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Matrix Metalloproteinase genes are transcriptionally regulated by E2F transcription factors: a link between cell cycle control and metastatic progression

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Matrix Metalloproteinase genes are transcriptionally regulated by E2F transcription factors: a link between cell cycle control and metastatic progression

By

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Dedication

To my caring husband Jan, who patiently listens to my frustrations, and enthusiastically celebrates my successes. Thank you again, and I promise to only get one Ph.D. . . .for your sake. To my supportive family, who taught me what it meant to work hard and never give up—because without hard work, there can only be limited play. To my incredible “friend-family”, I am so lucky to have found you all: my soul mates. I must have had some great karma stored up from something. To all—this is truly a product of your unconditional love and encouragement.

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Abstract

The Rb-E2F transcriptional regulatory pathway plays a critical role in the cell cycle. Rb is inactivated through multiple waves of phosphorylation, mediated mainly by cyclin D and cyclin E associated kinases. Once Rb is inactivated, cells can enter S-phase. Collectively, three Rb family members and ten E2F proteins coordinate every additional stage of the cell cycle, from quiescence to mitosis. However the Rb-E2F pathway is frequently altered in cancer. Aside from cell proliferation, the Rb-E2F pathway regulates other essential cellular processes including apoptosis, cell differentiation, angiogenesis and DNA damage repair pathways, but its role in invasion and cancer progression is less clear. We demonstrate here that matrix metalloproteinases genes (MMPs), which regulate the invasion, migration and collagen degradation activities of cancer cells during metastasis are transcriptionally regulated by the Rb-E2F pathway. Unlike E2F target genes involved in cell proliferation, which are solely regulated by the E2F activators (E2F1-3), additional E2F family members can regulate *MMP9, MMP14,* and *MMP15*. While we had previously shown that Raf-1 kinase physically interacts with Rb, and that disruption of this interaction with a small

molecule inhibitor of the Rb-Raf-1 interaction (RRD-251) can inhibit cell proliferation, angiogenesis, and growth of tumors in mouse models, we now show RRD-251 inhibits the expression of *MMP*s and the biological functions mediated by MMPs as well—including invasion, migration, and collagen degradation. RRD-251 also inhibits metastatic foci development in a tail vein lung colonization model in mice. These results suggest that E2F transcription factors may play a role in promoting metastasis through regulation of *MMP* genes. Conversely, another *MMP* gene connected to metastasis, *MMP2*, is transcriptionally repressed by E2F1 in lung cancer cells through a p53-KAP1-HDAC1-mediated mechanism. However, E2F1 cannot repress the *MMP2* promoter in cells that are lacking any component of this complex, such as *p53* mutant breast cancer cells. Therefore the role of the Rb-E2F pathway in *MMP* transcription and metastasis is cell type dependent. In addition to growth factors, nicotine can also induce cell proliferation, angiogenesis, EMT, and progression of lung cancer. In our studies, nicotine induced invasion, collagen degradation, and transcription of *MMP2, MMP9, MMP14,* and *MMP15* required nAChRs, and multiple E2F family members. Our studies also show that nicotine not only promotes tumor growth *in vivo* through the nAChR-E2F pathway—it also results in metastasis to the liver and brain. Taken together, these studies link the Rb-E2F pathway to the regulation of many facets of cancer.

Chapter 1: Introduction

1.The retinoblastoma gene

The speculation that humans likely carried genes which protected normal cells from becoming malignant was proposed long before the cloning of the first tumor suppressor gene. The retinoblastoma gene, *Rb1* or *Rb,* was the first tumor suppressor to be identified and subsequently cloned (1, 2). Studies following the inheritance patterns of a pediatric tumor of the retina, which primarily occurs in children between 9 months and 5 years, showed that deletion or mutation of chromosome 13q14.1-13q14.2 could result in retinoblastoma, and patients could also be victim of a secondary non-ocular tumor such as osteosarcoma (2, 3). These studies also pointed out that mutation or deletion of the *Rb* gene could be hereditary or arise during gametogenesis. The familial form of retinoblastoma is bilateral and multifocal, whereas the sporadic form is unilateral (2). Taken with Alfred Knudson's observation that two distinct genetic "hits" must occur thereby initiating cancer, these studies are the foundation for the current tumor suppressor paradigm.

Rb was cloned by chromosome walking from *Esterase D*, a gene that was linked to the retinoblastoma susceptibility locus. The *Rb* gene encoded a 928 amino acid nuclear phosphoprotein with weak DNA binding activity (4). It

was found that Rb protein could form stable protein-protein complexes with viral oncoproteins of adenovirus E1A and SV 40 large T-antigen, both of which cause tumors in rodents, in addition to the human papilloma virus (HPV) E7, which causes cancers in humans (5, 6). This led to the hypothesis that the *Rb* gene did not have to be mutated or deleted, but the Rb protein could also be inhibited by viral proteins to abrogate its tumor suppressor function, which instigates tumor formation (7).

1.1 Rb pocket proteins family members

Rb is one of three proteins in the pocket protein family, made up of Rb (p105), p107, and p130 (8). Although *Rb* is frequently mutated in a wide variety of human cancers, Burkitt's lymphoma, T-cell malignancies, gliomas, and some lung cancers have mutations of *p130,* whereas *p107* is mutated in several hematologic malignancies (9-11). The common pocket domain found in each of the Rb family proteins is used to bind to viral oncoproteins, transcription factors, and other proteins. The pocket is comprised of A and B pocket domains, which are separated by a spacer (Figure 1). The spacer region is utilized for cyclin binding, whereas other proteins including E2Fs, HDACs, and viral oncoproteins bind in region B (12). These proteins share the LXCXE motif as well. Further, p107 and p130 are more closely related to each other than to Rb, though they can still bind to certain E2Fs, and they are also regulated by phosphorylation from cyclin-dependent kinases. While Rb is commonly expressed in both proliferating and non-proliferating cells, p107 is mainly expressed in proliferating

cells, and p130 is expressed in arrested cells (13). p107 being expressed in proliferating cells is a phenomenon which is not well understood, given that overexpression of p107 results in G1 arrest in some cells (14).

