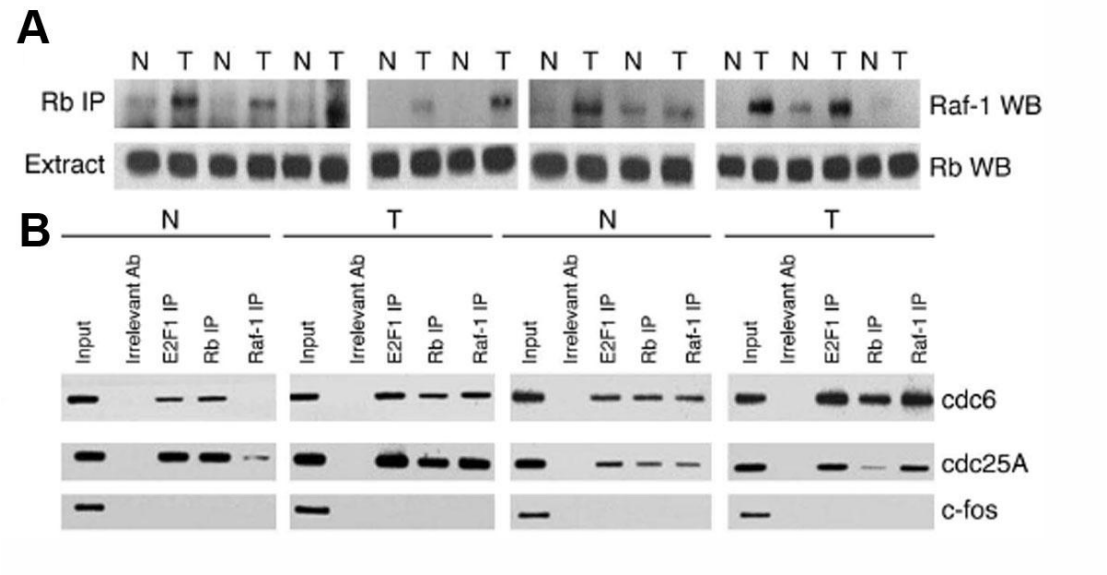


Figure 7. The Rb-Raf-1 interaction is elevated in tumor samples. (A) NSCLC tumors (T) contained more Rb-Raf-1 complexes than adjacent normal tissue (N). Rb-Raf-1 interaction was assessed by IP-WB on nuclear extracts. (B) ChIP assays on human NSCLC tumor samples show that more Raf-1 was present on both *cdc6* and *cdc25A* promoters in tumor samples compared to adjacent normal tissue. Adapted with permission from Dasgupta et al (215).

3.1.6 Disruption of the Rb-Raf-1 Interaction

The Rb-Raf-1 interaction was found to occur on amino acids 10-18 in the N-terminal region of Raf-1 (209). Raf-1 seems to function similar to viral oncoproteins; stable binding is required for inactivation of Rb and Raf-1 binds in the pocket domain of Rb. One major difference is viral oncoproteins dissociate E2F1 from Rb and Raf-1 does not. A peptide corresponding to amino acids 10-18 on Raf-1 was created to examine disruption of the Rb-Raf-1 interaction. The peptide sequence is ISNGFGFK, a C was added to the carboxyl terminal end to allow coupling to the carrier molecule penetratin. The Raf-1 peptide (1μM) could inhibit the Rb-Raf-1 interaction without inhibiting the binding of other proteins to Rb or Raf-1 (209). The Raf-1 peptide pen-conjugate could disrupt the binding of Rb-Raf-1 in cells; this was shown in confocal colocalization experiments as well as several other biochemical assays **(Figure 8)**. Kinetic experiments showed that the Rb-Raf-1 interaction occurred as early as 30 minutes from serum stimulation up to 4 hours, and this binding preceded the binding of cyclin D. Rb phosphorylation was also found at two hours of serum stimulation (time when Raf-1 is found to bind to Rb). More surprisingly, the inhibition of Rb-Raf-1 with the Raf-1 peptide pen-conjugate could significantly inhibit Rb phosphorylation even up to 16 hours post serum stimulation (209). Since Raf-1 binding to Rb does not cause E2F1 to dissociate yet could reverse Rb mediated repression of E2F1, it was examined how Raf-1 de-represses E2F1. Chromatin Immunoprecipitation assays (ChIPs) and Immunoprecipitation western blot

assays (IP-WBs) revealed that Raf-1 binding to Rb led to the dissociation of chromatin remodeling protein Brg1 from Rb. Although other corepressors are present, Raf-1 seems to specifically dissociate Brg1 from the promoters of E2F regulated genes. Treatment with the Raf-1 peptide pen-conjugate led to Brg1 recruitment on proliferative promoters. There was no change in the binding of HDAC1 and HP1. Disruption of the Rb-Raf-1 interaction with the Raf-1 peptide pen-conjugate also significantly inhibited proliferation (209). The peptide pen-conjugate could inhibit 50% of cells from entering S-phase. The peptide pen-conjugate efficiently inhibited angiogenic tubule formation in matrigel assays as well as adhesion, migration and invasion of human aortic endothelial cells (HAECs)(209). An anti-angiogenic and anti-proliferative agent can be expected to inhibit tumor growth since these are hallmarks of cancer. A549 human xenograft tumor growth was inhibited approximately 80% from treatment with the Raf-1 peptide pen-conjugate intratumorally (209). These results clearly demonstrated that disruption of Rb-Raf-1 interaction could efficiently inhibit tumor growth and angiogenesis in vivo. It can be assumed that small molecules that are capable of inhibiting the Rb-Raf-1 interaction have therapeutic potential for controlling proliferative disorders such as cancer. Essentially, the abovementioned studies led to the screening of small molecule libraries for compounds capable of inhibiting Rb-Raf-1 interaction, these experiments and more will be discussed in chapter 3 of this thesis.

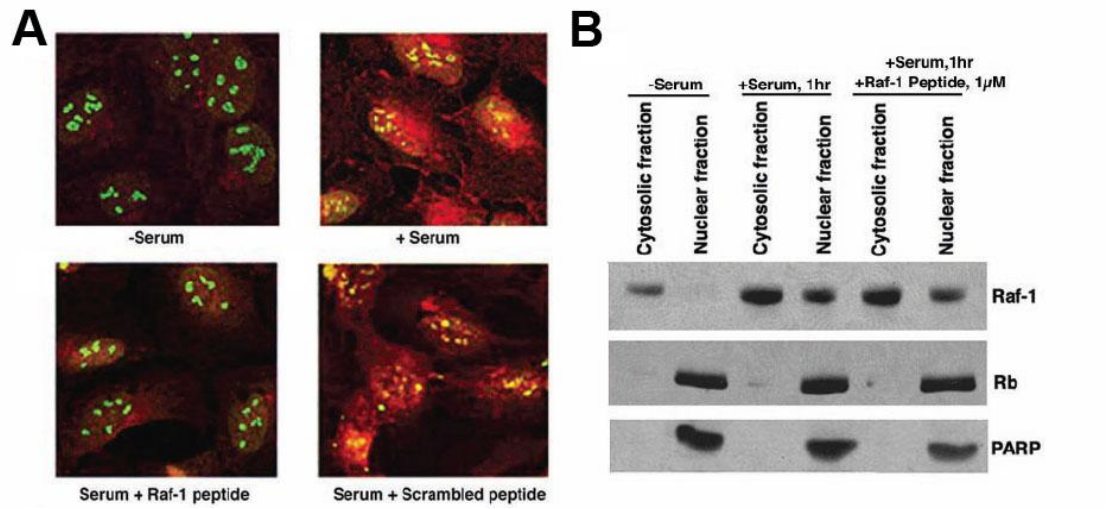


Figure 8. The penetratin-Raf-1 conjugate can inhibit Rb-Raf-1 interaction in intact cells. (A) U2-OS cells immunostained with Raf-1 (Red) and Rb (green) were visualized by confocal microscopy. Serum starved cells show no association of Raf-1 with Rb. Serum stimulation induces Raf-1 to translocate to the nucleus where it binds Rb, colocalization is seen in yellow. The presence of 1μM of the Raf-1 peptide conjugate could inhibit the binding of Raf-1 to Rb. The Raf-1 scrambled peptide has no effect on Raf-1 Rb binding. (B) Nuclear and cytosolic extracts revealed that Raf-1 peptide conjugate does not affect the nuclear translocation of Raf-1. Adapted with permission from Dasgupta et al (209).

3.2 Growth factor independent regulation of Rb

The Rb protein contains approximately 18 potential phosphorylation sites, cdk4/6 has been shown to target 4 residues C-terminal to the pocket domain (216,217). Cyclin D associated kinases; cyclin E-cdk2 complexes have also been shown to modulate Rb function. Although it has been shown that there is a clear link between growth factor stimulated Ras/MAPK pathway and Rb phosphorylation-cell cycle, other non-growth factors regulating this pathway have not been defined. Studies involving hormones and neurotransmitters have also revealed a link between Ras/MAPK signaling and Rb-E2F pathway. Treatment with Angiotensin II or Serotonin could induce phosphorylation of serine 795 on Rb and this activation was mediated by CDK4 and MAPK pathway (172). Stimulation with either Serotonin or Angiotensin II also resulted in dissociation of E2F from Rb (172). These studies support the idea that Rb is regulated during growth factor dependent stimulation as well as non growth factor activation.

3.2.1 Rb inactivation upon nicotine stimulation via nicotinic acetylcholine receptors (nAChRs)

Non-small cell lung cancer (NSCLC) is associated with 80% of the total number of lung cancer cases and is strongly associated with tobacco use. There are several carcinogenic compounds found in tobacco smoke such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN); these molecules can form DNA adducts leading to mutations in vital

genes like Ras, p53, and Rb (218,219). The carcinogen NNK that is structurally related to nicotine has been shown to induce proliferation and angiogenesis through nicotinic acetylcholine receptor subunits (nAChRs).

nAChRs are pentameric proteins consisting of nine α subunits (α2-α10) and three β subunits (β2-β4) in non-neuronal cells; and γ subunits are present in neuronal systems (220). Studies in recent years have shown that nicotinic receptors are also present in a wide variety of non-neuronal tissues, including human bronchial epithelial cells, human endothelial cells and astrocytes (220-223). These observations led to the realization that signaling through the nicotinic acetylcholine receptors could have functional roles in non-neuronal cells as well. The finding that nAChRs are present on non-neuronal cells was followed by the observation that nicotine could induce the proliferation of endothelial cells (221,224). Further, it was found that nicotine and structurally related carcinogens like NNK could induce the proliferation of a variety of small cell lung carcinoma cell lines (225,226). Studies from Dr. Schuller's lab revealed that nicotine stimulation induces the activation of Raf-1 (227). In addition to these studies, it has been shown that persistent nicotine exposure stimulates Ras signaling and MAPK activation in mouse epithelial cells (228). Nicotine was also shown to induce the cyclin D1 promoter and therefore cell cycle (228).

Recently, our lab has demonstrated the how nicotine signaling involves

the Rb-E2F pathway and promotes cell cycle entry. Nicotine stimulation of NSCLC cell lines leads to the binding of β-Arrestin to the α-7 nAChR, which in turn activates Src kinase (215). The activation of Src leads to the activation and binding of Raf-1 to Rb. Raf-1 can bind to Rb and initiate its inactivation facilitating cell cycle progression **(Figure 9)**. Nicotine stimulation resulted in dissociation of E2F1 from Rb and this correlated with the induction of cyclin/cdk activity as well as Rb phosphorylation. In response to nicotine stimulation, proliferative promoters *cdc6* and *cdc25A* were found to have more E2F1 and dissociation of Rb (215). Nicotine functions via the α7 nAChR upstream of Rb-E2F pathway facilitating cell cycle progression.

This led to the hypothesis that nicotine might be playing a direct role in the progression of human lung cancers. While there is no evidence that nicotine contributes to the induction of tumors, it has been demonstrated that nicotine promotes the growth of solid tumors *in vivo*, suggesting that nicotine might be playing a more important role in the progression of tumors already initiated (229,230). Chapter 4 focuses on the role of nicotine in tumor growth and metastasis.

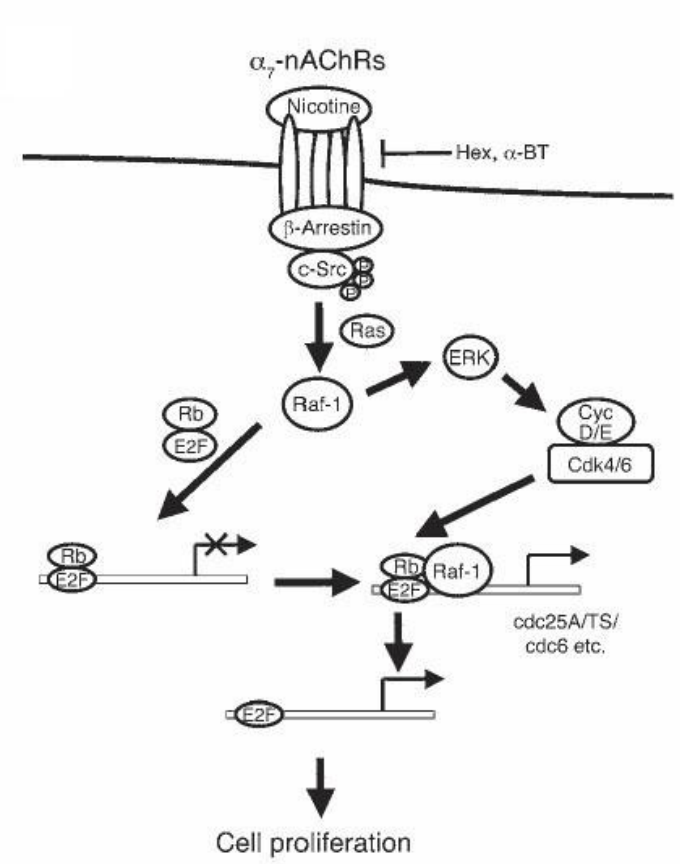


Figure 9. Schematic predicting the proliferative signaling by nAChRs in NSCLC cells. Nicotine stimulation causes the assembly of oligomeric complexes involving β-Arrestin, Src and nAChRs, facilitating the activation of Src. This leads to the activation of Raf-1, which binds to Rb; activation of MAPK and cyclins/cdks also occur. The activation of Src facilitates the binding of Raf-1 to Rb and multimeric complexes containing Rb, Raf-1 and E2F1 occupy proliferative promoters. Sustained mitogenic signaling leads to the dissociation of Raf-1 and Rb, while E2F remains bound to the promoter facilitating S-phase entry. Adapted with permission from Dasgupta et al (215).

4. Upstream regulators of Rb-E2F Function in Apoptosis

Several attempts have been made to understand how extracellular signals modulate Rb and E2F function to bring about cellular apoptosis. It has been suggested that suppression of apoptosis may be the primary function of Rb, independent of its anti-proliferative activity. It has been shown that Rb is inactivated upon apoptotic signaling as well as proliferative signaling (231-233).

4.1 Apoptotic Signaling Pathways Regulate Rb Function

In an earlier study, experiments were done to assess whether kinases involved in non-proliferative pathways like JNK and p38 affect Rb/E2F function (234). These two kinases were found to have opposite effects on E2F function: the stress-induced kinase JNK1 inhibits E2F1 activity whereas the related p38 kinase reverses Rb-mediated repression of E2F1. JNK1 could phosphorylate E2F1 *in vitro* reducing the DNA binding activity. Phosphorylation of Rb by p38 kinase upon Fas stimulation resulted in the dissociation of E2F and increased transcriptional activity. The inactivation of Rb by Fas was blocked by SB203580, a p38-specific inhibitor, as well as a dominant-negative p38 construct; cyclin-dependent kinase (cdk) inhibitors as well as dominant-negative cdks had no effect (235). These results suggest that Fas-mediated inactivation of Rb is mediated via the p38 kinase, independent of cdks. The Rb/E2F-mediated cell cycle regulatory pathway appears to be a normal target for non-mitogenic signaling cascades and could be involved in mediating the cellular effects of such

signals (234,236).

It has also been shown that the apoptotic signal-regulating kinase 1 (ASK1) kinase can modulate apoptotic signaling by affecting Rb function (237). It was found that ASK1 kinase had to overcome the anti-apoptotic effects of Rb to induce cell death. ASK1 was found to directly associate with Rb protein leading to its dissociation from specific pro-apoptotic promoters like p73. Release of Rb from pro-apoptotic promoters coincided with its enrichment on proliferative promoters; it appears that this is a mechanism to prevent inappropriate cell cycle entry in adverse conditions. This suggests that the ASK1-p38 kinases are able to modulate cellular apoptosis by modulating Rb function as well as the transcriptional activity of E2F1 **(Figure 10)**. It can be assumed that during mitogenic scenarios, Rb binds to prosurvival kinases such as Raf-1 and cyclins/cdks to promotes proliferation. In the presence of apoptotic stimuli, Rb binds to apoptotic kinase ASK1. Even though both of these interactions can inactivate Rb and activate E2F1 transcriptional activity, they induce different promoters: such as *p73* (apoptotic stimuli) or *cdc25A* (mitogenic stimuli)(237).

One very interesting finding is that the specific stress stimulus of tumor necrosis factor-alpha (TNF-α) regulates Rb function very differently depending on the cellular context. TNF-α is a pleiotropic inflammatory cytokine and has been shown to play two very important opposing roles in both inhibition of endothelial

cell proliferation and enhancement of apoptosis, yet stimulation of vascular smooth muscle cell proliferation and migration (238,239). TNF-α, like other chemoattractants such as PDGF, stimulates VSMC migration through the MAPK pathway (240). It has been shown that TNFα can induce Rb phosphorylation via p38 and ASK1 kinases, leading to apoptosis in most cells, including human aortic endothelial cells (HAECs). One intriguing exception to this is in vascular smooth muscle cells (VSMCs), where TNF-α is capable of inducing proliferation. Migration of VSMCs is a crucial event in the formation of vascular stenotic lesions. TNF-α is upregulated by VSMCs in atherosclerosis and following angioplasty. The VSMC response to TNF-α provides a therapeutic possibility to prevent VSMC proliferation and therefore block restenosis. Chapter 5 reveals new insights into TNF-α induced VSMC proliferation via Rb-Raf-1 and MAPK pathways.

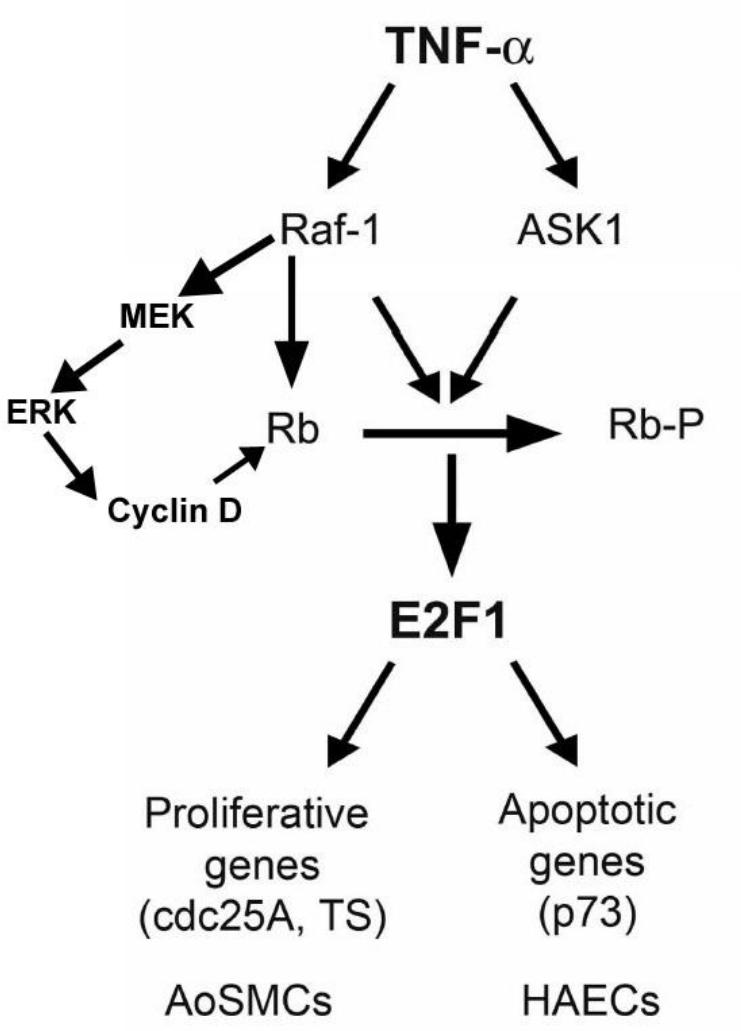


Figure 10. A model for the Rb/E2F pathway in cell proliferation and apoptosis in AoSMCs and HAECs upon TNF-α stimulation. In AoSMCs, TNF-α stimulates binding of Raf-1 to Rb, facilitating its inactivation and stimulating cell cycle progression. In HAECs, TNF-α stimulates the binding of ASK1 to Rb, leading to inactivation and E2F1 inducing apoptosis through p73. The inactivation of Rb releases E2F1, which can bind to proliferative or apoptotic promoters and make vital decisions on cell survival or death.

5. Summary

Rb plays a central role in cellular homeostasis. It acts as the main component of a very complex network in which cell cycle is regulated; it can be imagined that regulation of Rb is often disrupted in various diseases. Studies in mice genetically deficient in Rb in all hematopoietic cells revealed a significant role for Rb in hematopoiesis; suggesting that Rb is involved in many different types of cells in the body (241). Rb has also been linked to atherosclerosis and restenosis. In addition to cancer and heart disease, Rb/E2F pathway is found to be altered in human and mouse ulcerative colitis (Crohn's disease) (242). Studies from our laboratory have been focused specifically on Rb-E2F signaling pathways in lung cancer and heart disease, namely atherosclerosis.

Development of atherosclerosis is a stringently regulated and complex process that occurs as a result of aberrations in endothelial cell and smooth muscle cell (SMCs) function. Endothelial cells (EC) form the lining of the blood vessels and the heart, functioning as a barrier by regulating permeability, thrombogenicity, and production of growth inhibitory molecules (243). Endothelial cells also respond to mechanical forces. ECs are contact inhibited under normal conditions; but when endothelial cells sense an injury such as abrasion of a vessel, they proliferate and migrate leading to reendothelialization at sites of injury (244). At the same time, vascular smooth muscle cells proliferate and

migrate from the injured arterial wall into the vessel lumen leading to vessel thickening and occlusion, called restenosis (245). Intimal hyperplasia characterized by VSMC proliferation and extracellular matrix (ECM) deposition is a major process contributing to restenosis (246). Atherosclerotic lesions can be blocked if inhibition of VSMCs is effective (243). Several growth factors and cytokines are capable of stimulating VSMC migration and proliferation, such as platelet derived growth factor (PDGF), which plays a vital role in the development of restenosis (247). VSMC proliferation leads to downstream activation of Raf/MEK/ERK signaling pathway, which in turn inactivates Rb to induce cell proliferation. We have shown that an effective way to inhibit endothelial cell migration and invasion is through disruption of Rb-Raf-1. Targeting Rb-Raf-1 with small molecule inhibitors to prevent VSMC migration and invasion will serve as viable targets for drug therapy for vascular proliferative disorders.

The studies described indicate the role of Raf-1 binding to and regulating Rb's function. Mitogenic and non-mitogenic stimulation have been shown to induce this interaction in a variety of cell types (209,215). Raf-1 can bind and inactivate Rb and this facilitates further phosphorylation by cyclins/cdks and cell cycle progression. The Rb-Raf-1 interaction is elevated in NSCLC tumors suggesting this interaction plays a role in the oncogenesis of the tumors (215). The Rb-Raf-1 interaction may be regulating two very important hallmarks of cancer; proliferation and angiogenesis. Targeting the protein-protein interaction

with the Raf-1 peptide could prevent S-phase entry, inhibit angiogenesis and tumor growth in nude mice (209). Disruption of this interaction with peptides or small molecule inhibitors is a viable alternative to controlling proliferative disorders such as cancer and atherosclerosis **(Figure 11)**.

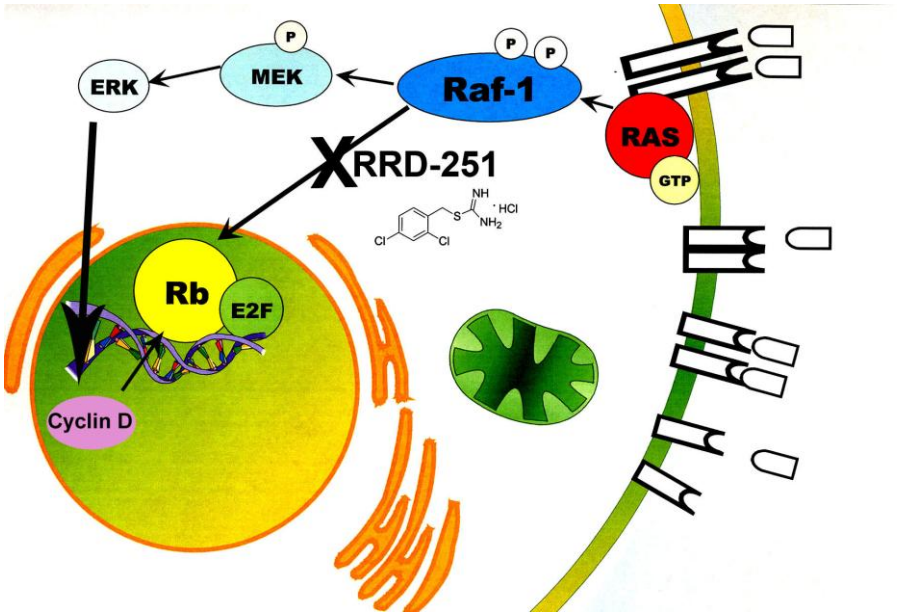


Figure 11. Schematic depicting the Raf-1-Rb signaling pathway. Small molecule inhibitors capable of disrupting the Rb-Raf-1 interaction is a viable strategy to prevent cell cycle progression, invasion, migration, angiogenesis and tumor growth.