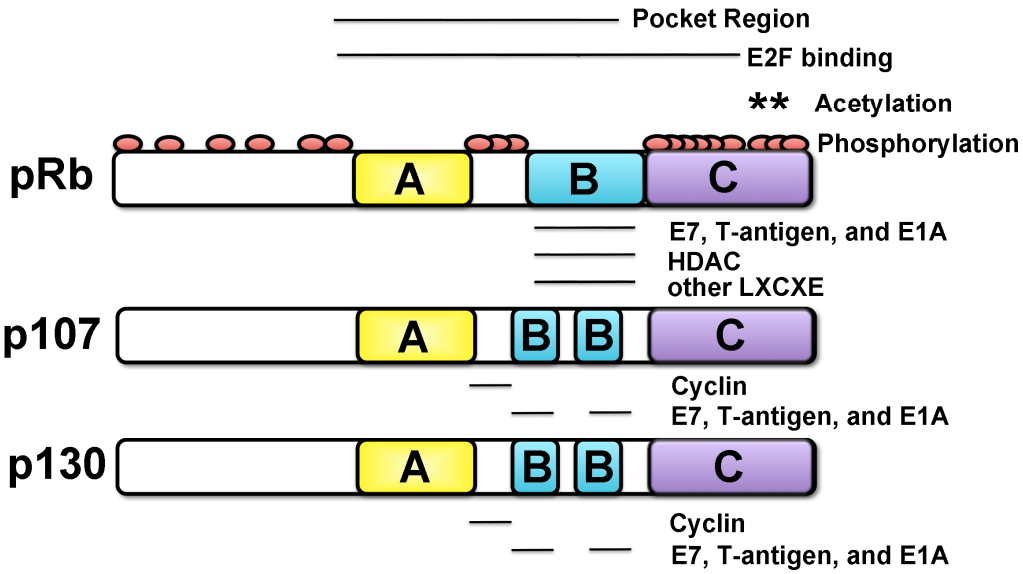

Figure 1. The family of Rb pocket proteins. The Rb protein family is comprised of 3 members: Rb, p107, and p130. The largest region of homology between these proteins lies in a pocket domain (regions A and B, separated by a spacer), which is required for their interaction with E2Fs and many other factors. Regions within the pocket, as well as a domain in the carboxy terminus of Rb, have been shown to be important for E2F binding. Red markers represent the many regulatory phosphorylation sites that have been mapped in Rb, and two acetylation sites (stars) have been mapped in the carboxy terminus of the protein. The cyclin binding site maps to the spacer that is unique to p107 and p130, as does a conserved domain that binds to viral oncoproteins (E1A, E7 and T Ag).

1.2 Rb interacting proteins

Studies have shown that Rb can bind to about 100 different proteins, including kinases, HDACs, HATs, histone demethylases, phosphatases and transcription factors—serving as a conduit between cell cycle machinery and promoter specific transcription initiation or repression (15, 16). These interactions assist Rb in the regulation of the G1 checkpoint, differentiation during embryogenesis and adult tissue development, regulation of apoptosis, and preservation of chromosomal stabililty (17). To this end, Rb can regulate the stability of cell cycle inhibitor p27 through an interaction with the anaphase-promoting complex/cyclosome (APC/C). This is an important part of Rb-mediated arrest in G1, aside from E2F-mediated regulation (18, 19). Rb can also regulate the transcriptional output of additional transcription factors besides E2F. Rb can associate with HES1 to promote stronger binding of RUNX2 to target gene promoters (20). Rb also associates with Sp1, HIF1α, and MYOD transcription factors to modulate gene transcription (21-23). Aside from being a transcriptional co-factor, Rb is also an adaptor protein that can recruit a variety of co-activators or co-repressors to target gene promoters.

1.2.1 Rb inactivation upon mitogenic and apoptotic stimulation

The cell division cycle comprises of essentially two distinct stages: the replication of DNA, collectively known as interphase, and the separation of DNA and other cellular organelles into two distinct daughter cells, known as mitosis.

The ability of cells to undergo the entire cell cycle is regulated by a variety of mitogens, which are factors that can initiate cell division, eventually pushing the cell into mitosis. Cells encounter a variety of mitogens that can initiate the cell cycle, including a variety of growth factors found in serum such as EGF, PDGF, TNF, TGF, and others (Figure 2). Under normal conditions and during cancer, cyclin-dependent kinases and their binding partners, cylins, promote cell cycle progression (24). Cdk1 is the most evolutionarily conserved Cdk; in the budding yeast *Saccharomyces cerevisiae*, a single Cdk equivalent to Cdk1 associates with multiple cyclins to regulate the cell cycle. Cdk1 is able to regulate all stages of the cell cycle in the absence of additional interphase Cdks (25). Although some cyclin/Cdk complexes have a wide array of substrates, Cdk4/6 in association with D-type cyclins is exclusive for the phosphorylation and inactivation of Rb family proteins, initiating the transition into S-phase (26, 27). Mitotic cyclins/Cdk complexes Cdk2/cyclin A and Cdk1/cyclin B phosphorylate Rb and other substrates to mediate the progression through S/G2/M phases of the cell cycle (28). Rb activation is reset in mitosis through the activation of phosphatase activity.

Rb can be phosphorylated by other kinases as well. Studies have shown that p38 and JNK1, both in the MAP kinase family, can affect Rb-E2F regulation of target gene promoters. p38 reverses Rb-mediated repression of E2F1; phosphorylation of Rb by p38 kinase upon Fas stimulation resulted in the dissociation of E2F and increased transcriptional activity during apoptosis (29). The inactivation of Rb by Fas was blocked by SB203580, a p38-specific inhibitor,

as well as a dominant-negative p38 constructs. Cdk-inhibitors as well as dominant-negative Cdks had no effect (30). Therefore, Fas-mediated inactivation of Rb during apoptosis is mediated via the p38 kinase, independent of Cdks. Other studies have shown that p38 directly phosphorylates Rb on Ser567, which is not phosphorylated during the normal cell cycle. Phosphorylation by p38, triggers an interaction between Rb and the human homolog of murine double minute 2 (Hdm2), leading to degradation of Rb, release of E2F1 and cell death, rather than proliferation (31).