Chapter 2: Materials and Methods

Cell culture and transfection

The human promyelocytic leukemia cell line U937 was cultured in RPMI (Mediatech) containing 10% fetal bovine serum (FBS; Mediatech). U2-OS, Saos-2, PANC1, CAPAN2, A375, DU145, SK-MEL-5, SK-MEL-28, MDA-MB-468, H1299 and MDA-MB-231 cell lines were cultured in Dulbecco modified Eagle Medium (DMEM; Mediatech) containing 10% FBS. A549 cells and A549 shRNA Rb cell lines were maintained in Ham F-12K supplemented with 10% FBS. ShRNA cells lines were maintained in media containing 0.5μg/ml puromycin. Line1, H1650, H596, H2172, PC-9, LNCap, PC3 and Aspc1 cell lines were cultured in RPMI (Gibco) containing 10% FBS. Human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) were obtained from Lonza and cultured in endothelial growth medium, supplemented with 5% FBS, according to the manufacturer's instructions. Human aortic smooth muscle cells were obtained from Lonza and cultured in smooth muscle basal medium, supplemented with growth factors and 5% FBS, according to manufacturer's instructions. U251MG and U87MG glioma cell lines were maintained in DMEM supplemented with non-essential amino acids, 50mM β-mercaptoethanol, and

10% FBS. Nicotine (Sigma) was dosed at 1μM concentration for all nicotine experiments. TNF-α (Promega) was added at 100ng/ml. PDGF (Biosource) was added at 100ng/ml concentration. ShRNA cell lines were made by stably transfecting A549 cells with two different shRNA constructs that specifically target Rb obtained from a shRNAmir library from Open Biosystems, Huntsville, AL.

In vitro drug library screening

ELISA 96-well plates (Nunc) were coated with 1μg/ml of GST Raf-1 (1-149aa) overnight at 4°C. Subsequently the plates were blocked for 1 hour. GST Rb at 20μg/ml was pre-incubated at RT for 30 minutes in the presence or absence of the compounds at 20μM. This GST Rb was added to the plate and incubated for 90min at 37°C. The amount of Rb bound to Raf-1 was detected by Rb polyclonal antibody (Santa Cruz) 1:1000 incubated for 60 min at 37°C. Donkey-anti-rabbit-IgG-HRP (1:10,000) was added to the plate and incubated at 37°C for 60 minutes. The color was developed with orthophenylenediamine peroxidase substrate tablets (Sigma) and the reaction was terminated with 3M H_2SO_4. Absorbance was read at 490nm. To determine disruption of Rb to E2F1, Phb, or HDAC1 the above protocol was used with the exception of coating GST Rb on the ELISA plate and adding the drugs in the presence or absence of GST E2F1, Phb, or HDAC1. E2F1 monoclonal antibody (Santa Cruz) (1:2000) was used to detect the amount of Rb bound to E2F1. Prohibitin monoclonal antibody (NeoMarkers) was used at 1:1000 to detect the amount of Rb bound to

Prohibitin. HDAC1 polyclonal antibody (Santa Cruz) was used at 1:1000 to detect the amount of Rb bound to HDAC1. For disruption of Mek-Raf-1 binding ELISAs, Raf-1 1μg/ml was coated on the plate and GST Mek (20μg/ml) was incubated in the presence or absence of the compounds for 30 minutes at room temperature. Mek1 polyclonal antibody (Cell Signaling) was used at 1:1000 to detect the binding of Raf-1 to Mek1. The IC_{50} concentrations for the Rb-Raf-1 inhibitors were determined by plotting with Origin 7.5 software.

Lysate preparation, immunoprecipitation, and Western blotting

Lysates from cells treated with different agents were prepared by NP-40 lysis as described earlier (209). Tumor lysates were prepared with T-Per tissue lysis buffer (Pierce) and a Fischer PowerGen 125 dounce homogenizer (248). Physical interaction between proteins *in vivo* was analyzed by immunoprecipitation-western blot analyses with 200μg of lysate and 1μg of the indicated antibody as previously described. Polyclonal E2F1, B-Raf, ASK1, Cyclin D and E were obtained from Santa Cruz Biotechnology. Monoclonal Rb and Raf-1 were supplied by BD Transduction laboratories. Polyclonal antibodies to phospho-Rb (807,811), phospho-Raf- (338), phospho-JNK, phospho ERK 1/2 and phospho Mek1/2 were supplied by Cell Signaling.

CAT assays

Assays for chloramphenicol acetyltransferase (CAT) and β-galactosidase

were performed using standard protocols (173). Cells were transfected by $CaCl_2$ and treated with drug asynchronously for 24 hours.

Chromatin Immunoprecipitation (ChIP) assay

A549 cells were rendered quiescent by serum starvation and re-stimulated with serum for 2h or 16h in the presence or absence of RRD 251 at 20μM. Cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. Subsequently, the cells were harvested and ChIP lysates were prepared (209). Immunoprecipitations were conducted using antibodies against E2F1, Rb, Raf-1, Brg1, HP1, and HDAC1 and the association with specific promoters detected by PCR as previously described. Rabbit anti-mouse secondary antibody was used as the control for all reactions. The sequences of the PCR primers used in the PCRs were as follows: *cdc6* promoter (forward primer), 5'-GGCCTCACAGCGACTCTAAGA-3'; and *cdc6* promoter (reverse primer), 5'-CTCGGACTCACCACAAGC-3' *TS* promoter (forward primer), and 5'-GAC GGA GGC AGG CCA AGT G-3' *TS* promoter (reverse primer). The cdc25A and *c-fos* primers are described in (209).

Real-time PCR

A549 cells were subjected to serum starvation or treatment with RRD-251. Unstimulated serum starved cells were used as a control. Total RNA was isolated by an RNeasy miniprep kit from QIAGEN following the manufacturer's protocol.

One microgram of RNA was DNase treated using RQ1 DNase (Promega), followed by first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). A fraction (1/20) of the final cDNA reaction volume was used in each PCR (249). Primers sequences are as follows: 5'-CTG CCA GCT GTA CCA GAG AT-3' (*TS* forward primer), 5'-ATG TGC ATC TCC CAA AGT GT-3' (*TS* reverse primer), 5'-CCC CAT GAT TGT GTT GGT AT-3' (*Cdc6* forward primer), 5'-TTC AAC AGC TGT GGC TTA CA-3' (*Cdc6* reverse primer), 5'-CTC AAC ACG GGA AAC CTC AC-3' (*18S* forward primer), and 5'-AAA TCG CTC CAC CAA CTA AGA A-3' (*18S* reverse primer). Real-time PCR was performed using a Bio-Rad iCycler.

In vitro kinase assay

The kinase reaction for Raf-1 was carried out with 100ng of Raf-1 (Upstate Signaling), 0.5μg of MEK1 (Upstate) as the substrate or 0.1μg Rb (QED Biosciences), 10μM ATP, 10μCi of [γ-^{32}P] ATP in the kinase assay buffer in the presence or absence of the drugs at 30°C for 30 minutes. 1μM of BAY-43-9006 was used as a control and 20μM RRD-251 was used. Cyclin D and E kinase assays are described in reference (209).

Proliferation assays

Bromodeoxyuridine (BrdU) labeling kits were obtained from Roche Biochemicals. Cells were plated in poly-D-lysine coated chamber slides at a

density of 10,000 cells per well and rendered quiescent by serum starvation for 24 hours. Cells were then re-stimulated with serum in the presence or absence of the indicated drugs for 18h. S-phase cells were visualized by microscopy and quantitated by counting 3 fields of 100 in quadruplicate. For nicotine treatments, Line1 cells were serum starved for 72 hours and subsequently stimulated with 1μM Nicotine (Sigma).

Soft Agar Colony Formation assays

Soft agar assays were done in triplicate in 12-well plates (Corning). First, the bottom layer of agar (0.6%) was allowed to solidify at room temperature. Next the top layer of agar was (0.3%) was mixed with 5,000 cells per well and the indicated drug. The drugs were added twice weekly in complete media to the agar wells. Colonies were quantified by staining with MTT 1mg/ml for 1hour at 37°C.

Matrigel Assays

Matrigel (Collaborative Biomedical Products) was used to promote the differentiation of HUVECs or HAECs into capillary tube-like structures (209). A total of 100μl of thawed Matrigel was added to 96-well tissue culture plates, followed by incubation at 37°C for 60 minutes to allow polymerization. Subsequently, 1 X 10^4 HAECs or HUVECS were seeded on the gels in EGM medium supplemented with 5% FBS in the presence or absence of 20μM

concentrations of the indicated compounds, followed by incubation for 24 hours at 37°C. Capillary tube formation was assessed using a Leica DMIL phase contrast microscope.

Ex-vivo Rat Aorta Ring Angiogenesis assays

Forty-eight well tissue culture plates were coated with 200μl of Matrigel and allowed to polymerize for 1 hour at 37°C. Thoracic aortas were excised from 8-10 week old male Sprague-Dawley Rats (250-300g) (250). The fibroadipose tissue was removed. The aortas were rinsed several times with EGM-2 (Clonetics), sectioned into 1mm rings and placed on the matrigel-coated wells. The rings were covered with an additional 200μl of Matrigel and allowed to polymerize. The rings were cultured in EGM-2 media in the presence or absence of 20μM of RRD-251. The media and drug were supplemented twice a week for one week. The Aortic rings were photographed on day 7 using a Leica phase contrast microscope. Quantitation of microvessel growth was done using Image Pro Plus (v.6.0) software and values are reported as microvessel area.

In vivo Matrigel Plug Angiogenesis assays

In vivo matrigel plug assays were carried as previously described (251). Cooled liquid matrigel (Collaborative Biomedical Products) (300μl) was injected subcutaneously into both flanks of nude mice. The next day, the mice were separated into two groups; one group received the vehicle (PBS/DMSO) every

day by i.p. injection and the second group received RRD-251 50 MPK daily by i.p. injection. The mice were treated for 7 days. At 7 days post matrigel injection, the mice were injected with 100μl of 100 MPK FITC-Dextran (Sigma) through the tail vein. 30 minutes later, the mice were euthanized and the matrigel plugs were removed and fixed in buffered formalin. Each sample was visualized and searched for areas of vessel formation. Two images were captured per matrigel plug. Samples were viewed with a Leica DMI6000 inverted microscope, TCS SP5 confocal scanner, and a 20X/0.7NA Plan Apochromat objective (Leica Microsystems, Germany). An Argon 488 laser line was applied to excite the samples and tunable filters were used to minimize background fluorescence. Image sections at 2.0 μm were captured with photomultiplier detectors 3D projections were prepared with the LAS AF software version 1.6.0 build 1016 (Leica Microsystems, Germany). Quantification of intensity and angiogenesis was performed using Image Pro Plus 6.2 (Media Cybernetics, Inc., Maryland). Average intensity per pixel is plotted as percent angiogenesis in each image, (n=12). Each image is representative of areas of vessel formation throughout entire matrigel plug. After confocal imaging, samples were paraffin blocked and stained with H&E. H&E images shown display ¼ of the matrigel plug.

Quantitation of VEGF

Asynchronously growing A549 cells were treated with RRD-251 (20 and 50μM) for 24 hours. Aliquots of media (1mL) were taken and stored at -20ºC for

later analysis. ELISA to human VEGF (Biosource, Invitrogen) was performed following manufacturer's protocols. Concentrations were plotted against the standard curve.

Animal Studies

Nude mice (Charles River, Wilminton, MA, USA) were maintained in accordance with Institutional Animal Care and Use Committee (IACUC) procedures and guidelines. A549 or H1650 cells were harvested and resuspended in PBS, and then injected s.c. into the right and left flanks (10×10^6 cells per flank) of 8-week old female nude mice as reported previously (209,248). For SK-MEL-28 xenograft experiments, SK-MEL-28 cells were resuspended in 1:1 PBS/Matrigel solution. When tumors reached about 100-200mm^3, animals were dosed intraperitoneally (i.p.) or orally by gavage with 0.1ml solution once daily. Control animals received a vehicle, whereas treated animals were given RRD-251 at the indicated doses. The tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume ($V = lw^2/2$) as described previously. Statistical significance between control and treated animals were evaluated using Student's *t*-test.

Immunohistochemistry staining

Upon termination of xenograft anti-tumor experiments, tumors were removed and fixed in 10% neutral-buffered formalin before processing into

paraffin blocks. Tissue sections (5μm thick) were cut from the blocks and stained with H&E, Ki-67, CD31, phospho-Rb, β-Catenin and E-cadherin antibodies. Paraffin sections were rehydrated to PBS and processed using the following protocols. Sections were rinsed in dH2O, and then subjected to microwave 'antigen retrieval' for 20 minutes on 70% power, with a 1 minute cooling period after every 5 minutes, in 0.01 M sodium citrate, pH 6.0. Sections were cooled for 20 minutes, rinsed 3 times in dH2O, twice in PBS and incubated in 5% normal goat serum for 30 minutes. Sections were incubated in primary antibody for 1 hour in 5% normal goat serum, rinsed 3 times in PBS. For color development the slides were treated with ABC kit from Vector labs, rinsed in dH2O, and developed using DAB as chromogen. After a final rinse in dH2O, sections were lightly counterstained in hematoxylin, dehydrated, cleared and coverslipped. Tissue sections were stained with hematoxylin and eosin (H&E) using standard histological techniques. Tissue sections were also subjected to immunostaining for CD31 (BD Biosciences, San Diego, CA, USA) using the avidin–biotin peroxidase complex technique. Mouse monoclonal antibody was used at 1 : 50 dilution following microwave antigen retrieval (four cycles of 5 min each on high in 0.1 M citrate buffer . Stained slides were scanned on an Ariol SL-50 Automatic Scanning System and whole tumor sections were quantitated using Image Pro Plus (v.5.1.0) software.

Statistical Analysis

Statistical analysis was performed using one tailed Student's t-test. Values were considered significant when the p value <0.01. Exact p values are reported.

In vitro binding assays

Glutathione S-transferase (GST) fusions of Rb and Raf-1 have been previously described (209,237). ^{35}-S labeled Raf-1 proteins were generated by in vitro transcription translation in rabbit reticulocyte lysate according to the manufacturer's instructions (Promega, Madison, WI). First, 8μl of the lysates were incubated with the GST-Rb beads in 200μl of protein binding buffer (20mM Tris [pH 7.5], 50mM KCL, 0.5mM EDTA, 1mM DTT, 0.5% NP-40, 3mg/ml BSA) at 4 C for 2 hours as described earlier (209,237). The input lanes contained approximately one fourth of that used in the binding assay. Peptide synthesis was carried out by Ted Gauthier at the USF Chemistry department. Alanine scan of Peptide consisted of the 8 amino acid peptide with an alanine replacing one amino acid at each position on the peptide.

Cell Viability Assays

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma and was constituted at 10mg/ml in sterile PBS. Cells were plated at densities of 3,000-5,000 cells per well in 96-well plates. Cells were treated asynchronously with several inhibitors at varying concentrations. DMSO was used as the control. 24-48 hours post-treatment, MTT was added to the

wells and allowed to metabolize for 30 min-2 hours. Media was carefully aspirated out from the wells and DMSO was added to solubilize the crystals. Absorbance was measured at 540nM on a Victor plate reader.

Apoptosis Assays

Cells (10,000/well) were plated in poly-D-lysine coated chamber slides. Drug treatment on asynchronous cells was for 18-24 hours and subsequently fixed with formalin. Apoptosis was measured using a TUNEL assay kit (Promega). Parallel experiments were also set up in 10cm tissue culture dishes and apoptosis was confirmed by immunoblotting for PARP (Cell Signaling).

Double Immunofluorescence Assays

U2-OS osteosarcoma cells were plated on poly-D-lysine coated chamber slides and rendered quiescent by serum starvation for 48 hours. Thereafter, the cells were re-stimulated with serum for 2 hours in the presence or absence of RRD-251 at 20μM concentration. Cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.2% Triton-X-100. Monoclonal anti-Rb (1:50) and polyclonal anti-Raf-1 (1:200) were added in blocking buffer, and incubated on the cells overnight at 4ºC. Secondary antibodies, goat anti-mouse (IgG)-Alexa Fluor 488 (green fluorochrome), and goat anti-rabbitt (IgG)-Alexa Fluor 548 (red fluorochrome) (Molecular Probes) were used as described previously (209). Nuclear staining was performed using DAPI. Immunostained Rb

and Raf-1 were visualized by confocal microscopy using a Zeiss scanning microscope model 510 system equipped with argon (458/488nm) and helium neon (543nm) laser systems.

NNK induced Carcinogenesis Animal Model

Two experiments were carried out using female A/J mice 4-6 weeks of age (Jackson Labs). NNK (NCI) (100mg/kg) was administered to all mice (*n*=16) once a week for 5 weeks. The mice were randomized into two groups; group one received the vehicle (PBS) (*n*=8) and group two received nicotine (*n*=8) by (i.p.) injection at a dose of 1 mg/kg three times a week for an additional 23 weeks. Nicotine levels in mice were analyzed using a cotinine ELISA kit. At the end of the experiment, the mice were euthanized and the lungs were fixed in 10% buffered formalin. The lungs were subsequently examined by stereoscope for number of lung tumors. The lungs were paraffin embedded and sectioned for IHC staining and pathological examination.

Line1 model of tumor growth and metastasis

Line1 Tumor Growth Experiments. Female BALB/c mice age 26-30 days (Charles River) were clipped and depilated using Nair for complete hair removal on the back and flanks. Line1 cells (1×10^6 per tumor) were harvested and resuspended in 100μl of PBS for injection (252). The mice were randomized 3-7 days after injection of tumor cells. Mice were separated into two groups

Vehicle (*n*=8) and Nicotine (*n*=8) (patch or i.p. injection). Mice received nicotine by i.p. injection at a dose of 1mg/kg three times a week. Nicotine was also applied using transdermal patches (Nico®Derm® CQ, GlaxoSmithKline) at a dose of 25 mg/kg daily. Patches (14mg) were cut into 30 equal sized squares representing 0.45mg of nicotine using a razor blade. Nicotine was administered for 2 weeks and tumor growth was measured thrice weekly. Nicotine levels in mice were analyzed using a cotinine ELISA kit.

Line1 Metastasis Experiments. Line1 cells (1×10^6 per tumor) were injected and the mice were subsequently randomized into two groups. Group one received the vehicle (*n*=16) and group two received nicotine (1mg/kg) (*n*=16) by i.p. injection thrice weekly. After 3 weeks of nicotine treatment, the tumors were removed under anesthesia and the skin was stapled, mice recovered on a warmed heating pad and the staples were removed after 7 days. Mice continued to receive nicotine or vehicle for an additional 2 weeks. At the end of the experiment, the mice were euthanized and the lungs were fixed in formalin.

Quantitation of Cotinine

Level of cotinine in urine was used as a marker for nicotine levels. Urine (100μl) was collected throughout the length of the experiments and stored in -20^{o}C for later analysis. Cotinine levels were determined by using the BioQuant Cotinine Direct ELISA kit (CalBiotech, Spring Valley, CA) following

manufacturer's protocols. Nicotine doses used in these studies correlated well with cotinine levels in urine of heavy smokers (253,254). Mice receiving 1mg/kg nicotine thrice weekly had an average urine cotinine concentration of 3000ng/ml. Mice that received 25mg/kg nicotine by transdermal patch had an average cotinine concentration of 5000ng/ml cotinine in their urine. Cotinine levels in urine are often in a wide range of concentration due to the variance of urine collection volumes. In human smokers, cotinine concentrations have been reported in values ranging from 1500ng/ml to 8000ng/ml (253-255).

Chapter 3: An orally available small molecule disruptor of Rb-Raf-1 interaction inhibits cell proliferation, angiogenesis and growth of human tumor xenografts in nude mice

Abstract

Though it is well established that cyclin-dependent kinases phosphorylate and inactivate Rb, the Raf-1 kinase physically interacts with Rb and initiates the phosphorylation cascade early in the cell cycle. We have identified an orally-active small molecule, RRD-251 (Rb – Raf-1 Disruptor 251), that potently and selectively disrupts the Rb/Raf-1 but not Rb/E2F, Rb/Prohibitin, Rb/Cyclin E and Rb/HDAC binding. The selective inhibition of Rb/Raf-1 binding suppressed the ability of Rb to recruit Raf-1 to proliferative promoters and inhibited E2F1-dependent transcriptional activity. RRD-251 inhibited anchorage-dependent and –independent growth of human cancer cells; and knockdown of Rb with shRNA or forced expression of E2F1 rescued from RRD251-mediated growth arrest. Oral treatment of mice resulted in significant tumor growth suppression only in tumors with functional Rb; and this was accompanied by inhibition of angiogenesis, inhibition of proliferation, decreased phospho-Rb levels, and inhibition of Rb/Raf-1 but not Rb/E2F1 binding *in vivo*. Thus, selective targeting of Rb-Raf-1 interaction appears to be a promising approach for developing novel

anti-cancer agents.

Introduction

The retinoblastoma tumor suppressor protein, Rb, is a vital regulator of the mammalian cell cycle and its inactivation facilitates S-phase entry (256,257). Rb is inactivated through multiple waves of phosphorylation during cell cycle progression, mediated by kinases associated with D and E type cyclins in the G1 phase (20,258). Rb is inactivated in most cancers, either by mutation or deletion of the gene, interaction with viral oncoproteins, or alterations in the levels and activity of upstream regulators of Rb function (1,38,259,260). Rb controls the G1/S boundary by repressing the transcriptional activity of the E2F family of transcription factors, especially E2Fs 1, 2, and 3 (30). Many genes necessary for DNA synthesis and cell cycle progression, such as *cyclins A* and *E*, *cdc2*, *thymidylate synthase*, *DHFR*, *ORC1* and *DNA polymerase* α require E2F for their expression (49,54,69,124). While cyclins and cdks phosphorylate Rb in mid to late G1 phase releasing transcriptionally active E2F (22,23,261), Raf-1 kinase binds and phosphorylates Rb early in the G1 phase (173). Disruption of this Rb/Raf-1 interaction by an eight amino acid peptide (corresponding to Raf-1 residues 10-18) prevented Rb phosphorylation even late in the G1 phase, suggesting that the binding of Raf-1 is necessary for the eventual inactivation of Rb (209). Further, the level of Rb-Raf-1 interaction is elevated in NSCLC tissue compared to the adjacent normal tissue (215), suggesting that this interaction

contributes to the oncogenesis of these tumors. These observations suggested that disruption of the Rb/Raf-1 interaction might have anti-cancer effects and raised the possibility that small molecules that can disrupt the Rb/Raf-1 interaction might be useful as novel anticancer drugs. Here we report a potent and selective small-molecule disruptor of Rb/Raf-1 interaction that significantly inhibits angiogenesis and tumor growth *in vivo* in an Rb-dependent manner.

Results

GFGFK, a pentapeptide corresponding to amino acids 13-18 of Raf-1 is sufficient for complete disruption of Rb-Raf-1 interaction

Previous work from our lab had shown that a peptide **ISNGFGFK** which corresponds to amino acids 10-18 on Raf-1 disrupts the Rb-Raf-1 interaction (209). In an effort to design peptide-mimics or disruptors of this interaction we first performed an alanine scan to determine the minimum requirements for Rb-Raf-1 disruption. To this end, eight amino acid peptides were synthesized with each position replaced with an alanine in the peptide's amino acid sequence. The eight amino acids synthesized were named A1-A8 corresponding to A for Alanine **(Figure 12A)**. Figure 12 shows that amino acids 10, 11 and 12 (I,S or N) are not required for Rb-Raf-1 disruption. However, replacement of any amino acids 13-15 (GFGFK) with alanine was detrimental **(Figure 12B)**. A peptide with the sequence FGFK could not disrupt the Rb-Raf-1 interaction indicating that amino acid 13 (G) was necessary for complete disruption **(Figure 12C)**. A peptide with

the amino acids ISNG also could not inhibit the Rb-Raf-1 binding. Thus, the minimum amino acids responsible for Rb-Raf-1 disruption were GFGFK. This peptide could disrupt Rb-Raf-1 as efficiently as the Raf-1 peptide **(Figure 12D)**. Although peptides are useful for targeting specific sequences of proteins *in vitro* to disrupt their interactions or enzymatic activity, they are of limited use as drugs *in vivo*. This is because they are degraded very quickly and delivery into cells is problematic. At the same time, information generated from studies on peptides can be fruitfully used to generate peptidomimetic drugs or other small molecules to target the interaction. Our future studies will use the GFGFK motif to generate new RB-Raf-1 disruptors. Given that the small peptide could disrupt the binding of two relatively large proteins, we embarked on identifying small molecules that can disrupt the binding of Raf-1 to Rb.

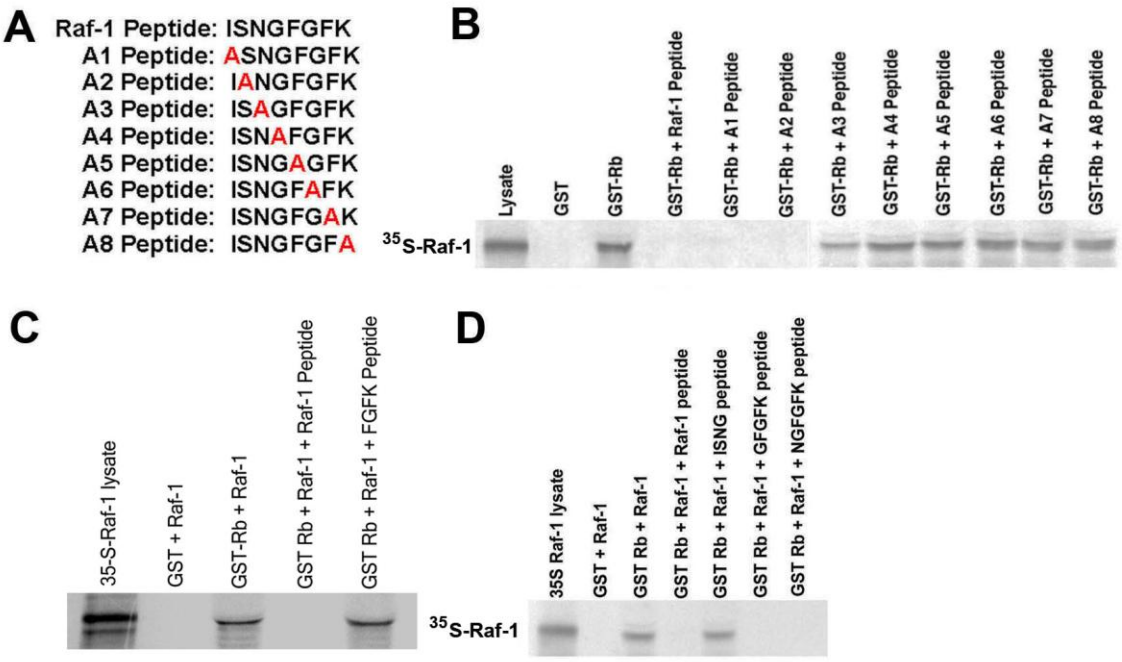


Figure 12. GFGFK pentapeptide is necessary for disruption of the Rb-Raf-1 interaction. (A) Alanine scan of 8 amino acid Raf-1 peptide. (B) Binding of ^{35}S-Raf-1 to GST Rb or unprimed GST in the presence or absence of 1μM of the peptides. (C) FGFK peptide is not sufficient to disrupt Rb-Raf-1 binding (D) GFGFK of the Raf-1 peptide is required for disruption of Rb/Raf-1 binding.

Identification of the small molecule Rb-Raf-1 disruptor, RRD-251

An ELISA was used to identify compounds that could inhibit the binding of GST-Rb to GST-Raf-1. Screening of the NCI diversity library of 1,981 compounds by Piyali Dasgupta identified two compounds, NSC-35400 and NSC-35950, which inhibited Rb-Raf-1 interaction 100% and 95% respectively at 20μM concentration. NSC-35400 and NSC-35950 each contained a benzyl-isothiourea derivative and a phenyl-based counter ion **(Figure 13A)**; to establish whether the benzyl-isothiourea derivative is the active component, Dr. Nick Lawrence's laboratory at the Moffitt Cancer Center synthesized RRD-251 **(Figure 13A)**, which was similar to NSC-35400 but contains chloride as the counter ion. ELISA analysis showed that NSC-35400 could disrupt the Rb-Raf-1 interaction with an IC_{50} of 81 ± 4nM; NSC-35950 had an IC_{50} of 283 ± 46nM, while RRD-251 had a value of 77 ± 3.6 nM **(Figure 13B)** suggesting that the benzylisothiouronium pharmacophore disrupts the Rb-Raf-1 interaction, IC_{50} assays were done by Piyali Dasgupta. ELISAs showed that the Rb/Raf-1 binding disruptors were highly selective for Rb/Raf-1 interaction over Rb/E2F1, Rb/HDAC1, Rb/prohibitin **(Figure 13C)** and Raf-1/Mek **(Figure 13D)** associations at a concentration of 20μM.

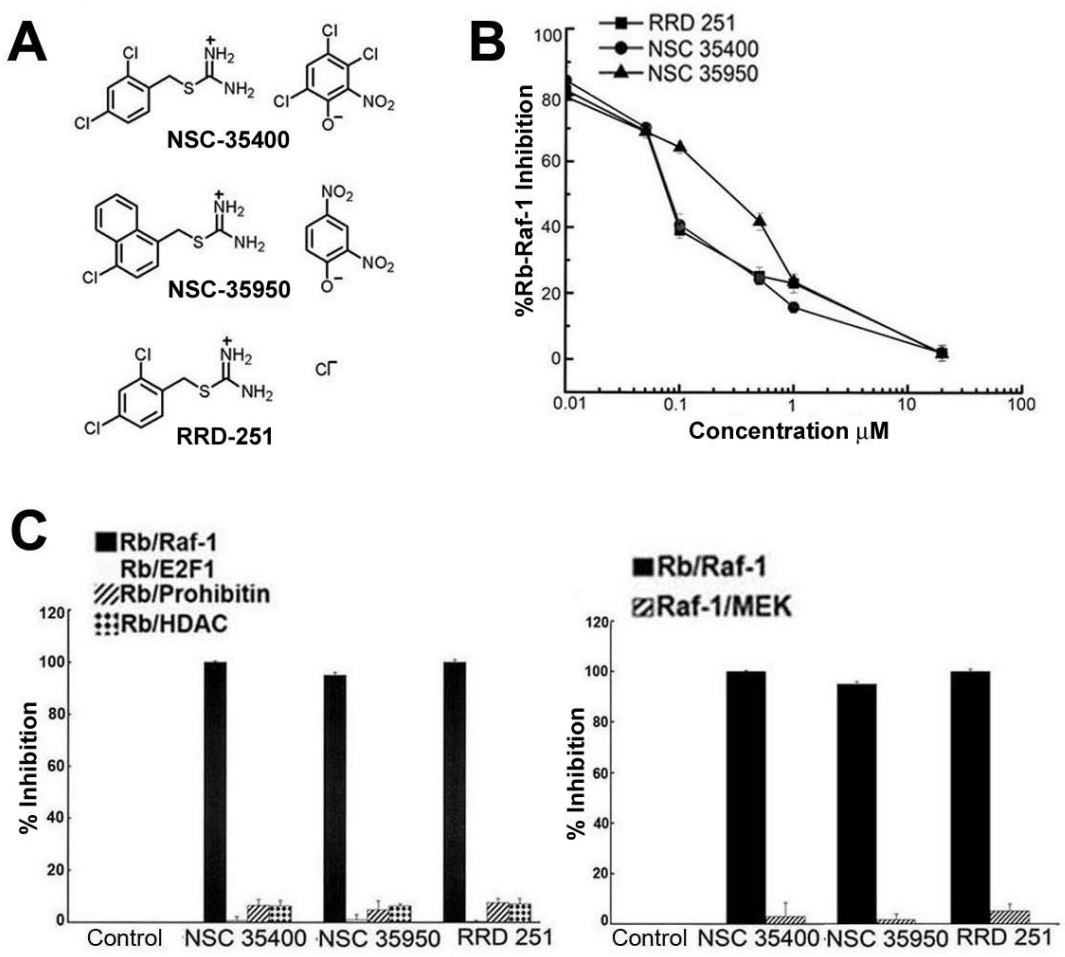
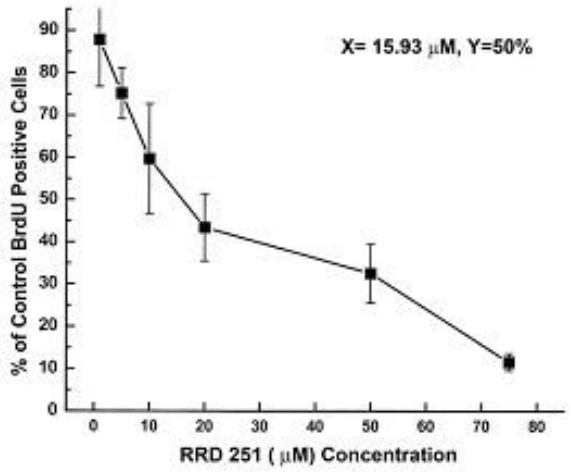


Figure 13. Identification of highly specific and selective Rb-Raf-1 inhibitors. (A) Chemical structures of compounds identified in the NCI diversity set that showed the highest inhibition of Rb-Raf-1 by ELISA. Highest scoring compounds NSC-35400 and NSC-35950 are both benzyl isothiourea derivatives. RRD-251 was synthesized to determine activity based on isothiourea structure. (B) NSC35400, NSC35950 and RRD-251 disrupt the Rb-Raf-1 interaction with high potency. IC_{50} values (81nM, 283nM and 77nM, respectively) were determined using ELISA. (C) Rb-Raf-1 inhibitors at 20μM concentration do not inhibit other binding partners to Rb (E2F1, prohibitin and HDAC1) and to Raf-1 (Mek).

RRD-251 inhibits cell proliferation in a wide range of cell lines

Since disruption of the Rb-Raf-1 interaction in cells via the Raf-1 peptide conjugate was capable of preventing S-phase entry, we evaluated the efficacy of RRD-251 to prevent S-phase entry in the A549 NSCLC cell line. RRD-251 could inhibit A549 S-phase entry with an IC_{50} of 15.93μM **(Figure 14A)**. It was next examined whether RRD-251 could inhibit the proliferation of cells that have mutations in the signaling pathways that impinge on Rb function, rather than in the Rb gene itself. RRD-251 could inhibit S-phase entry by 50 – 65% in pancreatic cancer cell lines such as Aspc1, PANC1, and CAPAN2 that harbor a non-functional *p16INK4a* gene (262) **(Figure 14B)**. RRD-251 also inhibited S-phase entry in two glioblastoma cell lines U87MG and U251MG, both of which are null for *p16* and *PTEN* (263). The metastatic human breast cancer cell line MDA-MB-231 harbors a *K-Ras* mutation and overexpresses EGFR (264); RRD-251 was able to inhibit its proliferation by 56% **(Figure 14B**). The A375 melanoma cell line harbors the V600E B-Raf mutation (265) and RRD-251 inhibited S-phase entry by 58%. Prostate cell lines LNCaP and PC3 both contain mutations in *K-Ras* and *PTEN* genes (266), and RRD-251 inhibited proliferation 86% and 35% respectively **(Figure 14B)**. These results indicate that treatment with RRD-251 could inhibit the proliferation of cell lines harboring a wide array of mutations in upstream signaling molecules and cell cycle regulators.

A



B

Cell Line/ Origin	Mutations	% Inhibition (BrdU) RRD-251 20μM
Aspc1/ Pancreatic	p16 (2bp deletion) Ras mut, p53 mut	65%
PANC1/Pancreatic	p16 (HD),Ras mut p53 mut, AKT2 amplification	53%
CAPAN2/Pancreatic	P16 mut, Ras, mut	51%
U87MG/ Glioma	p16 null, PTEN null	54%
U251MG/ Glioma	p16 null, PTEN null	35%
MDA-MB-231/ Breast	K-Ras mut EGFR (OE)	56%
A375/Melanoma	V600E	58%
LNCap/Prostate	PTEN mut, K-Ras mut	86%
PC3/Prostate	PTEN mut, K-Ras mut	35%

Figure 14. RRD-251 inhibits S-phase entry. (A) RRD-251 inhibits A549 S-phase entry in BrdU assays with an IC_{50} of 15.93μM. (B) BrdU incorporation assays showing the growth arrest mediated by RRD-251 in a variety of tumor cell lines harboring various mutations. RRD-251 could effectively arrest cells with mutations in EGFR, p16, PTEN, K-Ras and p53.

Inhibition of proliferation by RRD-251 is dependent on Rb status

Given the ability of RRD-251 to inhibit Rb phosphorylation, it was examined if it could inhibit cell proliferation and whether such an inhibition required a functional Rb gene. RRD-251 was effective at inhibiting serum-induced S-phase entry in parental A549 cells but had no effect on cells stably expressing sh6 and sh8, which lacked Rb (**Figure 15A).** We further examined RRD-251 treatment on cancer cell lines containing Rb mutations that render Rb non-functional. Osteosarcoma Saos-2 cells that have a loss of Rb (234) were not sensitive to treatment with RRD-251 while the U2-OS osteosarcoma cells carrying wild type Rb could be inhibited efficiently **(Figure 15B)**. In prostate cancer cell lines, RRD-251 was unable to inhibit proliferation in the Rb mutant DU145, yet could inhibit 60% of S-phase cells in PC3 cells (wt Rb) **(Figure 15B)**. RRD-251 did not inhibit proliferation in the lung cancer cell lines H596 and H2172, both of which harbor mutations in Rb, yet treatment with RRD-251 in H1650 and H1299 (wt Rb) could inhibit proliferation 90% and 70% respectively **(Figure 15B)**.

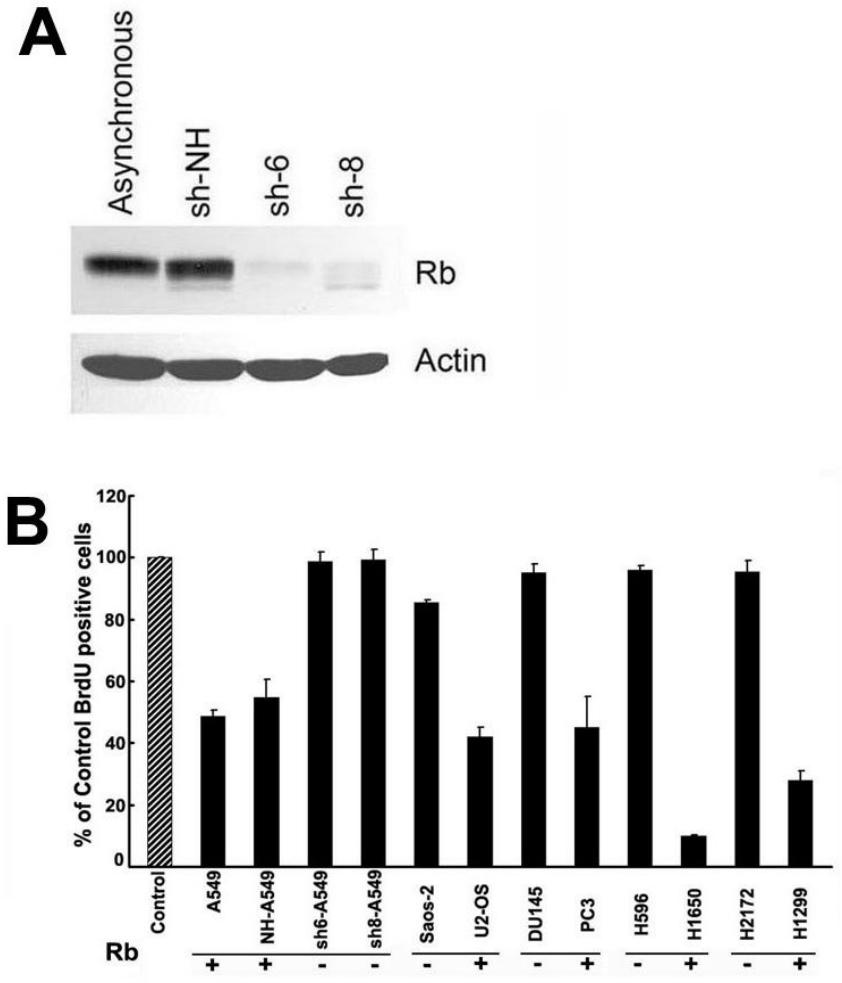


Figure 15. RRD-251 inhibits S-phase entry is dependent on Rb status. (A) A549 cells stably expressing shRNA to two different Rb constructs display almost complete knockdown of Rb protein (B) BrdU incorporation assay showing that 20μM of RRD-251 does not inhibit the proliferation of A549 cells over-expressing shRNA constructs to Rb, but arrests wild-type A549 cells and a non-homologous (NH) control shRNA. RRD-251 also does not inhibit S-phase entry in cancer cell lines that contain mutant Rb.

Melanoma and Pancreatic cell lines are sensitive to RRD-251

Next, the compounds were analyzed for inhibition of cell proliferation in several pancreatic cancer and melanoma cells lines, which have elevated MAP kinase activity as a result of Ras mutations or B-Raf mutations. RRD-251 was found to have the greatest effect on cell viability in three melanoma cell lines (SK-MEL-2, SK-MEL-5, SK-MEL-28) compared to agents that prevent the MAPK pathway such as BAY-43-9006 and PD-98089 **(Figure 16A-B)**. These results are independent of B-Raf status; SK-MEL-2 contains wild type B-Raf and SK-MEL-5 and SK-MEL-28 harbor V600E B-Raf mutation. BAY-43-9006 is a multi-kinase inhibitor that was found to target B-Raf, Raf-1, Flt-1, C-kit and several other receptor tyrosine kinases (RTKs). In addition, treatment of RRD-251 in melanoma cell lines was compared to the standard of care for melanoma Dacarbazine (DTIC); melanoma cells were significantly more sensitive to RRD-251 treatment compared to DTIC **(Figure 16C)**. The aforementioned melanoma cell lines were very sensitive to RRD-251 treatment in cell viability assays, this increased sensitivity was not observed in other cell lines such as A549 lung cancer cell line (data not shown). One surprising finding was that treatment of the melanoma cell lines resulted in apoptosis as shown by TUNEL staining **(Figure 17A-B)**. To confirm these results, PARP cleavage was assessed as a marker for apoptosis. SK-MEL-28, and not A549 displayed significant PARP cleavage as early as 4 hours from treatment with RRD-251 **(Figure 17C)**. Again, RRD-251 was more effective at inducing apoptosis in SK-MEL-28 compared to DTIC as

examined by PARP cleavage **(Figure 17D)**, experiments done in Figure 17C-D were performed by Sandeep Singh.

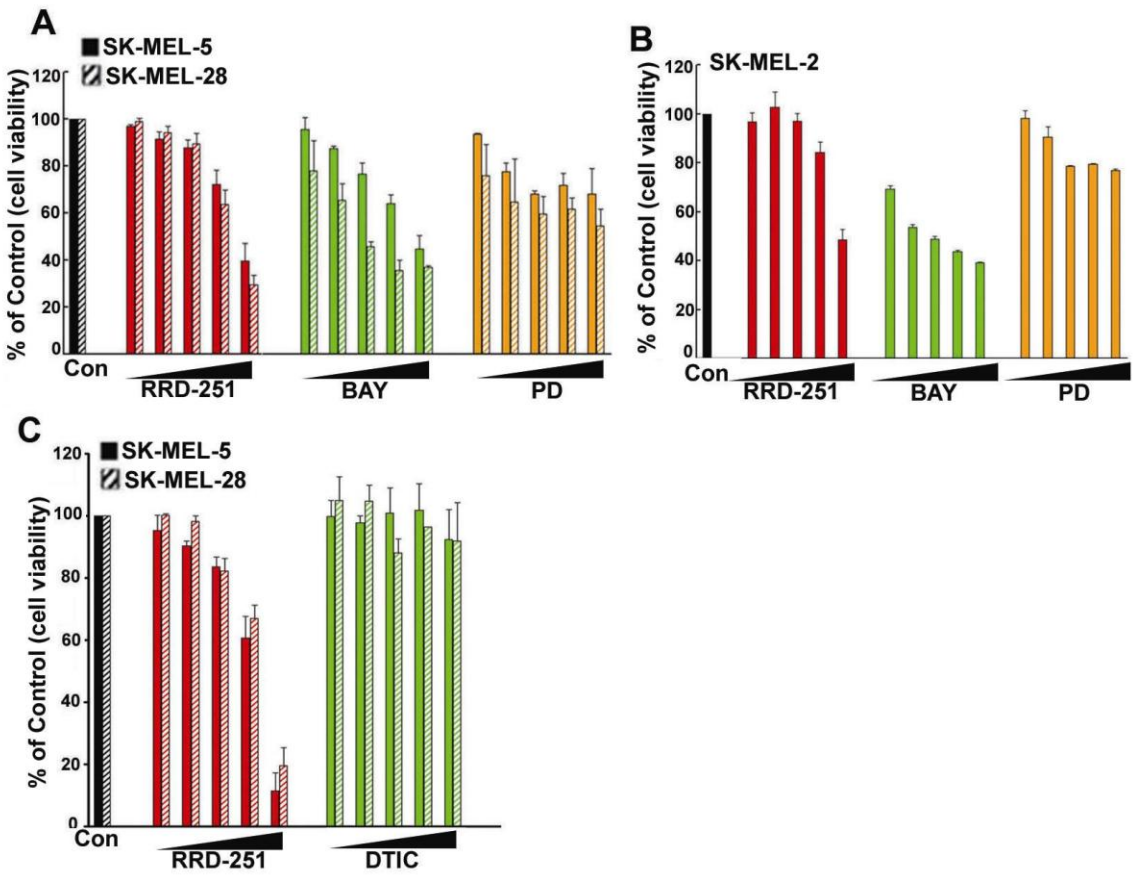


Figure 16. Melanoma cells are most sensitive to treatment with RRD-251. (A) Treatment with RRD-251 at increasing doses (1, 5, 10, 20 and 50μM) inhibits cell proliferation in comparison to BAY-43-9006 and PD98089 at the same concentrations in two B-Raf V600E mutant cell lines (B) RRD-251 displays significant inhibition of cell viability in SK-MEL-2 wild type B-Raf cell line (C) RRD-251 has a greater effect on melanoma cells compared to standard of care chemotherapy DTIC.

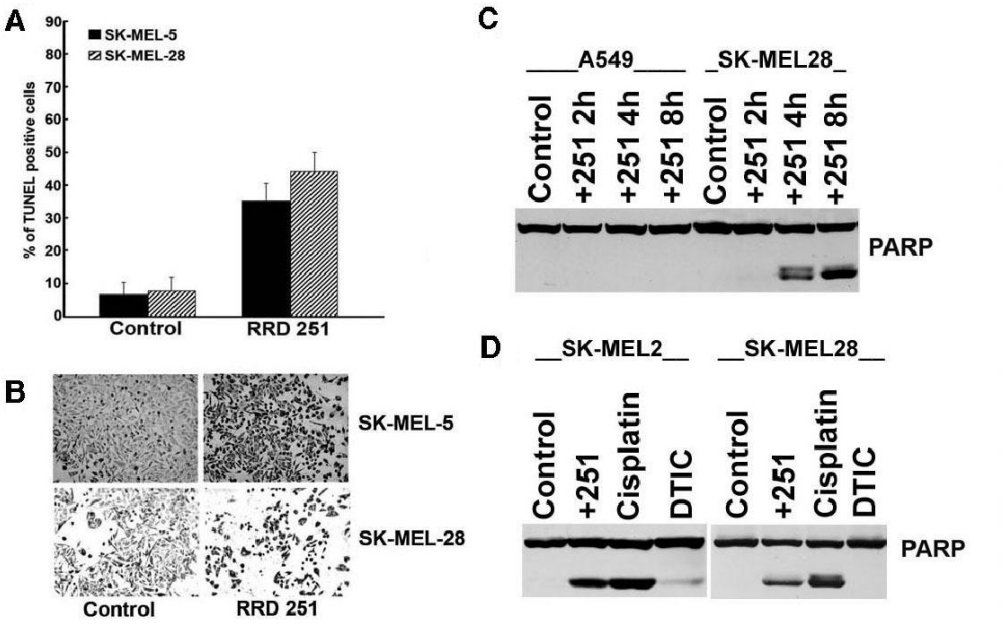


Figure 17. RRD-251 induces apoptosis in melanoma cell lines. (A) RRD-251 (20 μM) induces 40% apoptosis as shown by TUNEL assays. (B) Brightfield images of TUNEL staining on treated vs. non-treated melanoma cells (C). RRD-251 induced apoptosis at 4 hours in SK-MEL-28 and not A549 as shown by PARP cleavage. (D) RRD-251 induced significantly more apoptosis in SK-MEL-2 and SK-MEL-28 compared to DTIC, cisplatin was used as a control.

Another cell line found to have increased sensitivity to RRD-251 treatment in cell viability assays was the pancreatic cancer cell line, PANC1. We expanded this observation by comparing the treatment of RRD-251 on pancreatic cancer cell line PANC1 to the immortalized pancreatic ductal epithelial cell line HPDE6C7. PANC1 cancer cells were more sensitive to treatment with RRD-251 than HPDE6C7 **(Figure 18A-B)**. In addition to the cell line comparison, RRD-251 inhibited cell proliferation more than standard of care chemotherapy for pancreatic cancer, Gemcitabine and 5-Fluorouracil **(Figure 18A)**. RRD-251 treatment was also compared to inhibitors of the Ras/MAPK pathway (BAY-43-9006 and PD98059) and found to be more effective at inhibiting the cancerous PANC1 compared to the immortalized pancreatic ductal cell line HPDE6C7 **(Figure 18B)**.

Next, we examined the ability of RRD-251 to prevent soft agar colony formation. Ability to grow independent of a substratum is a feature of cancer cells and growth in soft agar measures the ability of cells to grow in an adherence-independent manner. Experiments were conducted on a panel of five cell lines to assess whether RRD-251 affected their growth in soft agar. Treatment with RRD-251 (100μM) twice a week could significantly inhibit the growth A549, H1650, PANC1, SK-MEL-5 and SK-MEL-28 colonies in soft agar **(Figure 19)**.