Further highlighting a role for Rb-E2F in apoptotic signaling cascades, the apoptosis signal-regulating kinase ASK1 has been shown to interact with Rb (32). The LXCXE motif on ASK1 is required for Rb binding, which correlates with increased E2F1 transcriptional activity and up-regulation of the proapoptotic protein p73. TNFα stimulation causes Rb to dissociate from the *p73* promoter, although conversely Rb binds to the mitogenic *cdc25A* promoter upon TNFα stimulation (32). The transcriptional induction of *ASK1* also appears to be dependent on E2F-mediated transcription of *Bim* through a positive feedback mechanism (33). In this study, ASK1 knockdown results in reduced E2F1 transcriptional activity, leading to decreased Bim induction after treatment with an HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA). Interestingly, TNFα can function as a mitogen, or a pro-apoptotic chemokine depending on cellular context. In human aortic endothelial cells, TNFα induces apoptosis, while in vascular smooth muscle cells (VSMCs) and aortic smooth muscle cells

(AoSMCs) it enhances the association of E2F1 with proliferative promoters like *thymidylate synthase* and *cdc25A*, along with Rb dissociation (34). Further, in smooth muscle cells treatment with TNFα or PDGF could stimulate the traditional MAPK signaling cascade in addition to stimulating the Rb-Raf-1 interaction, both ultimately leading to cell proliferation due to E2F recruitment at proliferative gene promoters (35). Blocking the Rb-Raf-1 interaction could inhibit S-phase entry induced by both mitogens in this study.

1.2.2 Rb inactivation by Raf-1

Experiments in yeast two-hybrid assays, *in vitro* binding assays, and immunoprecipitation western blot experiments revealed that Raf-1, a serine threonine kinase with a well characterized role in the MAP kinase pathway, could bind to Rb and p130, not p107 (36). Raf-1 translocates to the nucleus upon serum stimulation where it can bind to Rb as early as 30 minutes to 2 hours after mitogen stimulation (36). Raf-1 inactivates Rb and reverses Rb-mediated repression of E2F1 in transcriptional activity and S-phase entry assays. Further, the Rb-Raf-1 interaction was elevated in tumor tissue compared to normal adjacent tissue in eight out of 10 matched pairs, and more Raf-1 was recruited to proliferative promoters *cdc25a* and *cdc6* (37). This suggests that the Rb-Raf-1 interaction is a regulator of proliferation, and that enhanced activity through this signaling pathway might contribute to tumorigenesis.

Further studies have aimed at examining the consequence of inhibiting the Rb-Raf-1 interaction. To this end, a peptide corresponding to the Rb binding region of Raf-1, amino acids 10-18 (ISNGFGFK with a C added to the carboxyl terminal end to allow coupling to the carrier molecule penetratin), was created (38). The inhibition of the Rb-Raf-1 interaction by the Raf-1 peptide pen-conjugate could significantly inhibit Rb phosphorylation even up to 16 hours post serum stimulation (38). Importantly, B-Raf could also bind to Rb *in vitro,* however disrupting the Rb-Raf-1 interaction had no effect on the Rb-B-Raf interaction. A-Raf had no detectable interaction with Rb. Upon more investigation, Raf-1 was found to specifically dissociate BRG1, a component of the SWI/SNF chromatin modifiers, from the promoters of E2F regulated genes—treatment with the Raf-1 peptide pen-conjugate led to BRG1 recruitment on proliferative promoters, correlating with inhibited cell proliferation. Since peptides do not make good anti-cancer therapeutics due to their propensity for degradation *in vivo,* we sought to identify a small molecule disruptor of the Rb-Raf-1 interaction. We developed Rb-Raf-1 disruptor 251, RRD-251, which potently and selectively disrupts the Rb-Raf-1 but not Rb-E2F, Rb-prohibitin, Rb-cyclin E, and Rb-HDAC binding (39). Similar to the peptide, RRD-251 inhibited Rb phosphorylation, and anchorage-dependent and anchorage-independent growth of human cancer cells *in vitro* and *in vivo*. This was accompanied by inhibition of angiogenesis, proliferation, and phosphorylation of Rb (39).

1.2.3 Rb inactivation upon nicotine stimulation

Non-small cell lung cancer (NSCLC) comprises 80% of the total number of lung cancer cases and is strongly associated with tobacco use. Tobacco smoke contains carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). These molecules form DNA adducts leading to mutations in vital genes like *Ras, p53*, and *Rb* (40). The carcinogen NNK can induce proliferation and angiogenesis through nicotinic acetylcholine receptor subunits (nAChRs) in a variety of cell types (41). nAChRs are pentameric proteins consisting of nine α subunits (α2-α10) and three β subunits (β2-β4) in non-neuronal cells; ε, δ and γ subunits are present in neuronal systems (41). Studies have shown a functional role for nAChRs in non-neuronal cells as well. Nicotine can also induce proliferation of endothelial cells (42-44), small cell lung carcinoma cell (45, 46) and non-small cell lung cancer cell lines (37). Studies from our lab and others have demonstrated that nicotine signaling involves the Rb-E2F pathway and promotes cell cycle entry (Figure 2). One mechanism is through the induction of the *cyclin D1* promoter (47). Nicotine stimulation of NSCLC cell lines also leads to the binding of β-arrestin to the α7 nAChR, which in turn activates the Src kinase-Raf-1-Rb signaling cascade (37). This results in dissociation of E2F1 from Rb and the induction E2F target gene promoters, including proliferative promoters *cdc6, cdc25A, TS* and *survivin* (37, 48). E2F1 and p300 were recruited to these promoters when cells were treated with nicotine, leading to the induction of histone acetylation (48). Nicotine seems to be a tumor promoter *in vivo,* however cannot initiate tumorigenesis alone (49-

51). Nicotine can also initiate EMT and metastasis along with proliferation (41, 51). Overall, the inactivation of Rb by a variety of upstream signaling events can play a diverse role in cancer cells depending on the cell type and biological context.