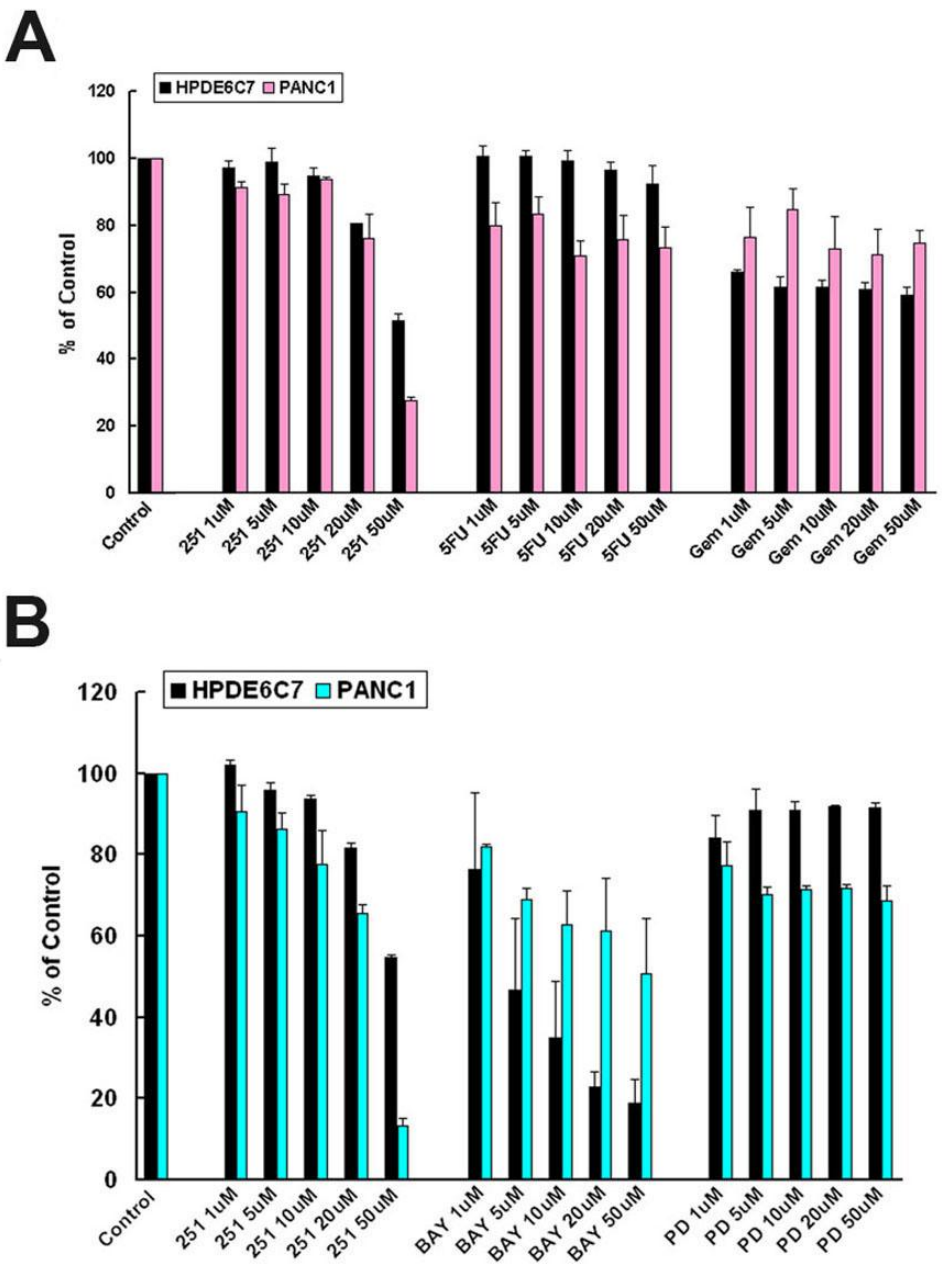


Figure 18. RRD-251 inhibits cell viability in PANC1 cancer cells. (A) RRD 251 inhibits cell proliferation in PANC1 cells better than standard of care therapy; 5-Fluorouracil or Gemcitabine (B) Inhibiting Rb-Raf-1 with RRD 251 is more effective than the multikinase inhibitor BAY-43-9006 or PD98059.

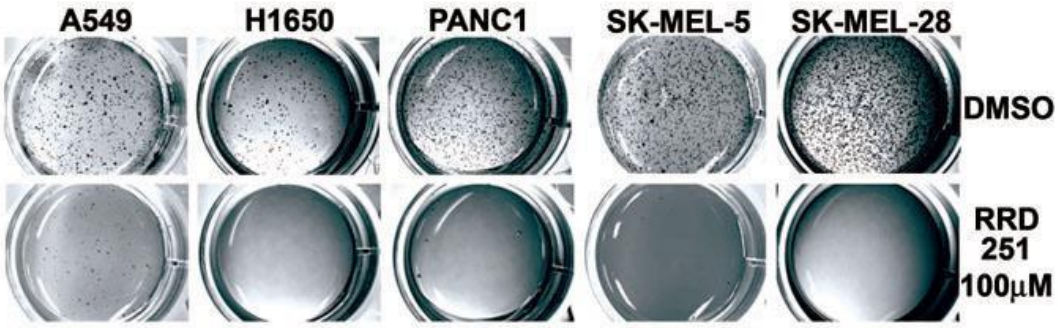


Figure 19. RRD 251 inhibits colony formation in soft agar. RRD 251 (100μM) dosed thrice weekly inhibits the adherence independent growth in A549, H1650, PANC1, SK-MEL-5, and SK-MEL-28 cells.

RRD-251 displays high specificity for Rb-Raf-1 interaction

The specificity of RRD-251 for the Rb-Raf-1 interaction in living cancer and normal cells was examined by immunoprecipitation-western blot analysis (IP-WB). H1650 or HUVEC cells were serum starved for 48 hours and subsequently serum stimulated for 2 hours in the presence or absence of RRD-251 at 100nM, 1μM, 5μM, 10μM and 20μM. In the NSCLC H1650 cell line, RRD-251 inhibited the Rb-Raf-1 interaction with an IC_{50} of 444nM (**Figure20 A-B)**. In the normal HUVEC cell line RRD-251 disrupted Rb-Raf-1 interaction with an IC_{50} of 903nM **(Figure 20 C-D)**.

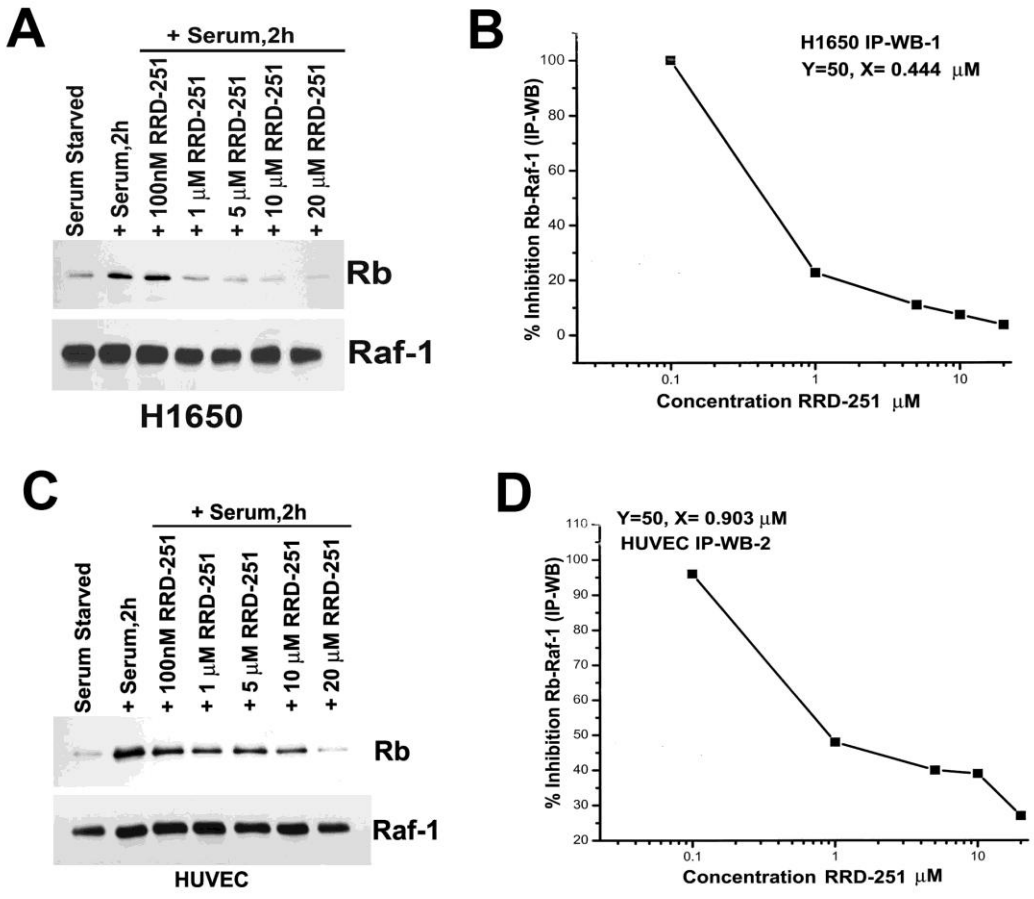


Figure 20. RRD-251 specifically targets Rb-Raf-1 interaction in living cells. (A) RRD-251 inhibits the Rb-Raf-1 interaction *in vivo* in H1650 cells. (B) The IC_{50} for disruption in H1650 was 444nM. (C) RRD-251 inhibits the Rb-Raf-1 interaction *in vivo* in HUVEC cells. (D) The IC_{50} for disruption in HUVEC cells was 903nM.

RRD-251 is selective for Rb-Raf-1 interaction

The selectivity of RRD-251 for Rb-Raf-1 interaction in living cells was next examined by IP-WB. A549 cells were serum starved for 72 hours and subsequently serum stimulated for 2 hours in the presence or absence of 20μM of NSC-35400, NSC-35950, and RRD-251; Raf-1 peptide conjugated to penetratin (209) was used as a positive control and a Raf-1 scrambled peptide was used as a negative control. It was found that the compounds inhibited the serum-stimulated binding of Raf-1 to Rb **(Figure 21A)**, but the binding of Rb to E2F1 was not affected, experiments done in Figure 21A were performed by Piyali Dasgupta. To further confirm the selectivity of RRD-251, cyclin E was immunoprecipitated from lysates of quiescent cells or those serum stimulated for 8 hours in the presence or absence of RRD-251; western blotting of the immunoprecipitates showed that RRD-251 did not inhibit the binding of Rb to Cyclin E **(Figure 21B)**. Since B-Raf has been shown to bind to Rb in *in vitro* pull down assays (209) a similar experiment was done on lysates from cells that were serum stimulated for 2 hours; RRD-251 did not inhibit the binding of B-Raf to Rb **(Figure 21C)**. Similarly, the binding of Raf-1 to Mek1/2 was not affected by RRD-251 **(Figure 21D)**.

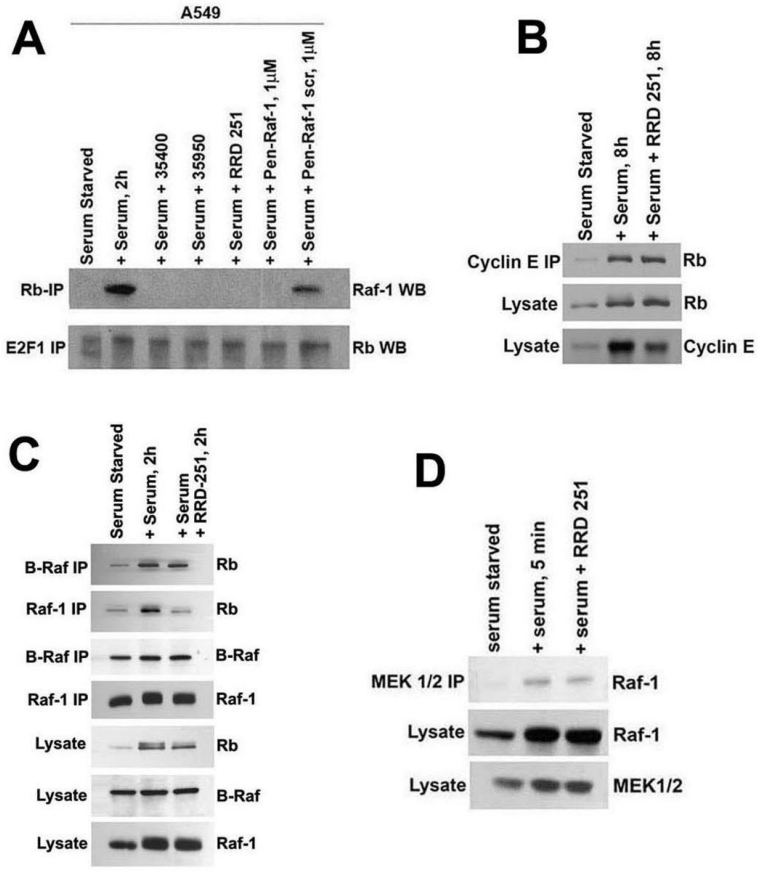


Figure 21. RRD-251 is selective for Rb-Raf-1 interaction in living cells. (A) Serum-stimulated binding of Raf-1 to Rb is inhibited by Rb-Raf-1 disruptors (20µM) as well as a Raf-1 peptide conjugated to penetratin, the drugs do not inhibit the binding of E2F1 to Rb. Further selectivity of the disruption was assessed by IP-western blots (B) RRD-251 does not inhibit Rb-Cyclin E interaction in cell serum-stimulated for 8 hours. (C) RRD-251 does not disrupt the Rb-B-Raf binding in IP-Western Blots. (D) Treatment of cells with RRD-251 for 5 minutes in the presence of serum does not affect the binding of MEK1/2 to Raf-1.

Next the ability of RRD-251 to disrupt Rb-Raf-1 interaction *in vivo* was examined by double immunofluorescence experiments in U2-OS cells. Serum starved cells display low amounts of Raf-1 (red) in the cytoplasm. However, upon serum stimulation for 2 hours, Raf-1 translocates to the nucleus where it binds to Rb (green), areas of co-localization can be visualized in yellow. Treatment with RRD-251 in the presence of serum displays no evidence of co-localization (yellow) **(Figure 22)**. This result verifies that RRD-251 can disrupt the Rb-Raf-1 interaction in intact cells.

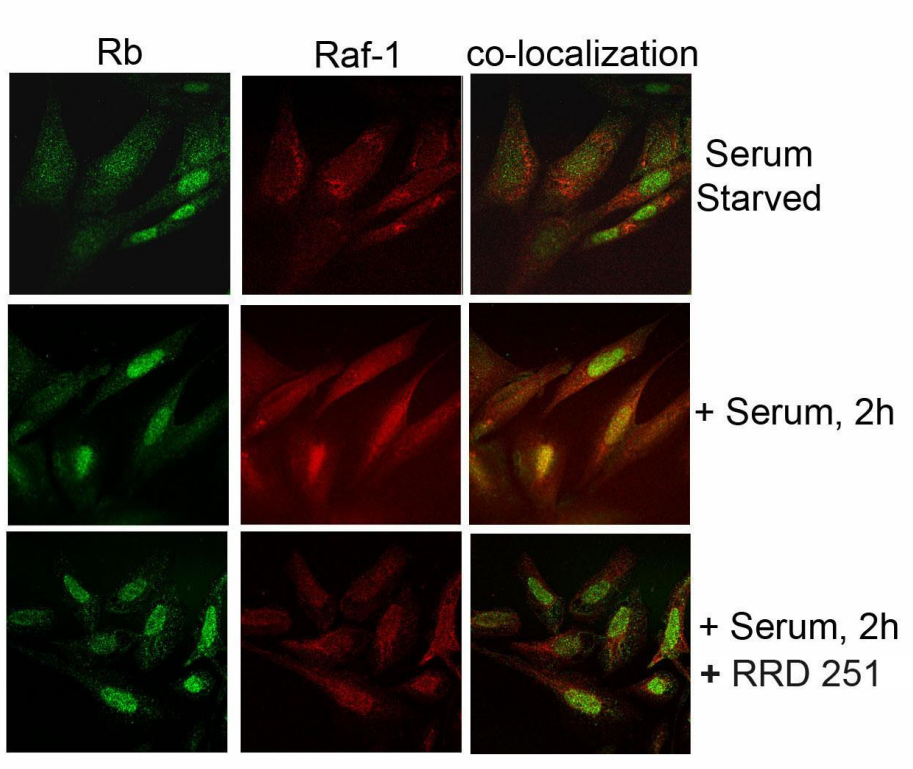


Figure 22. RRD 251 can inhibit Rb-Raf-1 colocalization. U2OS cells were immunostained with an anti-Raf-1 polyclonal antibody and an anti-Rb mouse monoclonal antibody, and the proteins were visualized by confocal microscopy.

RRD-251 inhibits Rb phosphorylation independent of kinase inhibition

Phosphorylation of Rb is necessary for inactivation of Rb and cell cycle progression to occur. Previous studies with the Raf-1 peptide revealed that inhibition of Rb-Raf-1 interaction resulted in inhibition of phosphorylation of Rb. Examination of lysates from cells serum stimulated for 2 hours (time point when Raf-1 binds and phosphorylates Rb) in the presence of RRD-251 showed a reduction in Rb phosphorylation, as seen by western blotting **(Figure 23A)**. At the same time, *in vitro* kinase assays showed that RRD-251 did not affect the kinase activities associated with Raf-1 **(Figure 23B-C)** on either MEK or Rb substrates, cyclin D on Rb substrate **(Figure 23D)**, or cyclin E on Histone H1 substrate **(Figure 23E)**, cyclin D and E kinase assays were performed by Piyali Dasgupta. These results suggest that the reduction in Rb phosphorylation in cells treated with RRD-251 is due to a disruption in the association of Raf-1 with Rb and that Raf-1 has to physically interact with Rb to inactivate it.

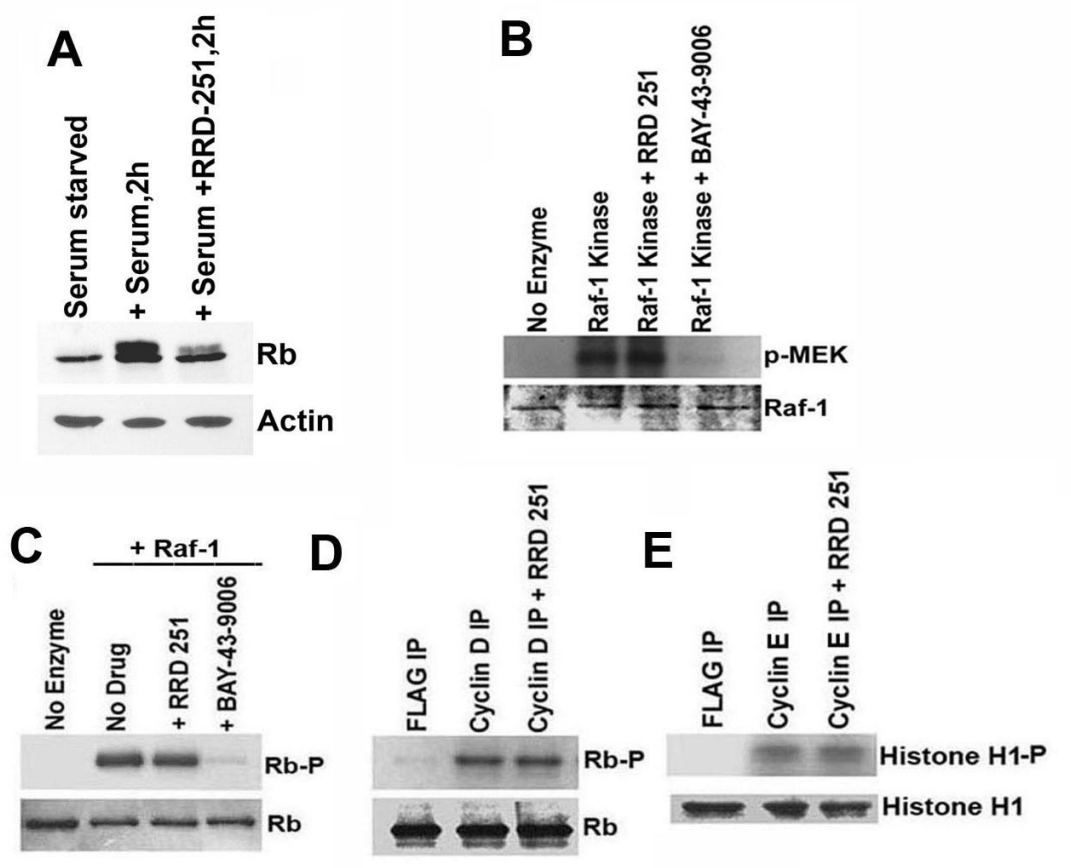


Figure 23. RRD-251 does not affect kinase activity. (A) RRD-251 inhibits Rb phosphorylation at the time point when Raf-1 binds to Rb, 2 hours (B-C) RRD-251 treatment does not inhibit Raf-1 kinase activity on MEK (B) or Rb (C) in *in vitro* kinase assays; BAY-43-9006 was used as a control. (D) RRD-251 does not inhibit cyclin D kinase activity in *in vitro* kinase assays. (E) RRD-251 does not inhibit cyclin E kinase activity in *in vitro* kinase assays.

RRD-251 inhibits E2F transcriptional activity

We next reasoned that if the disruption of the Rb/Raf-1 binding has functional consequences on cellular physiology, then RRD-251 should affect the transcriptional activity of E2F1. To examine this, transient transfection experiments were done in control A549 cells as well as A549 cells stably expressing two different shRNA constructs (sh6 and sh8) targeting Rb; these A549 cells had significantly less Rb protein compared to parental A549 cells. Transfection of E2F1 induced the expression of an E2-CAT reporter; treatment of the transfected cells with RRD-251 repressed E2F1-mediated transcription in a dose dependent manner (25-100μM) in wild type A549 cells but not in the A549 cells lacking Rb **(Figure 24A)**; this suggests that the presence of Rb is necessary for RRD-251 to function, E2-CAT reporter assays done in Figure 24A were done by Smitha Pillai. The effect of RRD-251 on the expression of two endogenous E2F-regulated proliferative promoters was next examined. A549 cells were serum starved for 72 h and serum stimulated for 24h in the presence or absence of RRD-251 (20μM) and the level of thymidylate synthase (*TS*) and *cdc6* gene expression was assessed by Real-time PCR. It was found that inhibition of the Rb-Raf-1 interaction correlated with the silencing of the *TS* and c*dc6* genes **(Figure 24B)**. We had reported that Raf-1 can be detected on proliferative promoters upon serum stimulation and these results indicate that RRD-251 probably affects E2F-mediated transcription by dissociating Raf-1 from the promoters. We had shown that the binding of Raf-1 to Rb resulted in the

dissociation of the co-repressor Brg-1 from E2F-responsive proliferative promoters (209); chromatin immunoprecipitation assays were carried out to examine whether RRD-251 affects this process. It was found that the association of Raf-1 to the above promoters upon serum-stimulation for 2 hours was disrupted by pre-treatment of cells with RRD-251 (20μM) **(Figure 24C)**. Furthermore, dissociation of the co-repressor Brg-1 from these promoters was also inhibited by RRD-251. This suggests that RRD-251 can modulate the transcriptional regulatory functions of Rb by modulating its phosphorylation status and affecting its interaction with chromatin remodeling proteins like Brg-1. The association of E2F1, HDAC1 and HP1 with these promoters was not affected by RRD-251, as seen by ChIP assays **(Figure 24C)**, ChIP assays done in Figure 24C were performed by Piyali Dasgupta.

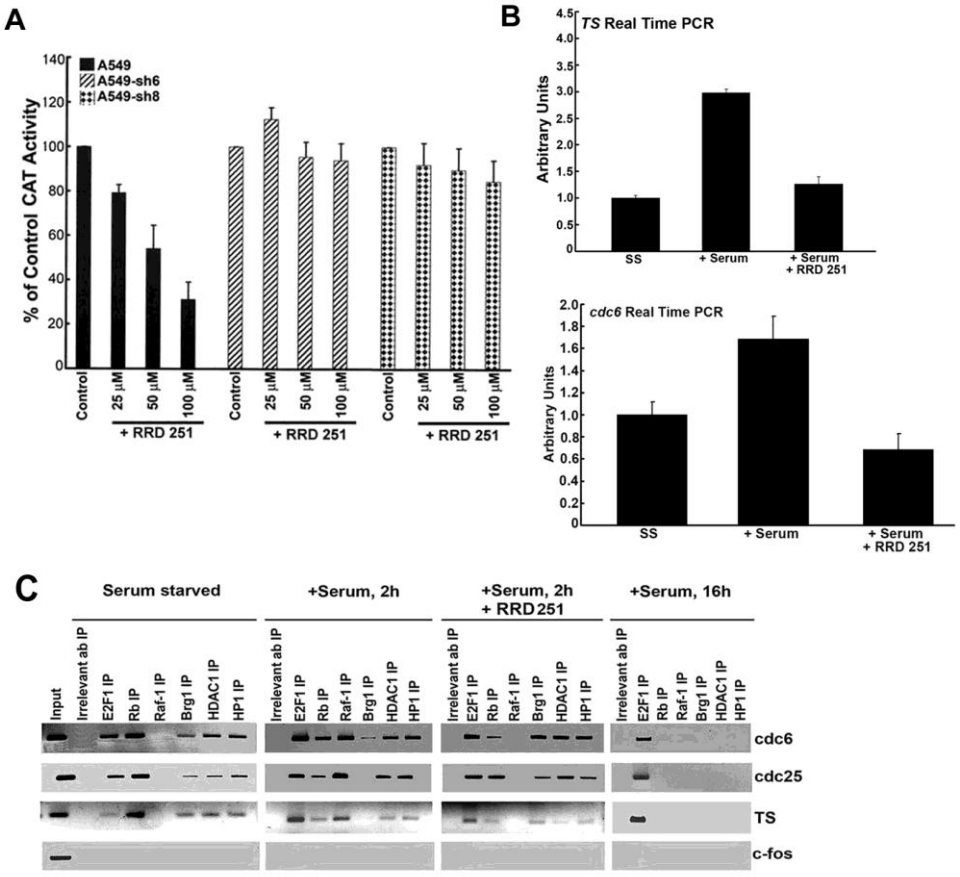


Figure 24. RRD-251 Inhibits E2F transcriptional activity (A) RRD-251 inhibits E2F1 mediated E2CAT transcription in CAT reporter assays. (B) RRD 251 inhibits *TS* and *cdc6* gene expression in real-time PCR experiments. (C) ChIP assays show that Brg1, not Raf-1 is present on quiescent A549 *cdc6, cdc25A,* and *TS* promoters. Upon serum stimulation, Brg1 dissociates from the promoters, correlating with Raf-1 binding. Serum stimulation in the presence of RRD-251 causes the dissociation of Raf and retention of Brg1 on E2F1 responsive promoters. Serum stimulation for 16 hours causes dissociation of Rb, Raf-1, Brg1, HDAC1 and HP1 from the promoters. An irrelevant antibody was used as a control for immunoprecipitations; *c-fos* promoter was used as a negative control.