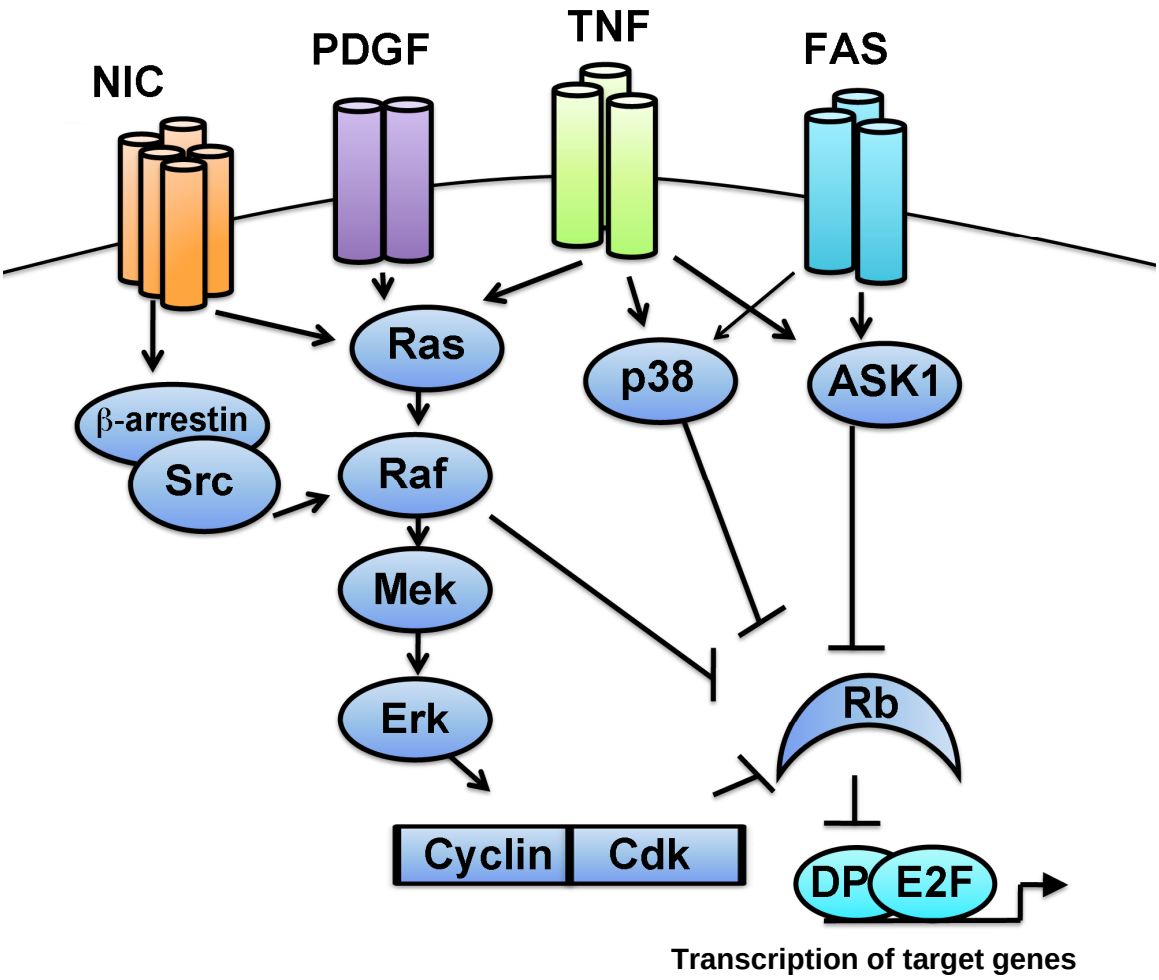

Figure 2. Mitogenic and apoptotic signals stimulate the inactivation of Rb. A variety of signals can initiate downstream signaling cascades, including those emanating from nAChRs, PDGFR, TNFR, and FASR. Rb is a major negative regulator of E2F-DP mediated transcription. Phosphorylation by cyclin/Cdk complexes, p38, Raf-1, and ASK1 leads to the inactivation of Rb, releasing it from E2F-DP heterodimeric complexes, and allowing sequential transcription to proceed.

1.3 Downstream mediators of Rb function: the E2F family

The E2F family comprises of 10 transcription factors sharing at least one conserved winged-helix DNA binding motif, while E2F7 and E2F8 have two. They bind the consensus sequence TTTCGCGC, as well as non-canonical sequences (Figure 3) (52). Since the initial discovery of E2F1 in humans, there has been an additional eight distinct *E2F* genes discovered (53-56). *E2F3a* and *E2F3b* are genes transcribed from different promoters, and vary only in their amino terminus (57, 58). In addition, although E2F3a is expressed in late G1/S phase, E2F3b is expressed equally in both proliferating, and quiescent cells, and is the E2F family member most highly associated with Rb in quiescence. *E2F7a* and *E2F7b* are splice variants from the same transcript, and result in a truncated carboxy terminus (59). E2F1-6 are recruited to target gene promoters along with dimerization partner proteins, TFDP1, TFDP2, and TFDP3, where this dimerization is mediated by the leucine zipper and marked box domains (60). The DP family of proteins also binds to canonical E2F binding sites, and bind to the C-terminus of Rb, accounting for added stringency in Rb-E2F repression (61). TFDP2 is similar to TFDP1, though is mainly associated with E2F4 and E2F5 (62-64). TFDP3 is functionally diverse from the other DP family members. It can inhibit transcription, prevent entry into S-phase, and abolish E2F1-mediated apoptosis when ectopically expressed (65).

E2F proteins are divided into the activators (E2F1, E2F2, E2F3a) and the repressors (E2F3b, E2F4, E2F5, E2F6, E2F7a, E2F7b, and E2F8). This classification of either activators or repressors is based on the proliferative output when each protein is overexpressed *in vitro.* E2Fs1-3 can significantly induce gene transcription and cell proliferation, however E2F4 and E2F5 can also activate certain gene promoters, though to a lesser extent than E2Fs1-3 (66). Studies have shown that dimerization with DP proteins can enhance the transcriptional activity of E2F4-5; however in quiescient cells these proteins are usually associated with repressor complexes that contain both pocket proteins and corepressors. Interestingly, the classical activator E2Fs, E2F1-3a, have been shown to form complexes with corepressor proteins including chromatin modifiers like HDAC1, SUV39H1, and BRG1, suggesting that in certain contexts these E2Fs might also mediate transcriptional repression (67-70). Further, the functions of E2Fs *in vivo* are highly tissue specific, making classification based on function quite difficult (56, 71-76). The remaining E2F family members, E2Fs6-8, function exclusively as repressors. E2Fs6-8 have different carboxy terminal features than the other E2Fs, lacking a transactivation domain and Rb binding domains. E2F6 can repress E2F targets when overexpressed in culture, and this activity is assumed to be through binding with a variety of polycomb group proteins (54, 77-79). E2F7 and E2F8 are the most unique structurally, having two DNA binding motifs, and are functionally an evolutionarily conserved branch of the E2F family for transcriptional repression (80, 81).

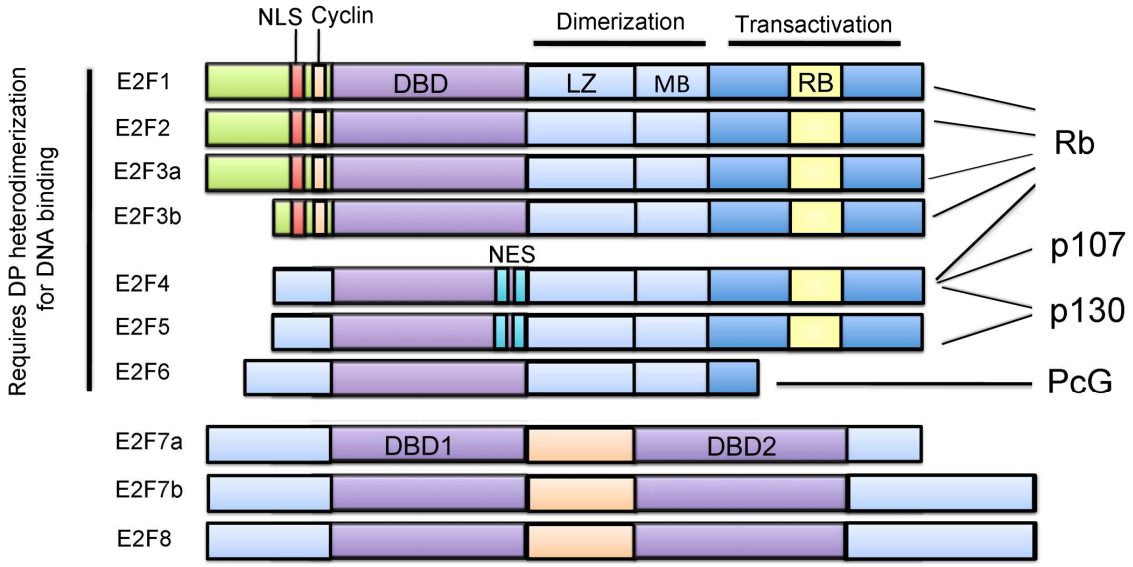

Figure 3. Protein structure of the E2F family. E2F1 though E2F6 contain one DNA binding domain (DBD) and the leucine zipper/marked box domain region that is required for DP heterodimerization (LZ and MB). E2F1 through E2F5 have a transactivation domain (dark blue), which also contains the Rb pocket protein binding domain. E2F1-E2F3b contain a specialized amino terminal sequence that harbors both a nuclear localization sequence (NLS) and region for cyclin binding. E2F4 and E2F5 are the only family members that contain a nuclear export sequence, and can therefore also be localized to the cytoplasm. The function of this localization is unknown. E2F6 cannot interact with Rb pocket proteins, but instead is repressed by polycomb group proteins. E2F7 and E2F8 have two DNA binding domains, separated by a spacer. E2F7a and E2F7b are splice variants, and differ only in their carboxy terminus. It is unclear whether corepressors or coactivators interact with E2F7 or E2F8.

1.4 Rb and E2Fs regulate the cell cycle

Cells receive a multitude of stimuli, which can be either growth promoting or inhibitory, and arise in a cell intrinsic or cell extrinsic manner. When E2F was initially discovered, it was identified as an entity that was required for the early region 1A (E1A) transforming protein of adenovirus to transcribe the viral E2 promoter (82-85). Nevins lab and others later discovered that E2F was targeted in normal cells by Rb protein (82, 83, 85). A large effort immediately followed identifying a role for E2F and Rb to control S-Phase entry.

To regulate the cell cycle, Rb must communicate with upstream kinases that impact protein binding ability of Rb, and with downstream effecter proteins, including the E2F family, that control gene transcription. Rb can negatively regulate the activation of these transcription factors by physically binding to both E2F and its dimerization partners (DPs) through its pocket domain and its C-terminal domain (61, 86, 87). As mentioned previously, Rb can also recruit co-repressors and chromatin modifiers to further repress transcription of target genes (88). Corepressors include HDAC1 (89, 90), BRG1/BRM (91, 92), HP1γ (93), SUV39H (94), Polycomb group proteins (95), DNMT1 (96), and various demethylases including UTX (97-99).

The repressive complexes often associated with Rb and Rb family members are predominantly found on inactive promoters, and in the case of cell

cycle regulated genes these complexes are found in quiescent cells (G_o). These complexes are often associated with the ubiquitously expressed, passive repressive family members, E2F4 and E2F5 (Figure 4) (66). E2F4 is the only E2F family member that can bind to all three Rb family members, including p130 and p107, whereas E2F5 preferentially binds to p130. Rb is also bound, along with co-repressors, to E2F1-3, either at inactive target gene promoters, or as sequestered complexes away from target sequences (8). Upon mitogenic stimulation, pocket proteins are phosphorylated, and dissociated from E2F1-3 at target promoters, and E2F4 and E2F5 are shuttled to the cytoplasm (100, 101). As previously discussed, the phosphorylation of Rb is mediated by kinases associated with D type cyclins, mainly Cdk 4/6, followed by kinases associated with cyclin E, mainly Cdk 2 (102). This leads to near complete inactivation of Rb by mid G1 phase (103). This inactivation also eliminates the transcriptional repression effect of Rb, and allows E2F-DP heterodimers to recruit coactivators to initiate transcription of genes required for entry into S-phase (104). Rb remains completely inactivated, and hyper-phosphorylated, for the remainder of the cell cycle. It becomes de-phosphorylated by protein phosphatases during mitosis (105). These events taken together are responsible for the initiation of a transcriptional program that can drive cells into S-phase (106). The completion of S-phase, and the entry into G2 is mediated by the actions of the repressor E2F family members, E2F6, E2F7, and E2F8 (59, 107, 108). Heteromeric and homomeric dimers of E2F7 and E2F8 can directly bind to the *E2F1* promoter, and effectively repress transcription (56). The proper transition through the cell

cycle is thereby executed by collective cooperation between E2F family members.