RRD-251 inhibits angiogenesis in vitro and in vivo

Raf-1 kinase has been shown to play a role in facilitating angiogenesis (210,267) and it has been suggested that Raf-1-mediated inactivation of Rb is involved in the process (209). We first examined A549 cells treated with RRD-251 for VEGF levels in culture media. Asynchronously growing A549 cells treated with RRD-251 for 24hours with either 20μM or 50μM displayed a significant decrease in VEGF levels **(Figure 25A)**. To examine whether angiogenic tubule formation could be inhibited by RRD-251, human umbilical vein endothelial cells (HUVECs) were grown in matrigel in the presence or absence of 20μM RRD-251; RRD-251 significantly inhibited the angiogenic tubule formation **(Figure 25B)**. These results were confirmed in an *ex-vivo* experiment using rat aortic rings. As shown in **Figure 25C**, 20μM RRD-251 was able to inhibit angiogenic sprouting from rat aortic rings grown in growth factor rich matrigel for 7 days. Quantitation of vessel area showed a significant reduction in angiogenesis **(Figure 25D)**. Because RRD-251 was able to greatly inhibit angiogenesis *in vitro*, we examined whether RRD-251 could inhibit angiogenesis in matrigel plugs *in vivo* (251). Aythmic nude mice were injected with cold matrigel in both flanks. Mice were administered either vehicle or RRD-251 50 mg/kg body weight (MPK) by intraperitoneal (i.p.) injection daily for one week. On the last day the mice were injected with 100 MPK FITC-Dextran via the tail vein. The mice were euthanized and matrigel plugs were fixed in formalin; angiogenesis in the entire plugs were assessed by confocal imaging. FITC images displayed growth of angiogenic

tubules in plugs from mice that received vehicle; in contrast, there was a remarkable inhibition of angiogenic vessel formation in the matrigel plugs from mice treated with RRD-251 **(Figure 25E)**. Quantitation of vessel intensity is plotted as relative angiogenesis per image and shows significant inhibition, p=0.0004 **(Figure 25F)**. Further examination of the matrigel plugs by H&E staining showed a complete inhibition of cells migrating into the matrigel for vessel formation **(Figure 25G)**.

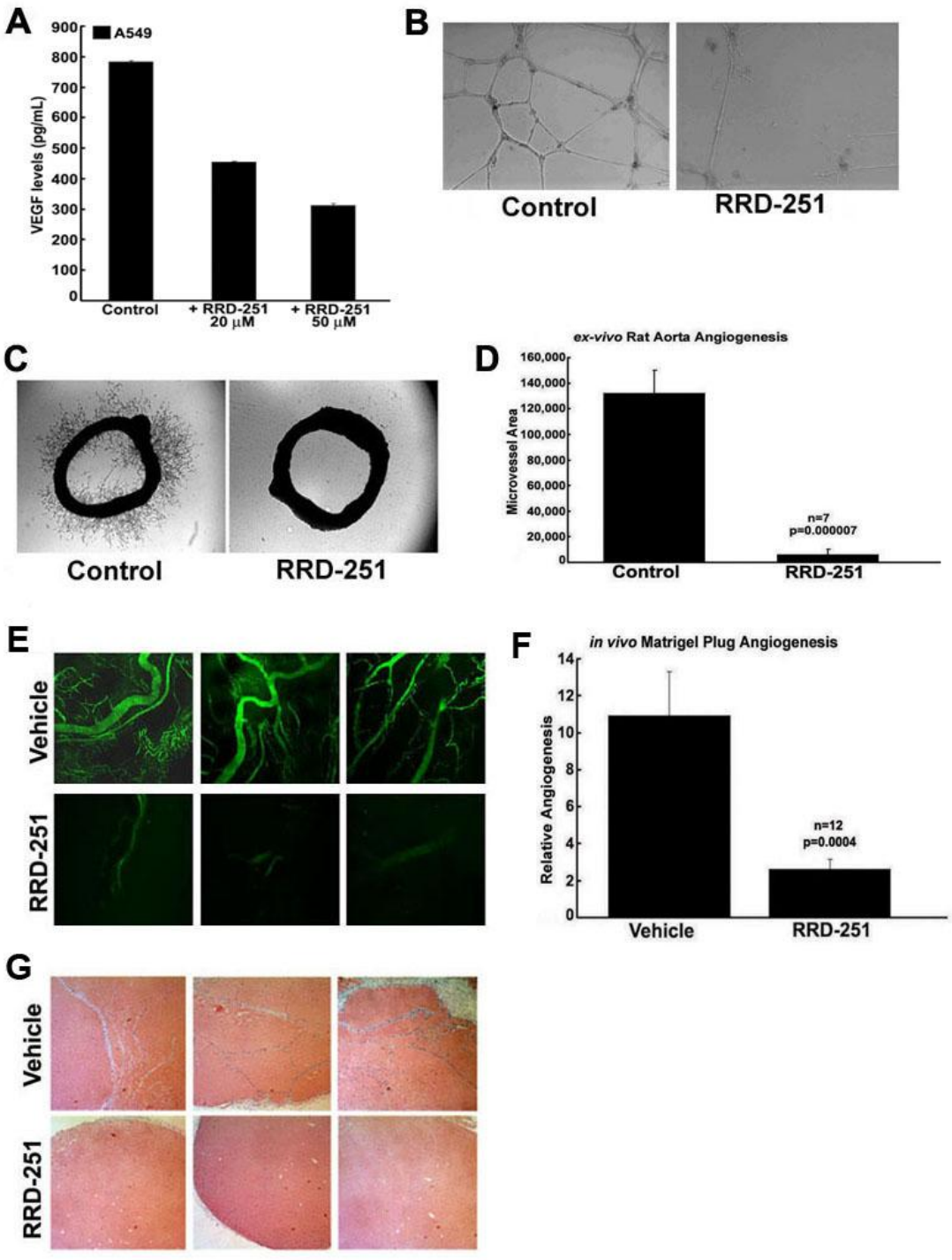


Figure 25. RRD-251 inhibits angiogenesis *in vitro* and *in vivo*. (A) RRD-251

inhibits VEGF levels in asynchronously growing A549 cells when treated at 20μM and 50μM. (B) RRD-251 inhibits Human Umbilical Vein Endothelial cell angiogenic tubule formation in matrigel. (C) RRD-251 inhibits angiogenesis in a rat aorta matrigel model. (D) Quantitation of vessel density. (E) Confocal FITC images of matrigel plugs from nude mice treated with Vehicle or RRD-251 50 MPK daily for one week. (F) Quantitation of FITC vessels in plugs. (G) H&E staining of matrigel plugs from nude mice treated with Vehicle or RRD-251 50 MPK. H&E images display ¼ of matrigel plug.

Antitumor activity of RRD-251

The ability of RRD-251 to inhibit cell proliferation, adherence-independent growth and angiogenesis demonstrates that it has desirable anti-cancer drug properties. This prompted us to assess whether RRD-251 could inhibit tumor growth *in vivo* in nude mouse xenograft models. Athymic nude mice were implanted s.c. with $1X10^7$ A549 cells bilaterally and the tumors were allowed to reach $200mm^3$ in size before oral or i.p. administration of RRD-251 or vehicle (209,248). Tumors from vehicle treated mice grew to an average size of 1040 ± 128 mm^3**;** in contrast, tumors in mice treated with RRD-251 did not grow and even regressed slightly (50 MPK-i.p.: 145 ± $20mm^3$; 150 MPK-oral 148 ± 32 mm^3) **(Figure 26A)**. Oral dose response experiments were carried out on A549 xenografts, which resulted in RRD-251 100 MPK and 150 MPK completely inhibiting tumor growth **(Figure 26B)**. Tumors from vehicle treated mice reached an average size of 996 ± 180 mm^3; in contrast, tumors in mice treated with RRD-251 (oral) responded in a dose dependent manner. Complete inhibition was seen in 100 MPK-oral: 293 ± $44mm^3$ and 150 MPK-oral: 237 ± 67 mm^3 **(Figure 26B)**. A549 xenograft assays in Figure 26A-B were done by Adam Carie. Similar results were observed with H1650 xenograft tumors; RRD-251 inhibited tumor growth significantly (2185 ± $326mm^3$ in vehicle treated animals compared to 557 ± $76mm^3$ in RRD-251 (50 MPK-i.p.) treated animals) **(Figure 26C)**. We also examined the efficacy of RRD-251 treatment in SK-MEL-28/matrigel xenografts since this cell line was most sensitive to treatment with RRD-251. SK-MEL-28

cells do not form tumors easily in mice and therefore were used in combination with matrigel (1:1) to allow the tumors to form. Mice treated with RRD-251 50 MPK-i.p. had significantly smaller tumors compared to vehicle treated mice (861±106mm^3 in vehicle treated mice compared to 341±42mm^3 in RRD-251 treated mice) **(Figure 26D)**. These results indicate that disruption of Rb-Raf-1 interaction is a viable method for inhibiting several types of tumors.

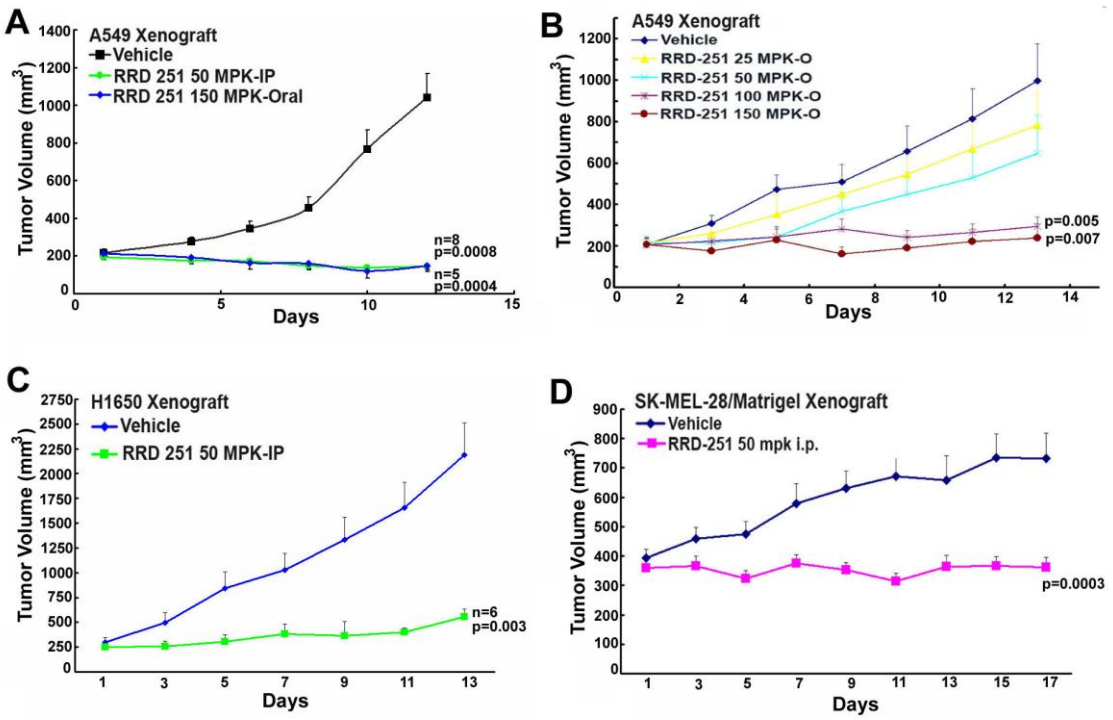


Figure 26. Intraperitoneal (i.p.) and oral administration of RRD-251 inhibits human tumor growth in nude mice. (A) A549 cells xenotransplanted bilaterally into the flanks of athymic nude mice were allowed to grow for 14 days until tumor volume reached 200mm^3; daily administration of RRD-251 at 50 MPK-i.p. and 150 MPK-oral completely inhibited tumor growth. (B) Dose response of RRD-251 administered by oral gavage, 100 MPK and 150 MPK could completely inhibit tumor growth. (C) RRD-251 inhibited H1650 xenograft tumor growth in nude mice. (D) RRD-251 inhibited SK-MEL-28 melanoma xenograft tumor growth in nude mice.

The A549 tumors (Figure 27A) were harvested at the end of the treatment and analyzed by immunohistochemistry by staining with hematoxylin and eosin (H&E), Ki-67, phospho-Rb (807,811), and CD-3, IHC staining was performed by Sandy Livingston in the University of South Florida IHC core. Histopathological analysis revealed a significant inhibition of proliferation in tumors from RRD-251 treated animals as seen by a reduction in Ki-67 staining **(Figure 27A)**; phosphorylation of Rb was also reduced as seen by staining with an antibody to phospho-Rb **(Figure 27A)**. The tumors also showed a reduction in microvasculature, as seen by CD31 staining **(Figure 27A)**. Quantitation of Ki-67 staining, phospho-Rb staining and CD31 staining is shown **(Figure 27 B-D)**. To assess whether RRD-251 reached its target, tumors were homogenized and lysates were prepared to assess the inhibition of Rb-Raf-1 interaction *in vivo*. RRD-251 was found to specifically inhibit Rb-Raf-1 but not Rb/E2F1 binding in the lysates from tumor xenografts of treated mice **(Figure 28)**.

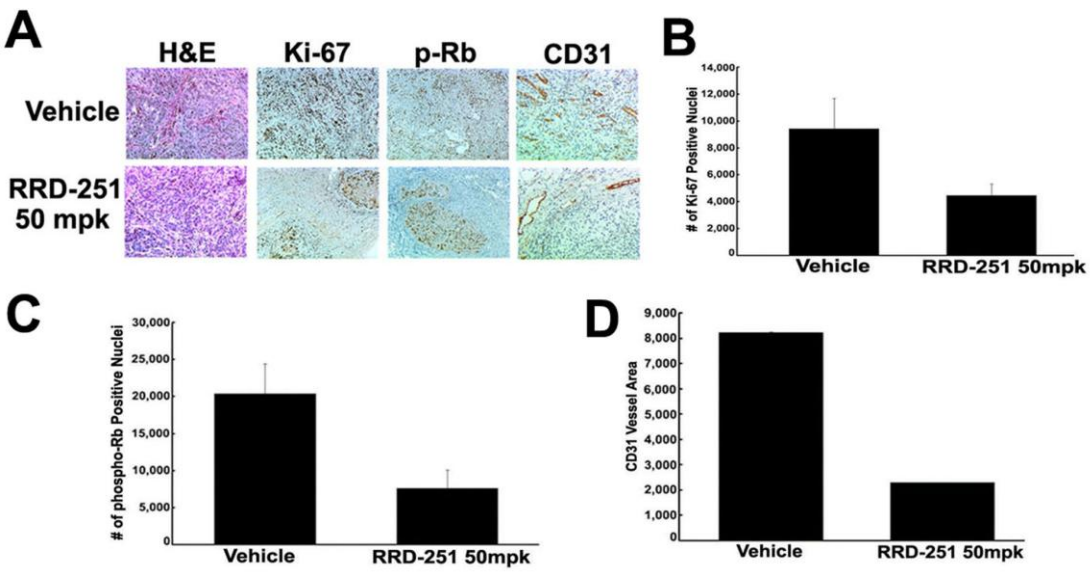


Figure 27. Tumors treated with RRD-251 display a decrease in proliferative and angiogenic markers (A) Immunohistochemical staining of tumors from mice treated with RRD-251. Tumors were stained with Ki-67 for proliferation, pRb for cell cycle, and CD31 for angiogenesis. (B-D) Quantitation of staining intensity for Ki-67, pRb and CD31.

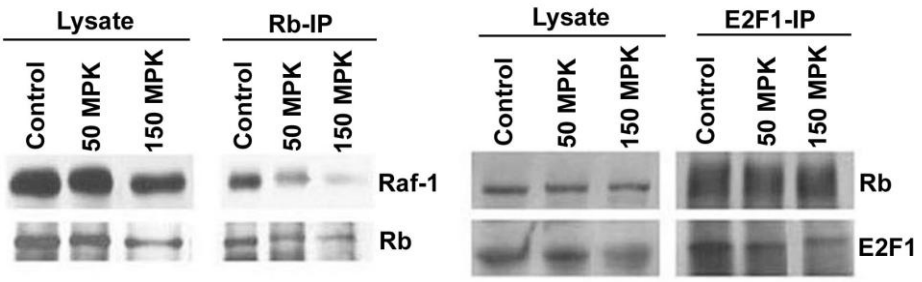


Figure 28. RRD-251 disrupts Rb-Raf-1 binding in xenograft tumors. Both doses of RRD-251 inhibit the Rb-Raf-1 interaction in tumor lysates without inhibiting Rb-E2F1 interaction, as seen by IP-Western blots.

Tumor Growth Inhibition by RRD-251 is Rb-dependent

Since RRD-251 did not inhibit the proliferation of A549 cells lacking Rb *in vitro*, experiments were done to assess whether tumors generated from these cells can respond to RRD-251 *in vivo*. Experiments in **Figure 29A** were carried out on nude mice carrying tumors from A549 cells stably expressing shRNAs for Rb (sh6 and sh8). Interestingly, these tumors did not respond to RRD-251 and continued to grow at the rate of the vehicle treated tumors **(Figure 29 A-B)** A549-sh6 and sh8 xenograft assays in Figure 29A-B were performed by Adam Carie and repeated by Rebecca Kinkade. To examine whether the sh6 and sh8 tumors maintained downregulation of Rb, lysates were made from the sh6 and sh8 tumors at the end of the experiment and a western blot was done for Rb. It was found that these tumors lacked Rb, further confirming that RRD-251 specifically targets the Rb-Raf-1 protein interaction to inhibit cell proliferation and tumor growth **(Figure 29C)**.

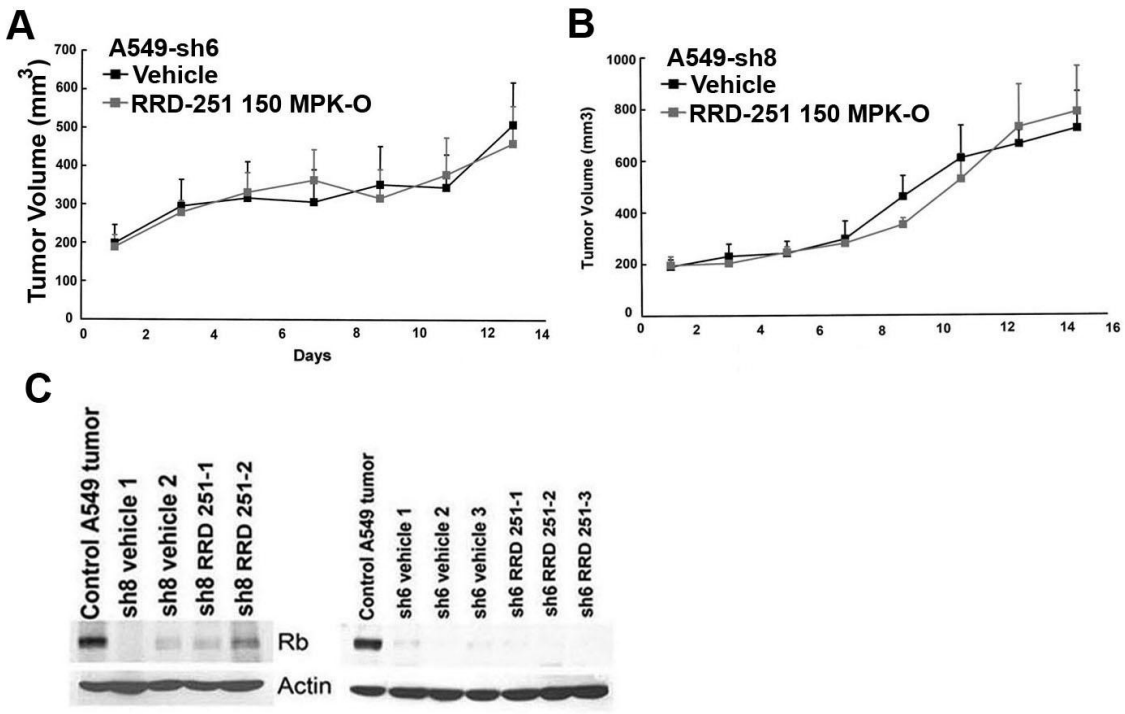


Figure 29. Inhibition of tumor growth is dependent on a functional Rb protein. A549-sh6 and A549-sh8 cells were implanted into the flanks of nude mice. (A-B) RRD-251 was unable to inhibit tumor growth in tumors lacking Rb protein. (C) Tumors maintain downregulation of Rb protein at the end of the experiment.

Discussion

The Ras/Raf/Mek/MAPK cascade is a proliferative pathway induced by a wide array of growth factors and is activated in many human tumors (175,176,268) and is an attractive target for the development of anti-cancer drugs (207,208,210,267). Raf-1 kinase itself has been targeted for cancer therapy and two clinical attempts have been made to inhibit Raf-1 activity in patients (190,269,270). It has been shown that signaling pathways through the MAP kinase cascade do not proceed in a linear fashion; instead they have been found to have substrates outside the cascade as well (173,271,272). In this context, the Rb protein appears to be an important cellular target of the Raf-1 kinase outside the MAP kinase cascade. Analysis of human NSCLC tumor samples revealed elevated levels of Rb-Raf-1 binding in tumor compared to adjacent normal controls (215), suggesting that Rb-Raf-1 interaction contributes to the oncogenesis of these tumors. While it is established that Rb gene itself is mutated in cancers like retinoblastoma, osteosarcoma and small-cell lung carcinoma, the majority of tumors harbor mutations in the upstream regulators of Rb function (1,38). These include genes like Ras, PTEN, p16INK4 as well as receptor tyrosine kinases (273-275). Our results show that the disruption of the Rb-Raf-1 interaction can be fruitfully utilized to inhibit the proliferation of cells harboring such mutations in the Rb regulatory pathway. Thus we believe that these molecules have the potential to target a wide variety of human cancers.

While inhibitors of cell proliferation, DNA damaging agents as well as microtubule disruptors have widely been used as anticancer agents, developments in the past decade have demonstrated that targeting angiogenesis is also an effective way of combating tumor growth (210). Thus humanized antibodies have been approved for use against certain cancers; further, recent studies suggest that growth factors like PlGF might be potential targets for anti-angiogenic therapy (276). In this context, our results show that RRD-251 can not only inhibit cell proliferation, but also inhibit neoangiogenesis *in vitro* and *in vivo*. Given the published reports that Raf-1 kinase contributes to angiogenesis and that VEGF can induce Rb phosphorylation, it is likely that RRD-251 is inhibiting angiogenesis by affecting these molecules (210,277). The ability of RRD-251 to inhibit both cell proliferation as well as angiogenesis might be acting in a two-pronged manner to inhibit the growth of tumors *in vivo*; these are desirable features in anti-cancer drugs.

Raf-1 has been shown to play a role in apoptosis, independently of MAPK activation. Raf-1 has prosurvival functions that regulate apoptosis; two different mechanisms have been established for this role (195,278). In one study, Raf-1 is targeted to the mitochondria by Bcl-2 protein promoting resistance to apoptosis (195). Another anti-apoptotic mechanism in which Raf-1 was shown to function was through its association with apoptosis signal regulating kinase 1 (ASK1) (278). It can be imagined that our results with apoptosis in melanoma cells may

be reflective of one of these two scenarios. ASK1 also binds to Rb to inactivate it so that it can overcome Rb's restraint on the cell cycle and therefore induce apoptosis (237). Since both Raf-1 and Rb bind to ASK1, it is possible that in certain types of cells (melanoma) these proteins function in an oligomeric complex where Raf-1 is bound to Rb and ASK1, when the Rb-Raf-1 interaction is disrupted, ASK1 can then induce apoptosis. Further studies are needed to examine the cell line and stimuli dependency of this interaction and will be useful for developing novel inhibitors capable of either inducing apoptosis or inhibiting cell proliferation, depending on the cellular context.

While it has been difficult to generate small molecule inhibitors of protein-protein (269) interactions that are clinically active, recent success in disrupting the hdm2-p53 (279) interaction shows that this is a viable strategy to develop novel anti-cancer drugs. Identification of RRD-251 as a cell–permeable, orally available, and highly selective inhibitor of the Rb-Raf-1 interaction is an example of the practicality of targeting protein-protein interaction for cancer therapy. Although we find that RRD-251 inhibits Rb-Raf-1 *in vitro* at nM concentrations in an *in vitro* ELISA assay, higher concentrations are needed to inhibit cell proliferation as well as growth of cells in soft agar; this finding is similar to what has been observed with other anti-cancer drugs such as BAY-43-9006, R547, and Iressa (212,280,281). At the same time, our *in vivo* studies show that concentrations can be achieved *in vivo* where RRD-251 has a significant

therapeutic benefit.

The finding that RRD-251 is effective in inhibiting the proliferation of cells harboring a wide variety of mutations in signaling cascades that inactivate Rb, but does not affect cells carrying mutated Rb or no Rb shows the specificity of this agent. Rb protein has been reported to interact with about one hundred proteins in the cell; it can be imagined that small molecules that can maintain the tumor suppressor functions of Rb by disrupting its physical interaction with other proteins would be a fruitful avenue to develop novel anti-cancer drugs.

Chapter 4: Nicotine Promotes Tumor Growth and Metastasis in Mouse Models of Lung Cancer

Abstract

Nicotine is the major addictive component of tobacco smoke. Although it is non-carcinogenic, it can induce cell proliferation and angiogenesis in non-neuronal cells. Here we show that nicotine significantly promotes the progression and metastasis of tumors already initiated. Nicotine administration either by intraperitoneal (i.p.) injection or transdermal patches caused a remarkable increase in the size of Line1 tumors implanted into BALB/c mice. Once the tumors were surgically removed, nicotine treated mice had markedly higher tumor recurrence as compared to the vehicle treated mice (59.7 % +/- 3.5 vs. 19.5 % +/- 7.7 respectively, p = 0.01, n=16). Nicotine also increased metastasis of dorsally implanted Line1 tumors to the lungs. While vehicle treated mice had an average of 0.9 +/- 0.2 lung metastases per mouse, nicotine treated mice had 8.1 +/- 1.7, p = 0.001, n=16. These studies on transplanted tumors were extended to a mouse model where the tumors were induced by the tobacco carcinogen, NNK. Lung tumors were initiated in A/J mice by i.p. injection of NNK; administration of 1 mg/kg nicotine three times a week led to an increase in the size as well as the number of tumors formed in the lungs. In addition, nicotine

significantly reduced the expression of epithelial markers, E-Cadherin and β-Catenin in the tumors of A/J mice. We believe that exposure to nicotine, either by tobacco smoke or nicotine supplements might facilitate increased tumor growth and metastasis.

Introduction

Lung cancer is the predominant cancer in the developed world and its onset is strongly associated with smoking habits (282,283). Despite the evident linkage of smoking to lung cancer, 30% of smokers diagnosed with lung cancer continue to smoke (284). Tobacco smoke contains a wide array of compounds that are deleterious to health; some of these compounds such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) are nicotine derivatives and are highly carcinogenic (218). These molecules can form adducts with cellular DNA, leading to mutations in vital genes like Ras, p53, and Rb (219). While nicotine is the addictive component in cigarette smoke, it is not a carcinogen and cannot initiate tumor formation in animals.

Nicotine exerts its cellular functions through nicotinic acetylcholine receptors (nAChRs), which are widespread in neurons and neuromuscular junctions (285). nAChRs are pentameric proteins consisting of nine α subunits (α2-α10) and three β subunits (β2-β4) in non-neuronal cells; delta and γ subunits

are present in neuronal systems (220). Recent studies have shown that nAChRs are also present in a wide array of non-neuronal tissues, including human bronchial epithelial cells, human endothelial cells and astrocytes (220-222). The finding that nAChRs are present on non-neuronal cells was followed by the observation that nicotine could induce the proliferation of endothelial cells (221) as well as lung carcinoma cell lines (226). In non-neuronal tissues, nicotine has been shown to induce the secretion of growth factors such as bFGF, TGF-α, VEGF, and PDGF (286). Nicotine has been shown to induce migration and invasion of cells via phosphorylation of calpain family members (287). Nicotine and its related carcinogens, like NNK, have been found to activate Raf-1, EGFR, Src, Akt and 5-lipooxygenase-mediated growth stimulatory pathways (227,288,289).

In addition, nicotine has also been found to inhibit apoptosis induced by opioids, etoposide, cisplatin, and UV irradiation in lung cancer cells (290,291). Nicotine's inhibitory effects have been attributed to its ability to activate and phosphorylate anti-apoptotic proteins like Bcl-2, induction of NF-κB complexes, activation of Akt pathway as well as inactivation of pro-apoptotic proteins such as Bad and Bax through phosphorylation in lung cancer cells (292,293). It was found that nicotine could prevent the apoptotic activity of gemcitabine, cisplatin and taxol, which are standard therapy for NSCLC, in a variety of human NSCLC cell lines. The protective effects of nicotine involved induction of IAP proteins,

XIAP and survivin in lung cancer cells (290). The anti-apoptotic effects of nicotine were mediated by activation of Akt which facilitated the stabilization of XIAP proteins and transcriptional activation of survivin. Nicotine stimulation increased the binding of E2F1 to the survivin promoter (290). These results further support clinical studies that demonstrate how patients who continue to smoke have worse survival profiles than those who quit before treatment (282). These studies also raise the possibility that patients who use nicotine supplements for smoking cessation might reduce the response to chemotherapeutic agents.

Recently, the mechanisms underlying the proliferative signaling of nAChRs have been discovered. It was found that nicotine functions like a growth factor, binding to nAChRs causing a recruitment of β-arrestin and Src to the nicotinic receptors resulting in the activation of MAPK and the subsequent binding of Rb-Raf-1 pathways (215). It was found that the levels of Rb-Raf-1 interaction were elevated in human NSCLC tumors compared to normal adjacent tissue (215) **(Figure 30A)**. This result suggested that Rb-Raf-1 pathways probably contribute to oncogenesis; the increased presence of Raf-1 on proliferative promoters in human NSCLC tumors supports this hypothesis (215) **(Figure 30B)**. It is likely that tumors exposed to nicotine have a proliferative advantage. Smokers have been found to be less responsive to chemotherapy and were also found to have increased metastasis of breast cancers to the lung (294-296).

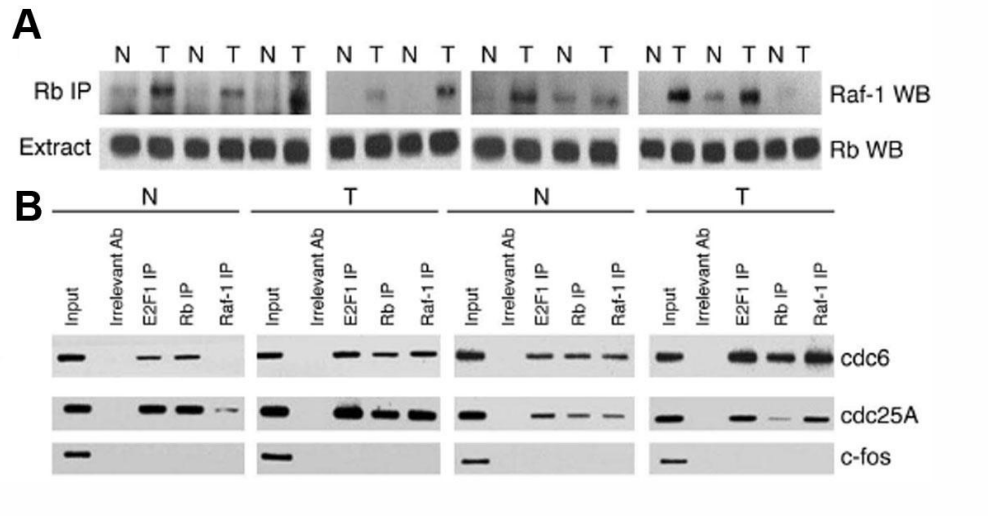


Figure 30. Rb-Raf-1 interaction is elevated in tumors. (A) NSCLC tumors *(T)* contained more Rb-Raf-1 complexes than adjacent normal tissue *(N).* Rb-Raf-1 interaction was assessed by IP-WB on nuclear extracts. (B) ChIP assays on human NSCLC tumor samples show that more Raf-1 was present on *cdc6* and *cdc25A* promoters in tumor samples compared to normal adjacent tissues. Adapted with permission from Dasgupta et al (215).

The key players mediating the mitogenic effects of nicotine are β-arrestin-1 and Src kinase. Src family kinases are involved in multiple receptor-mediated signaling pathways that regulate proliferation, survival, metastasis and angiogenesis. β-arrestin-1 is vital for nicotine mediated activation of Src and cell proliferation. β-arrestin-1 family members have been shown to act as scaffold proteins that recruit a variety of signaling molecules to membrane-bound receptors in a highly coordinated manner. β-arrestin-1 is required for nAChR-mediated activation of MEK/ERK pathway and proliferation of NSCLCs. Binding of nicotine to nAChRs causes a recruitment of β-arrestin-1 and Src to the nicotinic receptor resulting in activation of Rb-Raf-1 pathways (297). This signaling event causes the recruitment of E2F1, Raf-1 and Rb on E2F responsive proliferative promoters (215). Raf-1 inactivates Rb and facilitates Rb dissociation from the promoters and an increase in E2F1 therefore, inducing transcription of S-phase genes and further cell cycle progression (215). Understanding of the signaling pathways mediated by nAChRs in cancer cells may be a possible avenue for cancer therapy by targeting either β-arrestin-Src or Rb-Raf-1 interactions **(Figure 31)**. We have shown in Chapter 3 that inhibition of Rb-Raf-1 interaction is a viable mechanism for targeted cancer therapy.

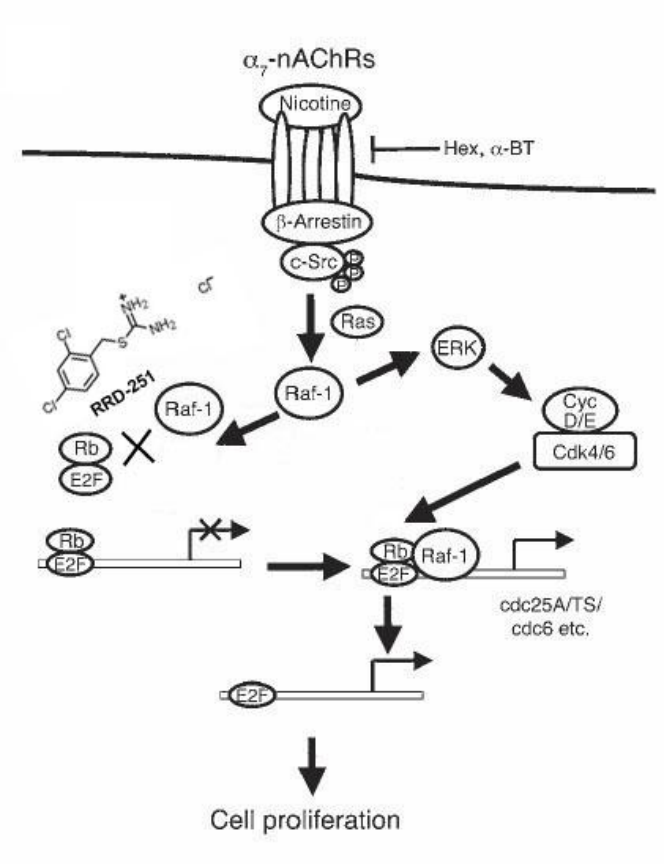


Figure 31. Schematic predicting the proliferative signaling by nAChRs in NSCLC cells. Nicotine stimulation causes the assembly of oligomeric complexes involving β-Arrestin, Src and nAChRs, facilitating the activation of Src. This leads to the activation of Raf-1, which binds to Rb; activation of MAPK and cyclins/cdks also occur. The activation of Src facilitates the binding of Raf-1 to Rb and multimeric complexes containing Rb, Raf-1 and E2F1 occupy proliferative promoters. Sustained mitogenic signaling leads to the dissociation of Raf-1 and Rb, while E2F remains bound to the promoter facilitating S-phase entry. Disruption of the Rb-Raf-1 interaction can block nicotine induced proliferation of NSCLC cells. Adapted with permission from Dasgupta et al (215).

Induction of cell proliferation, enhancement of cell survival and induction of angiogenesis are all effects seen from nicotine stimulation and they all contribute to the growth and progression of solid tumors in vivo. Studies from the Cooke laboratory have shown that nicotine can induce angiogenesis both in vitro and in vivo (222,223). It has also been shown that second hand smoke could induce tumor angiogenesis and growth (298). Nicotine induces angiogenesis through α7 nAChR subunits. Interestingly, inhibition of Src or Rb-Raf-1 interaction and not PI3K could efficiently inhibit nicotine induced angiogenesis (297).

Although tobacco carcinogens initiate and promote tumorigenesis, recent studies on nicotine raise the possibility that exposure to nicotine either by cigarette substitutes or nicotine supplements might confer a proliferative advantage for tumors already initiated. Recent studies from the Russo lab has shown that inhibition of nAChRs by α-cobratoxin can inhibit the growth of A549 tumors in immunocompromised mice (229); similarly, it has been shown that a combination of nicotine and estradiol can promote the growth of A549 tumors in athymic mice (230). While these studies suggest a role for nAChRs in tumor growth, there are no studies demonstrating the effect of nicotine as a single agent on tumor growth and metastasis in immunocompetent mice. Studies presented here show that nicotine by itself can induce the growth and metastasis of tumors in immunocompetent mice, independent of other tobacco carcinogens. Nicotine administered intraperitoneally or by commercially available transdermal

patches could promote tumor growth substantially. Further, mice exposed to nicotine showed significantly enhanced lung metastasis as well as tumor recurrence post surgical removal of the primary tumor. Similar effects were observed on implanted tumors as well as tumors induced by the tobacco carcinogen, NNK. These results imply that nicotine can enhance the growth and metastasis of pre-established lung tumors.

Results

Nicotine promotes the growth of tumors in mice

To determine the effects of nicotine on tumor growth and metastasis in immunocompetent mice, Line1 mouse adenocarcinoma cells were utilized. Line1 cells form subcutaneous (s.c.) tumors in BALB/c mice, which can metastasize to the lungs (252). To examine whether nicotine induced proliferation of Line 1 cells, the cells were serum starved for 72 hours and subsequently stimulated with 1µM nicotine for 18 hours. S-phase entry was measured using BrdU incorporation assays. Nicotine could efficiently stimulate Line1 cells into S-phase and treatment with the Rb-Raf-1 disruptor, RRD-251 abrogated nicotine induced proliferation in Line1 cells **(Figure 32)**. Next, it was examined how nicotine affects the growth and metastasis of Line1 cells implanted into the flanks of BALB/c mice **(Figure 33)**. Female BALB/c mice were injected with 1 million Line1 cells s.c. into each flank. The mice were randomized into two groups, with one group receiving vehicle (*n*=8) and the second receiving 1mg/kg nicotine (*n*=8) thrice weekly by intraperitoneal (i.p.) injection **(Figure 33)**.

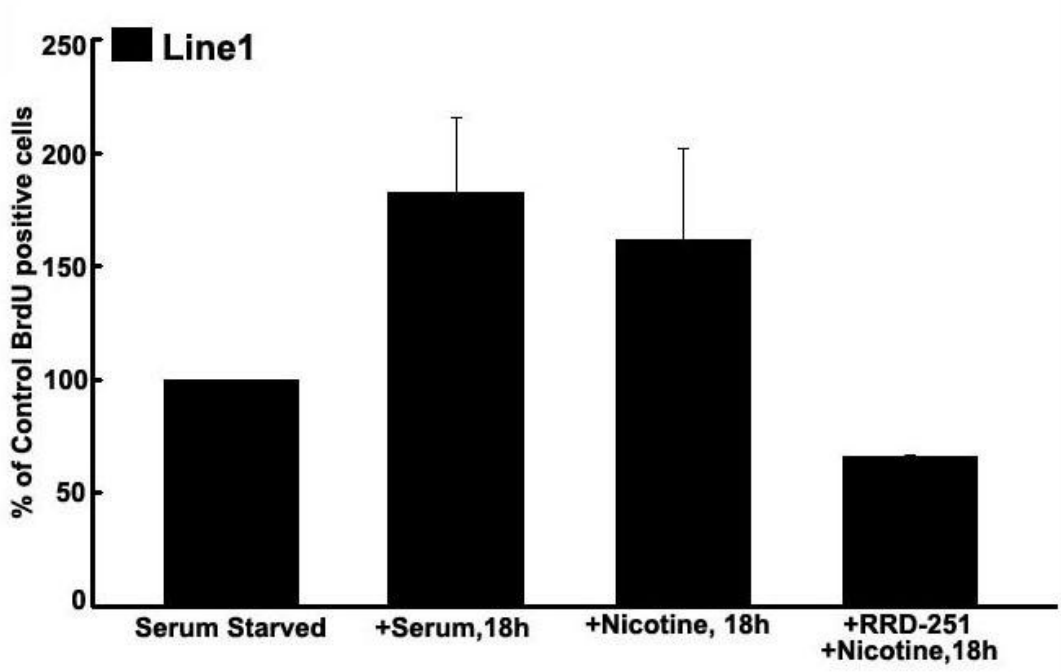


Figure 32. Nicotine (1μM) stimulates S-phase entry in Line1 mouse adenocarcinoma cells. Treatment with RRD-251 (20μM) abrogated nicotine-induced proliferation in Line1 cells.

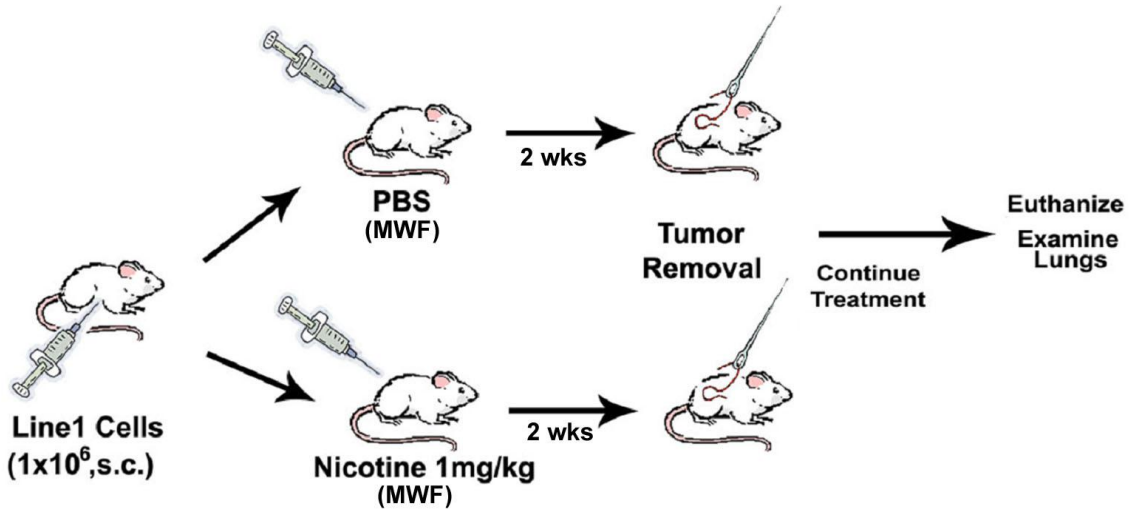


Figure 33. Schematic for the experimental design of Line1 tumor growth and metastasis in BALB/c mice. Line1 cells ($1x10^6$) are injected s.c. into the flanks of shaved BALB/c mice. Mice are randomized into two groups and administered either Vehicle (PBS) or nicotine 1mg/kg thrice weekly for 2 weeks. After two weeks, or when tumors reach 500-700mm^3, the tumors are surgically removed and the skin is stapled for one week. Mice continue to receive treatment for another 2 weeks or until tumors recurrence is evident.

Mice that received nicotine had significantly larger tumors compared to those receiving vehicle; tumor volumes averaged 695 +/- 98 mm^3 in vehicle treated mice, compared to 2267 +/- 369 mm^3 in nicotine treated mice **(Figure 34A)** p = 0.002. Based on the results with nicotine administered i.p., experiments were done to examine whether nicotine administered by over-the-counter transdermal patches could promote tumor growth. BALB/c mice (n=16) implanted with Line 1 tumors were randomized into two groups and nicotine patches were applied daily at a dose of 25 mg/kg nicotine. It was found that nicotine administered by transdermal patches could significantly increase the growth of Line1 tumors; control mice had an average tumor volume of 530 +/- 59 mm^3 whilst nicotine patch mice had an average volume of 871 +/- 106 mm^3 **(Figure 34B)**, p=0.019. Mice wearing nicotine patches also displayed changes in tumor shape, from oval with well-defined borders, to polygonal with irregular borders **(Figure 34C)**, potentially suggesting the nicotine treatment confers a more malignant phenotype. These experiments confirm that exposure to nicotine, even through nicotine supplements, might affect pre-established tumors.

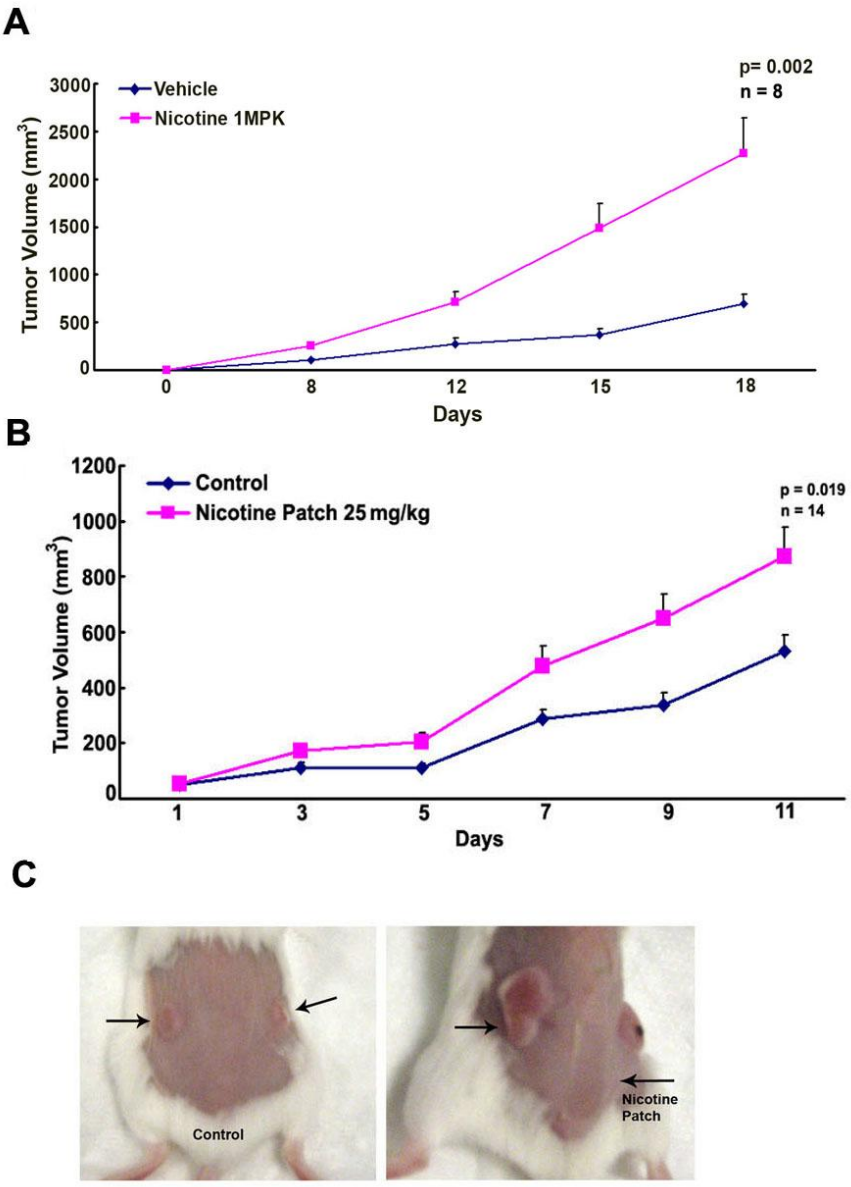


Figure 34. Nicotine promotes Line1 tumor growth. (A) Nicotine (1mg/kg) significantly promotes the growth of s.c. Line1 tumors when administered thrice weekly by i.p. injection. (B) Daily application of nicotine transdermal patches (25 mg/kg) enhanced tumor growth. (C) Mice bearing nicotine patches displayed irregular polygonal shaped tumors compared to control mice.

Nicotine promotes re-growth and metastasis of tumors in mice

Since nicotine was found to enhance tumor growth, experiments were conducted to assess its effect on tumor metastasis. In order to examine this, the implanted tumors were surgically removed after 14 days of treatment or once they reached 500-700mm^3. Tumors were removed to prevent discomfort from large tumors. Mice were anesthetized for tumor removal, and wounds were stapled closed. After the removal of staples, mice were administered vehicle or nicotine by i.p. injection for an additional 14 days. Interestingly, mice treated with nicotine showed a higher rate of tumor recurrence after the tumors were surgically removed **(Figure 35A)**; vehicle treated mice displayed an average of 19 +/-7% tumor recurrence, as compared to an average of 59 +/- 3% tumor recurrence in nicotine (1mg/kg) treated mice, p= 0.01. Tumor recurrence was calculated as percentage of recurring tumors out of the total number of tumors removed. Mice receiving the vehicle had an average of 0.9 +/- 0.2 metastatic foci in the lungs per mouse; in comparison, mice that received nicotine, 1 mg/kg thrice weekly, had an average of 8.1 +/- 1.7 foci in the lungs per mouse, p=0.001 **(Figure 35B)**. As shown in **Figure 35C**, nicotine treated mice also displayed significantly greater number of lung metastases as well as larger metastatic foci compared to those receiving vehicle. In addition, histologic examination of the lung tumors revealed larger metastatic foci in the nicotine treated mice **(Figure 35D)**.