The Rb-E2F proteins are inextricably linked to the expression of various genes required for S-phase entry and concomitant entry into the cell cycle. This canon holds in human cells, where overexpression of E2F1 can drive cells into S-phase (109), *Drosophila*, where dE2F1 overexpression induces S-phase (110, 111), and *Arabidopsis,* where differentiated and non-dividing leaf cells can re-enter S-phase upon AtE2F-a overexpression with AtDP-a (112). Further, activator E2Fs have been shown to overcome anti-proliferative signals, from TGFβ (70), in addition to their transformation capabilities when overexpressed in primary cells (109, 113-116). Of the genes identified that are rate limiting for cell cycle entry, *cyclin E* and *cyclin A* have been identified as true targets of E2F1-3 (117). Other targets have more specificity to which E2F family member is the main regulator depending on cell type, for example *cdc6, cdc25a, p107,* and *c-myb* are all strongly regulated by E2F1 and E2F3 in rat fibroblasts (117). Dihydrofolate reductase, Thymidylate synthase, cyclin D3 and Thymidine kinase, enzymes required for nucleotide synthesis, are also regulated by multiple E2F family members (64, 117, 118). Genes involved in G2/M are also E2F targets, including *cyclin B1*, *cyclin A2, cdc20, DBC2* (119) *Bub1* mitotic checkpoint protein kinase, *KRP1/2* mitotic motor proteins, and *AIM1,* a chromosome segregation kinase (120, 121). DNA replication genes including *MCM2-7*, multiple subunits of *DNA polymerase*, *FEN1*, *PCNA*, *Replication protein A2*, and

Topoisomerase 2α were also identified using microarray in various systems (120, 122, 123).

The expression of each E2F family member is crucial for the proper execution of the mammalian cell cycle, and this is also dependent on the association of these proteins with specific Rb family members. Several groups have sought to find ways to inhibit the inactivation of Rb, keeping it active as an E2F-repressor, and therefore inhibiting uncontrolled cell proliferation. This may be a possible therapeutic option for proliferative diseases.

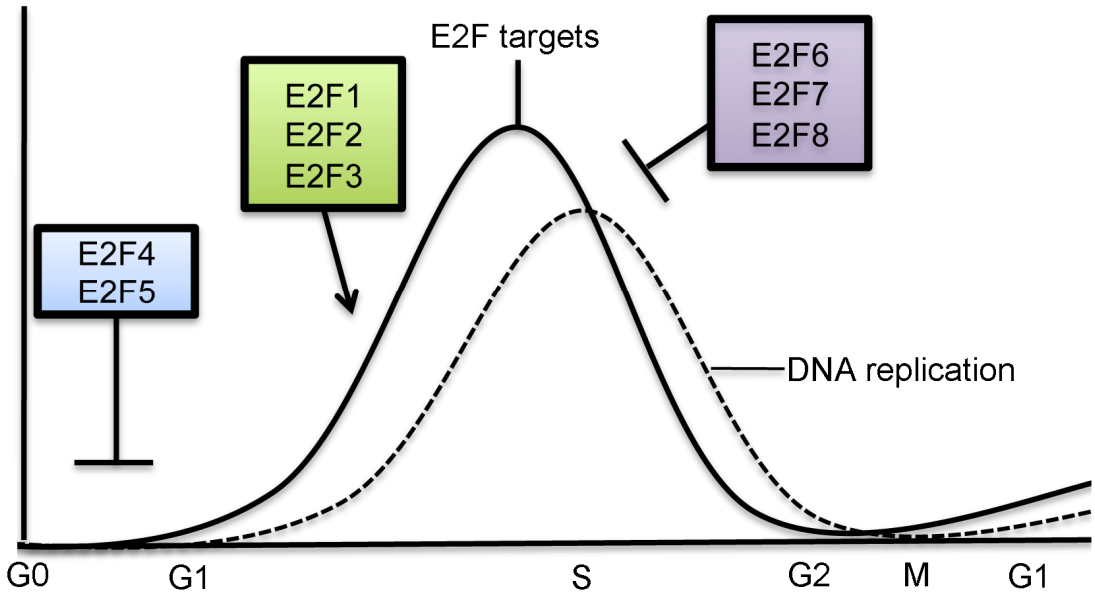

Figure 4. The contribution of E2Fs to cell cycle progression. In quiescient cells (G0), E2F4 and E2F5 associate with pocket proteins and other corepressors, preventing entry into the cell cycle. Upon mitogenic stimulation, Rb is phosphorylated, mainly by cyclin/Cdk complexes, and inactivated and the E2F repressor proteins are freed from E2F binding sites; E2F1-E2F3 take the place of E2F4-5. The recruitment of E2F1-3 allows for transcription of genes required for S-Phase entry and DNA replication (dashed line). Upon completion of S-phase, E2F proliferative promoters are inhibited by E2F6-8 independent of pocket protein binding. During late G2/M phase, Rb is dephosphorylated by protein phosphatases, enabling active Rb to again repress E2F-mediated transcription until it is further required.

1.5 Additional E2F target genes

With the development of precise high throughput gene profiling arrays and chromatin immunoprecipitation arrays (ChIP on chip), genes involved in apoptosis, signal transduction, transcriptional control, and membrane biology were identified as potential E2F targets (52, 120-129). Though E2Fs were originally assumed to be exclusive cell cycle regulators, these studies where among the first to allude to the possibility of E2Fs functioning in different biological settings and performing tasks unrelated to proliferation. This section aims to discuss the E2F target genes which play roles outside of proliferation.

1.5.1 E2Fs regulate apoptosis

The surprising observation that E2F1 knockout mice develop normally only to develop tumors of multiple locations as they age, including reproductive tract sarcoma, lung tumors, lymphoma and other tumor types along with tissue atrophy and glandular dysplasia was among initial evidence suggesting that *E2F1* was a tumor suppressor gene, in addition to an oncogene (71). Taken with the observation that thymocytes of *E2F1* null animals were hyperproliferative and had defects in apoptosis, it was established that, at least *in vivo*, E2F1 was a key mediator of apoptosis (71, 73). There are conflicting reports as to the degree of specificity for apoptotic inducers between E2F family members. Although it originally seemed that E2F1 was the sole inducer of apoptosis, (64, 130-132), E2F2 and E2F3 could also push cells towards apoptosis, though to a much

lesser extent than E2F1 (64, 117, 133, 134). Later studies demonstrated that E2F-mediated apoptosis was indeed through caspases 3, -7, -8, and -9 and could be through p53-dependent mechanisms as well (135-138). p53-dependent apoptosis is induced through $p19^{ARF}$ (139, 140) in mouse and human models, and therefore inhibits MDM2-mediated degradation of p53 protein (141, 142). In addition to caspase upregulation, E2F1 transcriptionally targets pro-apoptotic genes, including *p73* (143), *Apaf-1* (144), *Bid* (145), *SIVA* (146) and BH3-only genes such as *PUMA, Noxa, Bim*, and *Hrk/DP5* (147). E2F1 up-regulates the expression of genes that are pro-apoptotic cofactors of p53, such as *ASPP1* (apoptosis stimulating protein of p53) and *ASPP2*, thereby biasing p53 to activate pro-apoptotic genes and induce apoptosis (148-150). How cells determine whether to turn on pro-apoptotic genes or proliferative genes is still not clear, though JAB1 has been shown to only mediate the induction of pro-apoptotic E2F target genes (151). In addition to activation of pro-apoptotic proteins, E2F1 can also repress anti-apoptotic genes like *MCL-1* (152).

HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) have been shown to promote E2F1-mediated apoptosis through the induction of pro-apoptotic Bcl2 family member *Bim* through p53 and p73-independent mechanisms (153). As mentioned previously, p38 has been shown to phosphorylate Rb in response to stress and death receptor signaling in multiple cell types, such as endothelial cells, cerebellar neurons, Jurkat lymphocytic cells, colon cancer cells, and melanoma cells (29, 30, 154-157).

Interesting studies from Talianidis lab have shown that Set9 methylates E2F1 at lysine-185, which prevents E2F1 accumulation and activation of p73 (158). This methyl mark is removed by LSD1, which is required for E2F1 stabilization and apoptotic function. Overall, the induction of apoptosis by deregulated E2F1 is by now well established and the studies discussed above provide a number of components that mediate apoptotic activity. However, keeping in mind the pivotal role of the E2F family in regulating cell proliferation, it is imperative to better understand the mechanism determining whether the final outcome of E2F activity will be survival or death (159).

1.5.2 E2Fs regulate autophagy

The role of E2F-induced apoptosis in both normal development and disease is well-studied; other forms of cell death induced by E2F1 are less clear. Unlike the caspase cascades that mediate apoptosis, autophagy is an evolutionarily conserved vesicular-trafficking process that mediates the degradation of cytosolic proteins and organelles (160). Initial studies in Rb-null hepatocytes showed autophagic traits, and E2F regulated BH3-only protein BNip3 was required for hypoxia-induced autophagy (161). Also, activation of E2F1 upregulates *LC3, ATG1, ATG5* and *DRAM,* and enhances autophagy. In addition reducing endogenous E2F1 expression inhibits DNA-damage-induced autophagy (162, 163). *Beclin 1*, an essential autophagic gene, depends on E2F transactivation of the promoter, and upregulation of Beclin 1 by 14-3-3τ also

requires E2F1 (164). In the same study, depletion of E2F1 or 14-3-3τ inhibits autophagy. There is also evidence that in certain settings, Rb can induce autophagic-vacuole formation by inhibiting E2F function, and overexpression of E2F1 will bypass Rb-mediated autophagy, directing a cell for apoptosis (165). In fact, it seems that E2F transcriptional programs, which drive apoptosis and autophagy, may have overlapping functions. For example, the nutrient energy sensor *AMP kinase α 2 (AMPKα2*), which is an inducer of autophagy, is also a pro-apoptotic E2F1 target gene (161). An open question is whether E2F-induced autophagy results in cell death or cell survival—though like other E2F-mediated biological processes, cell type, stimulus, and temporal activation most likely direct the outcome.

1.6 Rb and E2F knockout mouse models

The generation of the *Rb* knockout mouse was the first successful mouse model with targeted deletion of a tumor suppressor gene (166-168). The most enigmatic finding in these animals is that they are not predisposed to retinoblastoma, which is observed in the human situation, however some display pituitary tumors. The *Rb* null mouse is embryonic lethal, exhibiting neuronal cell death and defective erythropoiesis. These developmental defects appear to be attributed to aberrant E2F activity; combined deletion with either *E2F1* or *E2F3a* animals can robustly suppress the *Rb*-mutant phenotype, and extend viability (68, 169). Studies on E2F knockout mice have provided some surprising information about the function of E2Fs *in vivo.* As mentioned previously, *E2F1*

knockout animals develop testicular atrophy, and tumors in several organs including sarcomas, lung tumors, and lymphomas (71). They also develop increased number of thymocytes, due to defects in apoptosis (73). *E2F2*$^{-/-}$ animals die early from autoimmune disease accompanied by widespread inflammatory infiltrates, glomerular immunocomplex deposition, and anti-nuclear antibodies (170). *E2F1*$^{-/-}$*;E2F2*$^{-/-}$ animals develop insulin-deficient diabetes and exocrine pancreatic dysfunction, dysplasia, and a reduction in the number and size of acini and islets, being replaced by ductal structures and adipose tissue (171, 172). Surprisingly, mutant pancreatic cells exhibit increased rates of DNA replication and apoptosis, ultimately resulting in pancreatic atrophy (171). *E2F3*-deficient mice arise at one-quarter of the expected frequency, demonstrating that *E2F3* is important for normal development, and mice which do survive are severely runted, primarily from insufficient proliferative gene transcripts including *B-myb, cyclin A, cdc2, cdc6,* and *DHFR* (173-175). Further, elegant knock-in studies demonstrate that *E2F3a*^{1ki} or *E2F3a*^{3bki} could suppress these postnatal phenotypes seen from *E2F3a* knockout (175). Dissecting the *E2F3a* versus *E2F3b* isoform functions, only loss of *E2F3a* (176), and not *E2F3b* gave a slight decrease in white adipose tissue. However *E2F1*$^{-/-}$*;E2F3a*$^{-/-}$ double mutant animals die around one month with under-developed sex organs, reduced pancreatic exocrine cells and other developmental defects (175, 176). *E2F1*$^{-/-}$*;E2F3b*$^{-/-}$ animals are viable and fertile, demonstrating the crucial role of the *E2F3a* isoform (175). Triple knockout mice, *E2F1*$^{-/-}$*;E2F2*$^{-/-}$*;E2F3*$^{-/-}$*,* are embryonic lethal, with no clear disturbances to proliferation (177, 178). Mice lacking *E2F4*

surprisingly show no abnormalities in cell proliferation or cell cycle arrest. However, *E2F4* is essential for normal development—mice lacking *E2F4* have several developmental defects including impaired erythroid proliferation, hematopoietic lineages maturation defects and craniofacial abnormalities, making animals more susceptible to opportunistic infections (179-181). *E2F5* null mice develop normally with no defects in cell proliferation, however newborn mice develop non-obstructive hydrocephalus, suggesting excessive cerebrospinal fluid (CSF) production by the choroid plexus (75). *E2F6*$^{-/-}$ mice have a normal lifespan, similar phenotype to *PcG* null mice (76). Further, *E2F6* is essential for the long-term somatic silencing of certain male-germ-cell-specific genes, but it is dispensable for cell cycle regulation (76). *E2F7* and *E2F8* null mice have no observed phenotype, though the combined knockout, *E2F7*$^{-/-}$*; E2F8*$^{-/-}$*,* animals are embryonic lethal with widespread apoptosis, vascular dilation and hemorrhage. One can conclude that, at least in mice, the "repressive" arm of the E2F family is crucial for development but are dispensable for tumor formation (56).