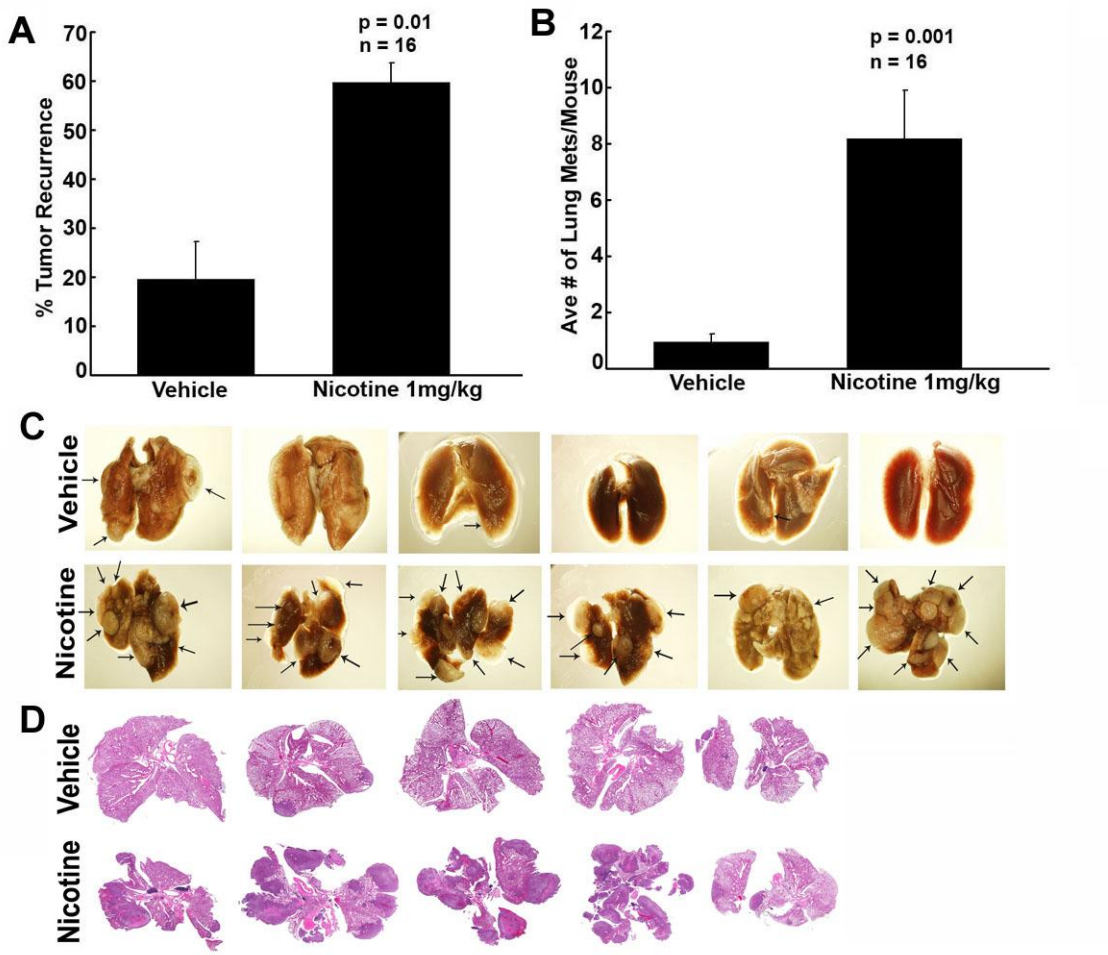


Figure 35. Nicotine increases metastatic potential. (A) Nicotine treated mice (1mg/kg) displayed higher incidence of tumor recurrence following surgical removal of tumors compared to the vehicle control group *p*=0.01, *n*=16. (B) Graph displaying the average total number of lung tumors per mouse in vehicle and nicotine treated mice, *p*=0.001, *n*=16. (C) Nicotine treated mice display significantly more lung metastasis from primary Line1 subcutaneous (s.c.) tumors. (D) H&E staining of lungs from vehicle and nicotine treated mice, nicotine treated mice display larger tumors.

Nicotine enhances the growth of tumors induced by tobacco carcinogens

Experiments were designed to examine the effects of nicotine on tumors induced by the tobacco carcinogen, NNK; this experimental system mimics a situation where tumors are initiated by a carcinogen, followed by exposure to nicotine alone. Towards this purpose, A/J mice (*n*=16) were treated with 100mg/kg NNK once a week for five weeks to initiate tumor formation and subsequently they were randomized into two groups. One group of mice received the vehicle (PBS) (*n*=8) whilst the second group received nicotine 1mg/kg (*n*=8) thrice weekly by i.p. injection; mice were treated with nicotine or vehicle for 28 weeks **(Figure 36)**. At necropsy, lungs from both vehicle and nicotine-treated mice had tumors **(Figure 37A-B)**. H&E stained lung sections, from both groups, were scanned and a pathologist (Dr. Domenico Coppola, Moffitt Cancer Center Pathology) outlined the tumor. The size and number of tumor foci were quantitated. Mice that received PBS after NNK injections had an average of 10 +/- 3 lung tumors per section and mice that received nicotine 1mg/kg had 16 +/- 3 tumors per section **(Figure 37C)**, p =0.01. Tumor size was also increased in nicotine treated mice **(Figure 37D)**. This suggests that exposure to nicotine of pre-established tumors can result in enhanced tumor growth.

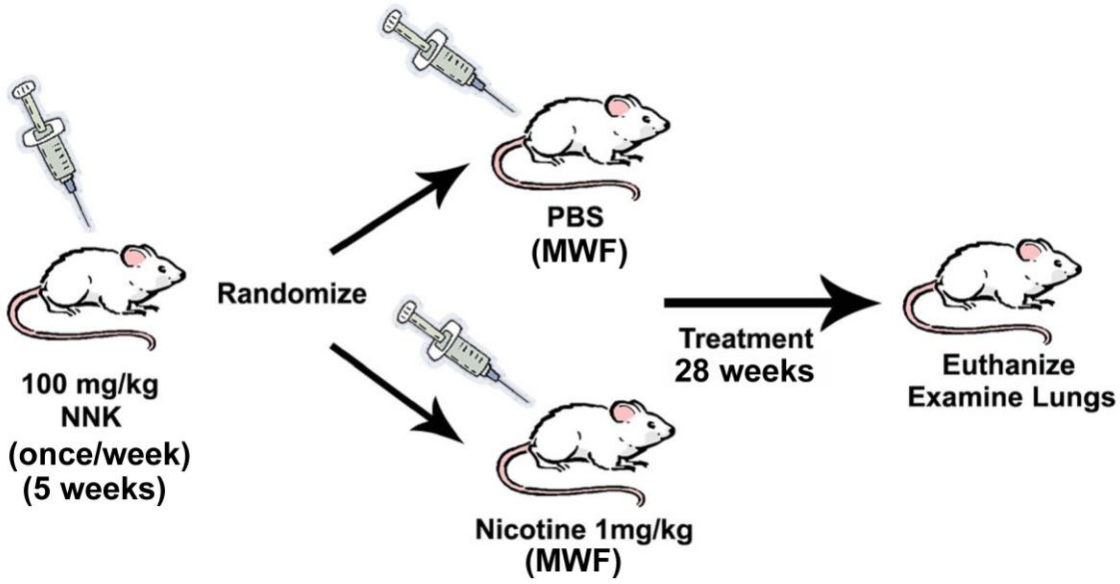


Figure 36. Schematic for NNK induced carcinogenesis experimental design. A/J mice were administered 100 mg/kg NNK once a week for 5 weeks and subsequently randomized into two groups. The control group received the vehicle (PBS) thrice weekly and group two received nicotine 1mg/kg thrice weekly by i.p. injection. Treatment with nicotine or PBS continued for 28 weeks. At endpoint, mice were sacrificed and lungs were examined for tumors.

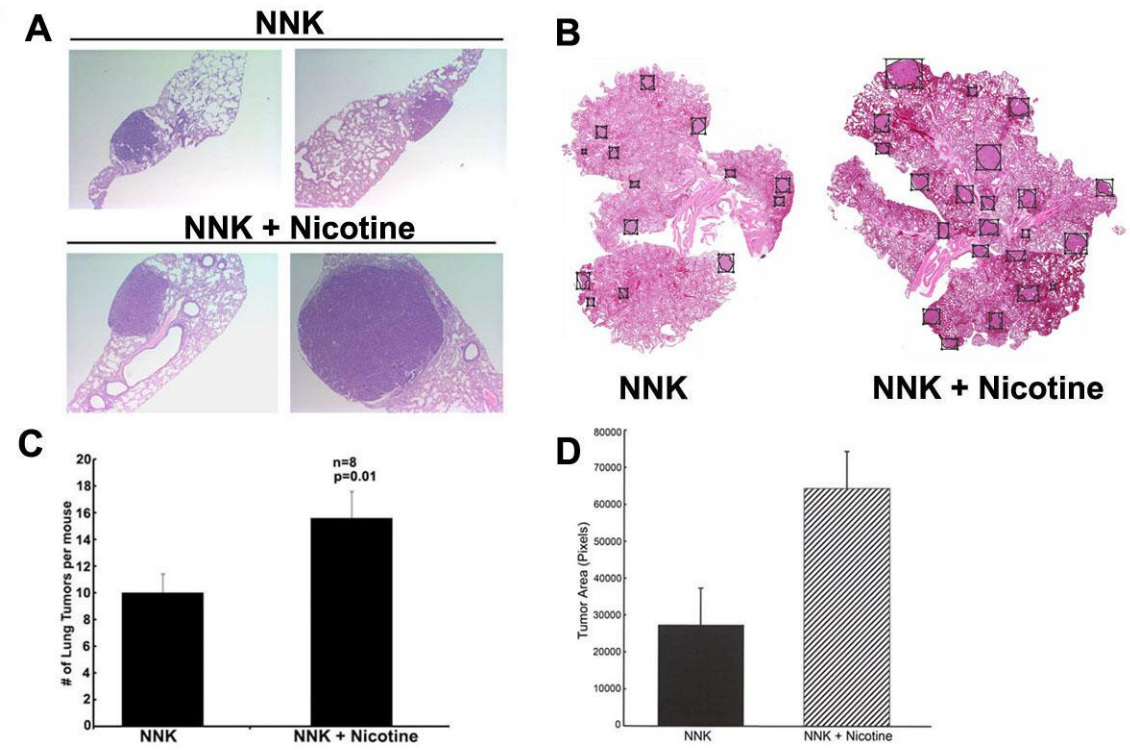


Figure 37. Nicotine (1 mg/kg) increases number and size of NNK induced lung tumors. (A) H&E staining of transverse sectioning of lungs. (B) Representative scanned images of H&E stained coronal lung sections. (C) Nicotine increases the average number of lung tumors per mouse p=0.01, n=8. (D) Nicotine increased tumor area.

Nicotine facilitates EMT-like changes in lung cancers

Given the observation that nicotine can induce tumor growth and promote metastasis, attempts were made to understand the molecular events mediating these processes. Epithelial-mesenchymal transition (EMT) is a phenomenon by which cells lose their epithelial phenotype and acquire more mesenchymal features that facilitate detachment and migration. We examined the tumors in A/J mice for changes consistent with an EMT-like phenomenon, using immunohistochemical staining for E-cadherin and β-Catenin, two proteins involved in the adhesion of epithelial cells. β-Catenin binds to E-Cadherin to facilitate cell adhesion and to exert its signaling functions. E-cadherin levels were found to be significantly decreased in the tumors of mice treated with nicotine **(Figure 38A)**, E-Cadherin staining was performed by Sarmistha Banerjee; the results are quantified in **Figure 38B**. The same mice revealed a loss of the typical β-Catenin membranous staining pattern in their lung tumors **(Figure 38C)**, β-Catenin staining was performed by Sandy Livingston in the USF IHC core; the results are quantitated in **Figure 38D**.

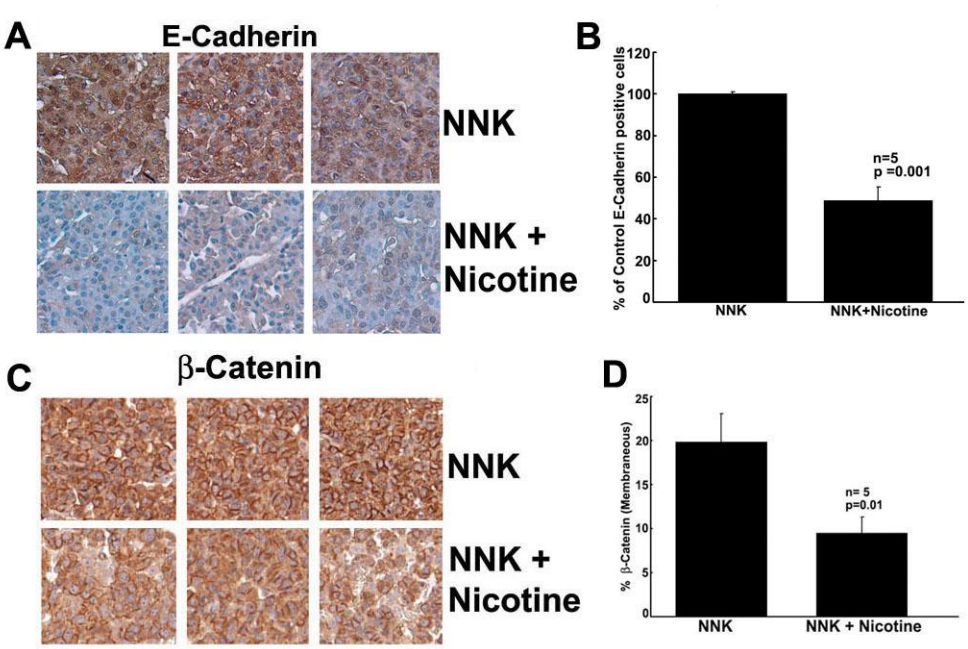


Figure 38. Nicotine reduced expression of epithelial markers. (A) E-cadherin staining of A/J lung tumors induced by NNK or NNK+ Nicotine. (B) Quantitation of E-cadherin intensity in tumors. (C) β-Catenin staining of A/J lung tumors induced by NNK or NNK and nicotine. (D) Quantitation of membranous β-Catenin.

Discussion

Several observations suggest that those exposed to tobacco carcinogens are more likely to develop larger, more vascularized tumors with a high propensity for metastatic spread and resistance to chemotherapy (296). In addition, about 30% of lung cancer patients who are smokers continue to smoke after they have been diagnosed (282). This is problematic, as smokers who continue to use tobacco after a cancer diagnosis or return to smoking, experience increased adverse medical consequences, such as: increased tumor progression, development of a second cancer, greater recurrence following successful treatment, greater cancer-related mortality, and reduced quality of life (299,300). While these studies strongly demonstrate a role for tobacco carcinogens in the initiation, growth and progression of cancers, the relative contribution of nicotine by itself to these processes is not known. This is a significant aspect, since the use of nicotine supplements is usually part of most cigarette smoking cessation programs. Nicotine supplementation through patches, nasal sprays, chewing gum, etc., is now widely used to assist in smoking cessation. The serum concentrations of nicotine achieved with these modalities vary, but the transdermal delivery of nicotine can result in serum concentrations of nicotine that are observed in active smokers (301). Although it is known that nicotine is not carcinogenic, the risks associated with long-term nicotine supplementation are unknown.

While nicotine has been demonstrated to induce cell proliferation, angiogenesis and growth of tumors implanted in immunodeficient mice (229), the studies presented here show for the first time that nicotine could indeed promote tumor growth in two fully immunocompetent mouse models. Further, our results show that the presence of nicotine can enhance the growth of lung tumors induced by a tobacco carcinogen. Essentially, the A/J mouse model is reflective of a situation where a smoker who has tumors initiated in the lung quits smoking and uses nicotine supplements to overcome the craving. Our results also show that a commercially available nicotine transdermal patch can promote the growth of tumors implanted into mice.

The finding that epithelial adhesion molecules like E-Cadherin and its binding partner β-Catenin are affected by nicotine provides a molecular basis for these findings. It can be imagined that nicotine, through the nAChR signaling pathways, induces changes in gene expression patterns to facilitate EMT and tumor metastasis. Indeed, it has been reported that the expression pattern of nAChR subunits are different in tumors from smokers and non-smokers (302). Given the ability of nicotine to affect various aspects of tumor growth and metastasis, it is possible that antagonists of nAChR signaling might prove beneficial in controlling the growth and progression of lung cancers; certain studies support this contention. Further, such agents that modulate the function of nAChRs such as varenicline, an agonist of $\alpha 4\beta 2$ nAChRs, might be better

alternatives for smoking cessation than nicotine itself.

We have shown that RRD-251 is capable of inhibiting nicotine-induced proliferation in line1 cells. It has previously been shown that treatment with the Raf-1 peptide to disrupt the Rb-Raf-1 function can inhibit nicotine induced cell proliferation, migration, invasion, and angiogenesis *in vitro* (215,297). In addition to targeting Rb-Raf-1, inhibition of Src activation could also prevent nicotine-induced angiogenesis *in vitro* (297). Targeting the key molecules in nicotine mediated tumor progression and metastasis may be a better alternative for smokers with NSCLC.

Chapter 5: TNF-α stimulates proliferative pathways in vascular smooth muscle cells

Abstract

Atherosclerosis is characterized by hyperplastic neointima and an inflammatory response with cytokines such as TNF-α. TNF-α is a pleiotropic cytokine that mediates inflammatory, proliferative, cytostatic, and cytotoxic effects in a variety of cell types, including endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). Interestingly, TNF-α has been shown to play two very opposing roles in these cell types; it mediates the inhibition of EC proliferation and induction of EC apoptosis while facilitating stimulation of proliferation and migration in VSMCs. Here we show that TNF-α is capable of stimulating proliferation in rat VSMCs as well human VSMCs in a Raf-1/MAPK dependent manner. TNF-α could increase the expression of E2F regulated proliferative *cdc6* and *cdc25A* genes in AoSMCs, as seen by real time PCR assays. Surprisingly, we find an activation of the stress-induced kinase, JNK1, in VSMCs upon treatment with TNF-α. TNF-α was capable of inducing the Rb-Raf-1 interaction and treatment with the Rb-Raf-1 inhibitor, RRD-251, could prevent TNF-α induced S-phase entry in AoSMCs. In addition, inhibition of Raf-1 or Src kinases using pharmacologic inhibitors could also prevent S-phase entry, while

inhibition of JNK was not as effective. These results suggest that inhibiting the Rb-Raf-1 interaction is a potential avenue to prevent VSMC proliferation associated with atherosclerosis.

Introduction

Development of atherosclerosis is a stringently regulated and complex process that results from aberrations in endothelial cell and vascular smooth muscle cell (VSMCs) function. Endothelial cells (EC) form the lining of the blood vessels and the heart, functioning as a barrier by regulating permeability, thrombogenicity, and production of growth inhibitory molecules (243). Endothelial cells also respond to mechanical forces. ECs are contact inhibited under normal conditions; but when endothelial cells sense an injury such as abrasion of a vessel, they proliferate and migrate leading to re-endothelialization at sites of injury (244). At the same time, vascular smooth muscle cells proliferate and migrate from the injured arterial wall into the vessel lumen leading to vessel thickening and occlusion, called restenosis (245). Intimal hyperplasia characterized by VSMC proliferation and extracellular matrix (ECM) deposition is a major process contributing to restenosis (246). Atherosclerotic lesions can be blocked if inhibition of VSMCs is effective (243). Several growth factors and cytokines are capable of stimulating VSMC migration and proliferation, such as platelet derived growth factor (PDGF), which plays a vital role in the development of restenosis (247).

PDGF can stimulate VSMC proliferation and migration at sites of stress (303). It has been shown that suppression of PDGFR activation can inhibit VSMC proliferation by decreasing activation of its downstream signaling molecules (304-306). PDGF is a potent mitogen that mediates arterial response, and stimulates proliferation and matrix production (307). PDGF signaling leads to downstream activation of proliferative genes that contribute to atherosclerosis and restenosis.

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic inflammatory cytokine. Accelerated atherosclerosis is characterized by hyperplastic neointima and an inflammatory response with cytokines such as TNF-α. TNF-α has been shown to play two opposing roles in inhibition of endothelial cell proliferation and enhancement of apoptosis, while stimulating vascular smooth muscle cell proliferation and migration (243,308). Although there are conflicting reports on the ability of TNF-α to stimulate VSMC proliferation, there is compelling evidence defining the migration stimulating activity of this cytokine (309,310). TNF-α, like other chemoattractants such as PDGF, stimulates VSMC migration through the MAPK pathway (240).

The apoptosis induced by TNF superfamily requires binding of a ligand to its receptor leading to oligotrimerization of receptors (311-313). This results in aggregation of death domain containing proteins allowing recruitment of TRADD (TNF receptor 1-associated death domain protein). TRADD binds FADD (Fas

associated death domain- containing protein) and TRAF-2 (TNF receptor 1-associated protein 2) proteins, which in turn lead to activation of procaspase-8 and apoptosis signal-regulating kinase 1 (ASK1), respectively (311,314-316). TNF-α treatment leads to simultaneous activation of the ASK1-JNK/p38 death signal (315-318). Reports on the effects of TNF-α on apoptosis or proliferation in VSMCs are conflicting (308). Several investigations report that TNF-α itself does not induce VSMC proliferation while other studies suggest TNF-α induces proliferation of VSMCs through NF-κB mediated transcription mechanisms (308,319). Regarding apoptosis, there are also inconsistent reports. Certain studies have shown that TNF-α could induce apoptosis in VSMCs via caspase-3 activation while others found no pro-apoptotic activity for TNF-α in these cells (309). Further investigations revealed that activity for TNF-α in VSMCs is dependent on two distinct cell phenotypes: spindle and epithelioid VSMCs, which respond distinctly to diverse stimuli. While TNF-α induces proliferation in spindle VSMCs, it induced apoptosis in epithelioid VSMCs (308).

Although PDGF and TNF-α have very different signaling intermediates, their downstream functions require MAPK activation. It is therefore important to identify the upstream mechanisms contributing to increased proliferation by these two stimuli. Stimulation of VSMCs with PDGF leads to downstream activation of Erk1/2 via the Ras/Raf/MAPK (mitogen-activated protein kinase/extracellular-signal-regulated-kinase) pathway. Activated ERK1/2 rapidly translocates to the

nucleus where they target transcription factors that regulate cell cycle progression, such as cyclin D1 (320). Cyclin D1 binds cdk4/6 and together they facilitate S-phase entry through phosphorylation of the retinoblastoma (Rb) protein (275). Stimulation of VSMCs with TNF-α has been shown to enhance proliferation through ERK1/2 (321) although the exact mechanism is not known. The Rb-Raf-1 pathway has been shown to play a role in response to mitogens as well as non-mitogens and enhance S-phase progression of a wide variety of cell lines (209,215). Here, we show that TNF-α stimulates proliferation in VSMCs by activating Raf-1/MEK/ERK pathway and facilitating Rb-Raf-1 interaction.

Results

TNF-α stimulates proliferation of vascular smooth muscle cells

Because of conflicting reports on the effects of TNF-α induced proliferation of VSMCs, we examined the effects of serum, TNF-α, and PDGF on VSMCs by BrdU incorporation assays. Rat A10 cells, which are immortalized vascular smooth muscle cells, were serum starved for 24 hours and subsequently re-stimulated with serum, TNF-α (100ng/ml), or PDGF (100ng/ml) for 18 hours and S-phase entry was measured using standard BrdU incorporation assays. TNF-α could stimulate proliferation in vascular smooth muscle cells to a certain extent **(Figure 39A)**. In the same manner, we examined the effects of TNF-α in primary human aortic vascular smooth muscle cells (AoSMCs). As shown in **Figure 39B**,

TNF-α could stimulate S-phase entry comparable to PDGF; serum was used as the positive control.

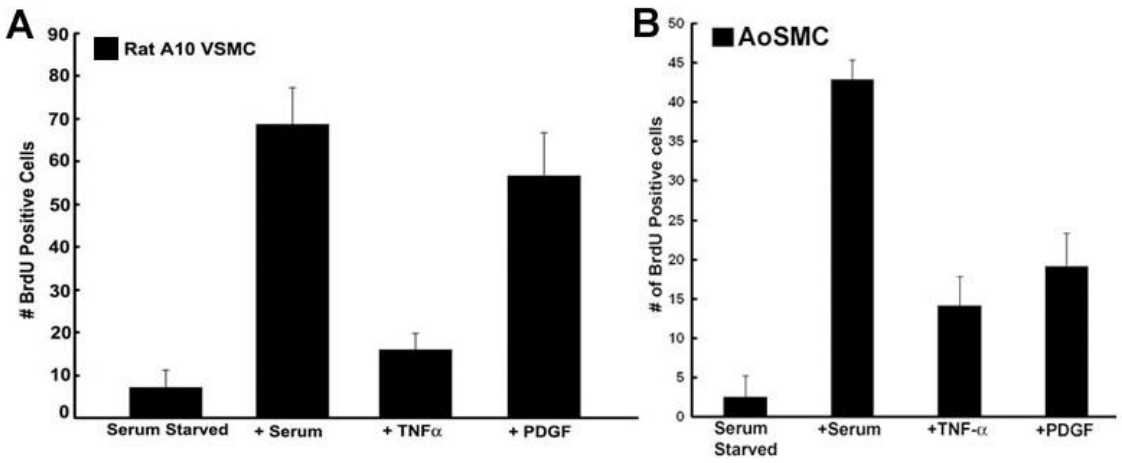


Figure 39. TNF-α stimulates proliferation in vascular smooth muscle cells. (A) Rat A10 VSMCs were serum starved and subsequently stimulated with serum, TNF-α or PDGF for 18 hours and BrdU incorporation was measured. (B) Similar assay was done using human AoSMCs.

TNF-α activates Raf/MAPK pathway in vascular smooth muscle cells

TNF-α has been shown to induce migration of vascular smooth muscle cells through ERK1/2 activation (240). We wanted to examine if TNF-α treatment had any effects on Raf-1 kinase in these cells. Treatment of vascular smooth muscle cells with TNF-α for 10 min, 30 min, 1h and 2 hours led to a shift in Raf-1 migration indicative of Raf-1 phosphorylation, as seen by western blotting **(Figure 40A)**. Indeed, phosphorylated Raf-1 can be seen by western blotting for serine 338 on Raf-1; activation was highest at 1 hour of TNF-α treatment **(Figure 40A)**. ERK1/2 activation was seen in response to TNF-α and was highest at 30 minutes of treatment **(Figure 40A)**. The stress activated protein kinase/ Jun-amino terminal kinase SAPK/JNK is a member of the MAPK family that is potently and preferentially activated by stresses such as UV irradiation, ceramides and cytokines like TNF-α (313). In certain instances, JNK can be activated by growth factors (322-324). We wanted to examine if TNF-α was capable of activating stress kinases in a proliferative scenario. To this end, AoSMCs were serum starved and stimulated with TNF-α or PDGF for 30 minutes (time point when Raf-1 activation and ERK1/2 activation was present). Western blotting for JNK activation using an antibody that recognizes phosphorylated Thr183/Tyr185 residues revealed TNF-α and PDGF activated JNK in VSMCs **(Figure 40B)**. Time course studies showed that activation of ERK1/2 and JNK1 occurred at 10 minutes of stimulation and was highest at 30 minutes, activation went down to basal levels at 2 hours of TNF-α treatment **(Figure 40C)**.

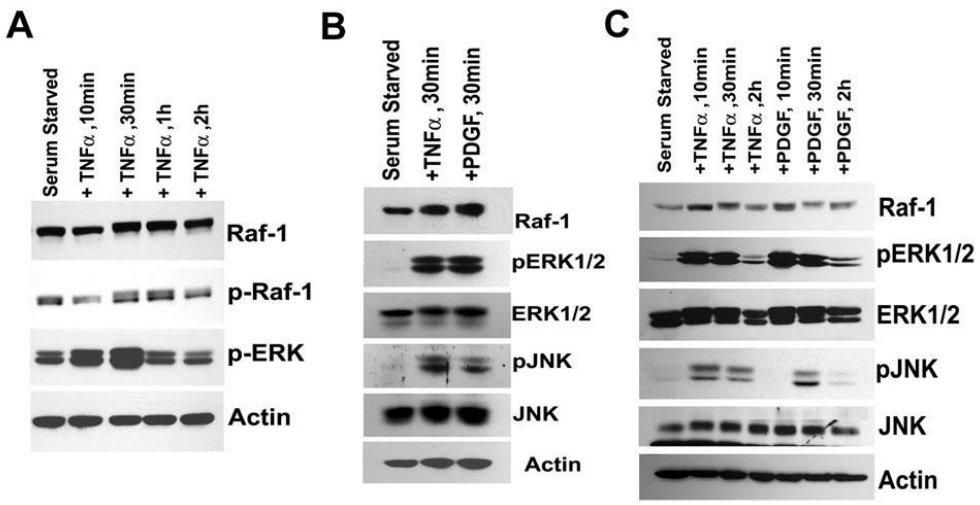


Figure 40. TNF-α activates Raf/MAPK pathway in VSMCs. (A) Time course stimulation of AoSMCs results in Raf-1 activation highest at 1 hour and ERK1/2 activation peaks at 30 minutes. (B) Activation of ERK1/2 coincides with JNK1 activation from 30 minutes of TNF-α treatment. (C) PDGF and TNF-α time course stimulation shows ERK1/2 and JNK activation occurs simultaneously from the different stimuli.

TNF-α induced AoSMC proliferation is abrogated by targeting upstream activators of Raf-1

Since TNF-α led to activation of Raf-1 in vascular smooth muscle cells and Raf-1 has been shown to play a very important role in cell proliferation we examined if vascular smooth muscle cell proliferation could be inhibited via targeting Raf-1 or kinases that activate Raf-1. Both Src and PKC kinases are known to activate Raf-1 in response to growth factor signaling (175). To evaluate the importance of these kinases in TNF-α induced proliferation, the Src inhibitor PP2 and the PKC inhibitor Ro-31-8220 were used in BrdU incorporation assays. AoSMCs were serum starved and subsequently stimulated with PDGF or TNF-α in the presence or absence of the aforementioned inhibitors for 18 hours. BrdU incorporation assays revealed that both inhibition of Src and PKC could efficiently block TNF-α or PDGF induced S-phase entry **(Figure 41A)**. Targeting upstream of Raf-1 or Raf-1 itself using the multikinase inhibitor BAY-43-9006 could completely inhibit S-phase entry induced by PDGF or TNF-α **(Figure 41B)**. Next we examined if inhibition of downstream activation of JNK could prevent S-phase entry induced by PDGF or TNF-α. Inhibition of downstream JNK activation did not significantly block proliferation, suggesting that in order to efficiently block proliferation, activation of Raf-1 should be inhibited **(Figure 41B)**.

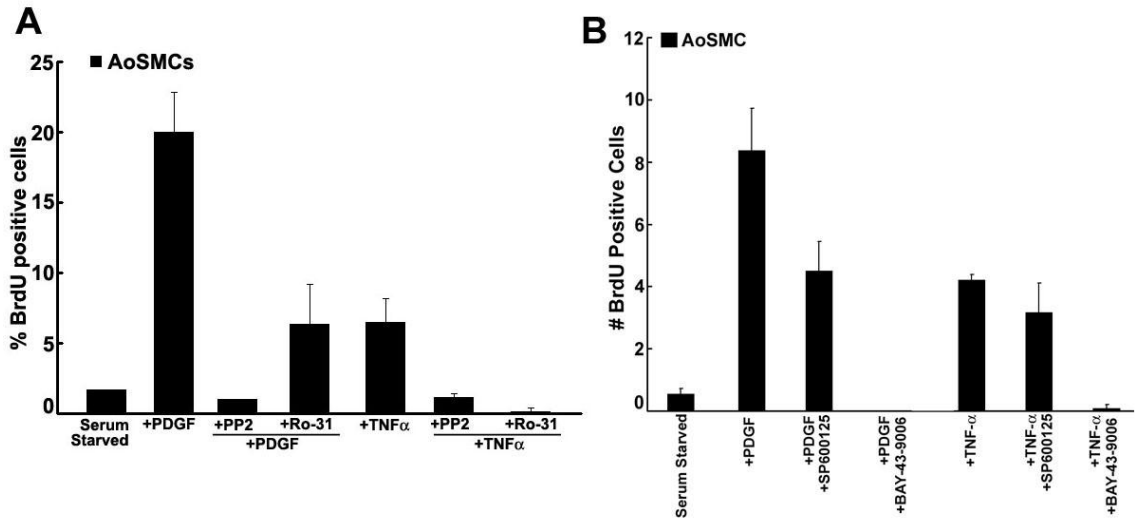


Figure 41. Targeting Raf-1 activation blocks AoSMC proliferation. (A) Src inhibitor (PP2) and PKC inhibitor (Ro-31-8220) block TNF-α and PDGF induced proliferation. (B) Multi-kinase inhibitor BAY-43-9006 that targets Raf-1 can completely inhibit PDGF and TNF-α induced proliferation while the JNK inhibitor (SP600125) does not significantly affect TNF-α induced proliferation.

TNF-α treatment induces E2F regulated genes involved in proliferation

Since TNF-α was functioning similar to a growth factor in stimulating cell cycle, we examined if this was in an E2F dependent mechanism. To this end, Real Time PCR was performed on two E2F responsive genes from AoSMCs that were serum starved and subsequently stimulated with either TNF-α or PDGF for 18 hours. TNF-α could induce *cdc25A* and *cdc6* gene expression 3.5 and 3 fold respectively **(Figure 42A-B)**. Next, we examined if in fact E2F1 was present on the proliferative promoter *cdc25A* in response to TNF-α. Treatment with TNF-α or PDGF for 18 hours led to an increase in E2F1 on the *cdc25A* promoter and a dissociation of Rb **(Figure 42C)**. In quiescent cells, we consistently observed a faint band for E2F1 on the *cdc25A* promoter and the presence of Rb was also detected in starved cells on this promoter. The *c-fos* promoter was used as a negative control.

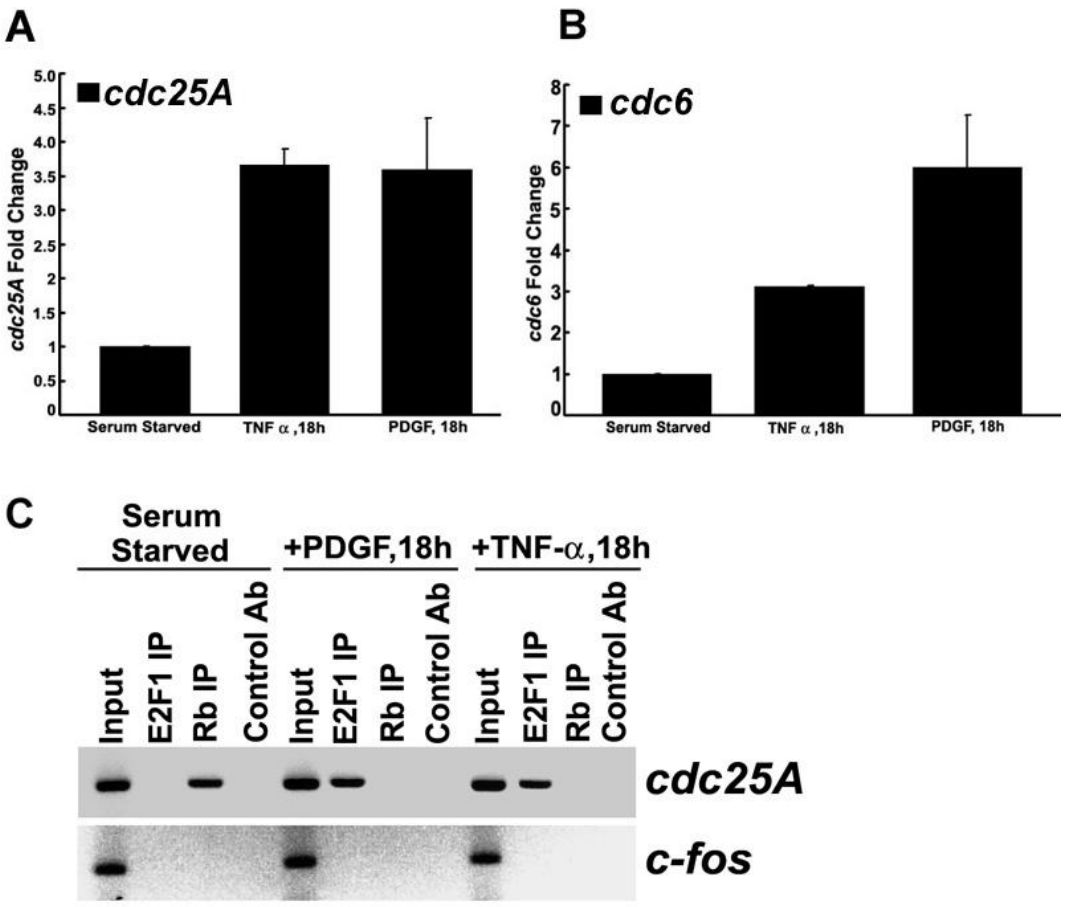


Figure 42. TNF-α and PDGF induce E2F regulated genes in AoSMCs. (A) Treatment with TNF-α and PDGF for 18 hours led to 3.5 and 4 fold increase, respectively in *cdc25A* gene expression in real time PCR assays. (B) Treatment with TNF-α and PDGF for 18 hours led to 3.5 and 7 fold increase, respectively in *cdc6* gene expression in real time PCR assays. (C) Treatment with TNF-α or PDGF led to an increase in E2F1 and dissociation of Rb on the proliferative promoter *cdc25A* in ChIP assays, *c-fos* was used as the negative control.

TNF-α induced AoSMC proliferation involves Rb-Raf-1 interaction

Our lab has shown the importance of the Rb-Raf-1 interaction in mediating proliferation in a wide array of cell lines. Since Raf-1 activation is evident in response to TNF-α induced proliferation in AoSMCs, we examined if Raf-1-Rb interaction is involved in mediating these effects. Treatment with the Rb-Raf-1 inhibitor RRD-251 in the presence of TNF-α or PDGF for 2 hours could efficiently reduce Raf-1 levels in both AoSMCs and rat A10 cells **(Figure 43A-B)**. Next, we examined if TNF-α stimulation of AoSMCs could induce the Rb-Raf-1 interaction, this was done by IP-WB analysis. Treatment with TNF-α and PDGF for 2 hours led to an increase in Raf-1 bound to Rb; in addition there was less E2F1 associated in the TNF-α and PDGF stimulated complexes **(Figure 43C)**. We next examined if RRD-251 could prevent serum, TNF-α or PDGF induced proliferation in AoSMCs. AoSMCs were serum starved and subsequently stimulated with serum, TNF-α, or PDGF in the presence or absence of 20μM RRD-251. In response to all three stimuli, RRD-251 was capable of inhibiting S-phase entry in AoSMCs **(Figure 43D)**. These results suggest that inhibiting Rb-Raf-1 interaction and signaling might be a viable alternative to prevent atherosclerosis.

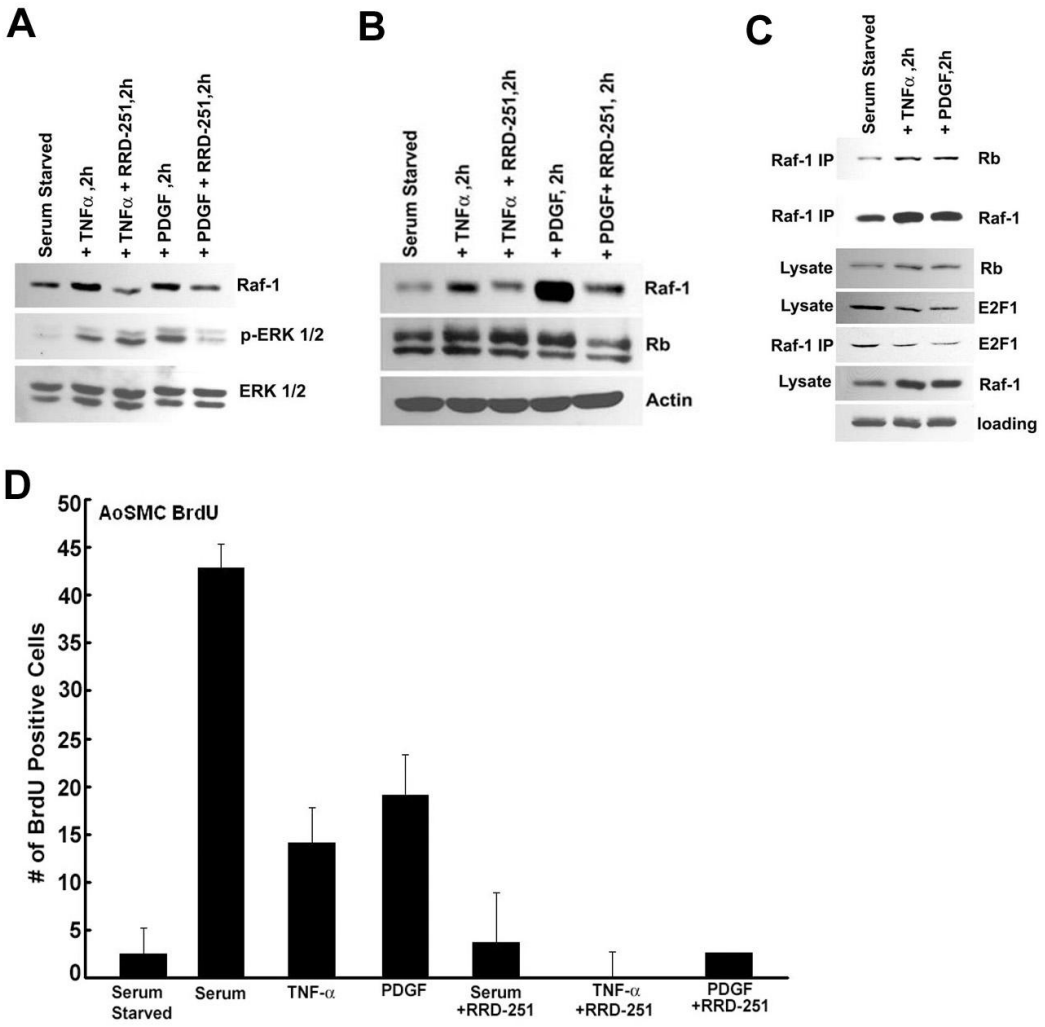


Figure 43. Inhibition of Rb-Raf-1 interaction prevents AoSMC proliferation. (A-B) treatment with TNF-α or PDGF in the presence of RRD-251 inhibits Raf-1 levels in AoSMCs (A) and A10s (B). (C) TNF-α and PDGF treatment induced Rb-Raf-1 binding in AoSMCs. (D) Treatment with RRD-251 inhibits AoSMC proliferation induced by serum, TNF-α and PDGF.

Discussion

The dynamics of endothelial and vascular smooth muscle cells play the predominant role in the progression of atherosclerosis and restenosis. Migration, proliferation, and differentiation of ECs as well as VSMCs are important pathological responses that contribute to the development of vascular lesions. The 'switch' of VSMCs from the quiescent phenotype to the proliferative and migratory phenotype is a vital event in the pathogenesis of atherosclerosis and restenosis post-angioplasty. Therefore, VSMC proliferation and migration both serve as suitable targets for drug therapy in vascular proliferative disorders. This study provides evidence that TNF-α and PDGF evoke similar signaling mechanisms that contribute to VSMC proliferation. Although they are not equally efficacious in activating these pathways, TNF-α is capable of activating growth factor receptor signaling pathways. S-phase entry assays revealed that TNF-α is capable of stimulating cell cycle progression in vascular smooth muscle cells. The proliferative response also increased E2F regulated genes *cdc6* and *cdc25A*, in fact TNF-α stimulation led to an increase in E2F1 on the proliferative promoter *cdc25A*. One interesting finding was in response to TNF-α, Raf/MAPK activation occurred and this coincided with an activation of the stress kinase JNK1. Inhibition of TNF-α or PDGF induced cell proliferation with pharmacologic inhibitors targeting Raf-1, upstream of Raf-1 or JNK displayed inhibition of S-phase entry only when targeting Src, PKC or Raf-1 not JNK. This suggests that

JNK activation most likely is not responsible for the proliferative responses seen with TNF-α.

Studies from our lab have shown that upon TNF-α treatment, ASK1 is responsible for Rb inactivation as an initial signaling event in Ramos and Jurkat cells (237). We observe similar response in HAECs where Rb is inactivated on TNF-α treatment in addition to upregulation of the pro-apoptotic proteins like p73 (unpublished data). The role of p53 in TNF-α induced apoptosis has been controversial (325,326). We also find that TNF-α has no affect on p53 expression however p73 levels were found to be upregulated implicating p73 to be major contributing factor to endothelial apoptosis induced by TNF-α. We have observed that TNF-α signaling in ECs functions in an E2F1 regulated apoptotic pathway (unpublished data). Especially in this context the studies from our lab show that Rb interacts with ASK1 upon apoptotic stimuli, and ASK1 has to overcome Rb function to execute its pro-apoptotic functions suggesting that Rb acts as a critical connector between apoptotic and proliferative pathways, by interacting with the functionally distinct kinases like Raf-1 and ASK1 (237). Thus the role of Rb phosphorylation by specific kinases is pertinent for directed signaling for apoptotic or proliferative pathways (234).

The contrasting observation in AoSMCs, where TNF-α treatment resulted in a lack of apoptotic response and increase in proliferation suggests that TNF is involved in multiple pathways depending on the cellular context. We observed

activation of Raf-1 and ERK, which are also indicative of a proliferative response. It has been shown that a colocalization of TNF-α and ERK1/2 occurs and ERK 1/2 activation induces the expression of Ets-1, Egr-1, and c-fos in neointimal lesions from rat aortae 2 weeks post balloon injury (327). The ChIP and RT-PCR experiments showed recruitment of E2F1 to proliferative promoters suggesting that E2F1 is a key mediator in the TNF-α induced proliferative or apoptotic pathways in VSMCs or ECs, respectively.

Rb-Raf-1 interaction was found to play a vital role for serum, PDGF and TNF-α induced proliferation in VSMCs. Targeting Rb-Raf-1 interaction using RRD-251 could completely inhibit S-phase entry in these cells. Our lab has previously shown that disrupting the Rb-Raf-1 interaction can prevent endothelial cell adhesion, migration and proliferation. Taken together, the importance of this interaction in both endothelial cell as well as vascular smooth muscle cell physiology in atherosclerotic lesions needs further evaluation and may provide useful tools in development of therapies for heart disease. This study is an attempt to delineate mechanisms underlying the differential effects of E2F-1 in different cellular activities with regard to the involvement of proliferative and apoptotic genes. The divergent responses of AoSMCs and HAECs to TNF-α thus provide unique therapeutic possibilities: simultaneously targeting the cell cycle of two different cell types, within same tissue microenvironment resulting in opposite and biologically complimentary effects.

Summary and Conclusions

Rb plays a vital role in cell proliferation and its inactivation facilitates S-phase entry (257). It has been well accepted that inactivation of Rb occurs through a cascade of phosphorylation events mediated by kinases associated with D and E type cyclins (328). Rb is known to have growth suppressive properties and an inhibition of Rb phosphorylation can lead to a G1 arrest (329). Several studies have suggested that mitogenic signaling pathways converge on the Rb dependent g1/S checkpoint (330,331). Members of the Ras/Raf/MAPK pathway have been shown to be involved in the upregulation of cyclinD1 and Rb phosphorylation (171,330,331). Furthermore, it has been shown that Ras mediated transformation and stimulated cell cycle progression requires inhibition of Rb activation through cyclin D (332). It is well established that most cancers inactivate Rb function by regulating the phosphorylation events that govern its function. Studies from our laboratory have shown that Raf-1 is capable of binding to Rb and facilitating its inactivation and this occurs prior to the binding of cyclins and cdks (173,209). We find that the Rb-Raf-1 interaction facilitates mitogenic and non-mitogenic stimulation and disruption of this interaction has great therapeutic potential for controlling proliferative disorders.

Although it has been difficult to generate small molecule protein-protein interactions that translate to the clinic the recent successes in disrupting the hdm-2-p53 protein-protein interaction clearly show that this is a viable strategy for developing novel drugs (269,279). We have described the discovery and characteristics of a novel protein-protein inhibitor for the disruption of the Rb-Raf-1. We have shown that blocking the Rb-Raf-1 interaction can prevent S-phase entry in a wide range of cancer cell lines including lung, breast, prostate, brain, pancreatic, and melanoma; indicating that the Rb-Raf-1 interaction may be involved in mediating cell cycle progression is several cancers of varying origin. RRD-251 also prevented tumor growth in vivo in both lung and melanoma xenografts.

Our lab has specifically focused on the Rb-Raf-1 interaction in NSCLC, mainly because nicotine and tobacco carcinogens such as NNK have been shown to stimulate the binding of Raf-1 to Rb in normal lung cells as well a lung cancer cells (215). In addition, non-small cell lung cancer (NSCLC) is associated with 80% of the total number of lung cancer cases and is strongly associated with tobacco use. Our lab and others have shown that the Rb-Raf-1 interaction is found to be elevated in human NSCLC tissue samples compared to adjacent control suggesting that this pathway contributes to the oncogenesis of these tumors (215). Blocking the Rb-Raf-1 interaction with either the Raf-1 peptide or

RRD-251 could prevent nicotine induced proliferation and angiogenesis *in vitro* (297). Future *in vivo* experiments could reveal whether blocking the Rb-Raf-1 interaction is necessary to prevent nicotine induced lung metastasis. These studies along with other in vivo models will open the door to developing novel therapeutic for treatment of NSCLC in smokers.

In another scenario, we find the Rb-Raf-1 interaction to mediate both endothelial cell and vascular smooth muscle cell proliferation. In endothelial cells, mitogenic as well as nicotine stimulation induced the Rb-Raf-1 interaction and cell proliferation. In vascular smooth muscle cells, mitogenic as well as non-mitogenic (cytokine-TNF-α) stimulation induced the Rb-Raf-1 interaction and cell proliferation. ECs and VSMCs in the heart respond to a variety of stimuli that decides if and when these cells will either proliferate or die (apoptosis). The proliferative response of these cells contributes to vessel thickening (occlusion) often known as restenosis or atherosclerosis. Inhibition of the Rb-Raf-1 interaction with RRD-251 prevented both EC and VSMC proliferation.

Based on the above findings we propose that inhibition of Rb-Raf-1 interaction is a viable mechanism for the treatment of proliferative disorders. We have shown that treatment with the Rb-Raf-1 disruptor RRD-251 could prevent cell cycle progression in response to a wide range of cell signals. In addition, smokers may have elevated levels of Rb-Raf-1 interaction and disrupting this interaction may help prevent the progression of NSCLC.

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