1.7 Alterations of the Rb-E2F pathway in cancer

The oncogenic capacity of E2F was highlighted when it was shown to transform cells when overexpressed with other oncogenes such as *RAS* and *c-MYC* (115, 182). Although mutations in *Rb* have been found in a variety of human cancers, mutations in *E2F* family members remain fewer than *Rb* mutations (66). Being a classic tumor suppressor gene, both copies of the *Rb*

gene locus are mutated in both sporadic and inherited retinoblastoma. The retinoblastoma gene often undergoes point mutations and partial gene deletions, resulting in an mRNA species and a truncated protein product (183). Mutations in the *Rb* gene have been identified in various tumor types, including osteosarcoma (90%), breast cancer (20%), small-cell lung cancer(>90%), non-small cell lung cancer (30%), prostate cancer (20%), melanoma (rare, but inherited mutation predisposes to melanoma), bladder cancer (20-50%), CML (20%), and gliomas (15-30%) (184-194). Although not by mutation, human papillomavirus (HPV) is thought to initiate cervical carcinoma and squamous cell carcinoma of the head and neck in part by inactivating Rb with the E7 protein (195). Further, the Rb pathway is mutated in most cancers, either through targeting upstream regulators such as cyclin D or p16, or downstream effectors such as E2F proteins themselves. Amplification of the *cyclin D1* gene is observed in human laryngeal squamous cell carcinomas, breast cancer, anal and esophageal squamous cell carcinomas, mantle cell lymphomas, and some lung cancers (196-200). In all cases over-amplification of the *cyclin D* or *cdk4* kinase can lead to Rb phosphorylation, inactivation, and thereby release its role as an active tumor suppressor. Negative regulators of cyclin-Cdk activity are also frequently mutated in cancer, such as *p16, p21*, or *p27* (201-204).

In human cancers, there is also sufficient evidence of aberrant *E2F* expression in divergent tumor types, hinting at the important role for E2F activity in various organs (66, 205). In fact there are reports linking *E2F1* or *E2F3* locus

amplification to hepatocellular carcinoma (206-209), bladder cancer (210-213), retinoblastoma (214, 215), and liposarcoma (216, 217). In the case of bladder cancer, this amplification imparts cells with rapid growth and more invasive capacity (218). Similarly, malignant melanoma has increased copy number of E2F1 (219). Overexpression of *E2F1-3* has been observed in glioblastoma (220), lung (221-225), ovarian (226, 227), breast (228, 229), gastric (230-232), and colon cancer (233, 234). Chromosomal deletions of *E2F1, E2F2,* and *E2F3* genes have also been detected in several cancers including neuroblastoma (235, 236), thyroid (235), and pancreatic cancer (237, 238). Despite *E2F4* and *E2F5* being part of the repressive arm of the E2F family, there are few mutations, deletions, or silencing events of these genes identified in human cancer. One study highlights that the *E2F5* gene is amplified along with *MOS* and *MYC* in breast cancer, and minimal common region 8q21.3-8q23 in osteosarcoma (239, 240) (241). There is also increased expression of E2F4-8 in several cancers, including breast (241, 242), colon (243, 244), ovarian (226, 227, 245), and skin (246). Whether these mutations are oncogenic remains unclear.

1.8 Rb and E2Fs in angiogenesis and tumor progression

Angiogenesis, the formation of new blood vessels from the pre-existing vasculature, is a critical step in normal embryonic development, wound healing, inflammation, and a precursor for tumor progression. By extending the vasculature within a tumor, adequate supply of oxygen and metabolites enable the mass to grow. In early stages of angiogenesis, endothelial cells and tumor

cells secrete proangiogenic factors such as vascular endothelial growth factor, VEGF, and basic fibroblast growth factor (bFGF) and interleukin-8 (IL-8), which stimulate endothelial cell proliferation, migration, and differentiation into the tumor bed (247-249). pRb is necessary for cyclin A1 to induce autocrine expression of VEGF (250), and mice defective of *p130* had impaired new vessel growth, and therefore less tumor xenograft growth (251). Several genes that are responsive to VEGF and required for angiogenesis are regulated by E2Fs; these direct E2F target genes include human metallothionein 1G *(hMT1G)* (252), VEGF receptor 1 *(FLT-1),* VEGF receptor 2 *(KDR),* angiopoieten 2 *(ANGPT2)* (253) and platelet-derived growth factor receptor α *(PDGFR-α).* Thrombospondin-1 *(TSP-1),* an anti-angiogenic glycoprotein, is also an E2F1 target gene (254). E2F1, E2F2 and E2F3 can activate the *fibroblast growth factor receptor 2 (FGFR-2)* promoter, contributing to malignancy (255, 256).

Several studies have provided initial evidence that Rb-E2F pathway may regulate tumor progression. Reconstitution of pRb in various cancer cell lines suppresses tumorigenicity in nude mice and confers less tumor cell invasion *in vitro* (257). Overexpression of Ad-E2F1 in NIH3T3 fibroblasts resulted in a down-regulation of several genes involved in proteolysis (122) whereas vascular endothelial growth factor B *(VEGF-B),* matrix metalloproteinase 16 *(MMP16),* and fibroblast growth factor 2 *(FGF-2)* are induced through direct and indirect mechanisms upon E2F activation in another study (258). In addition, high levels of E2F1 were found in lung metastasis of colon cancer and associated with high

levels of *TS* (259). E2F overexpression in head and neck carcinoma cell lines also conferred more invasive properties *in vitro*; there was also an additive effect on proliferation (260). There is evidence that E2F activity is required for integrin α6β4-mediated invasion of breast cancer cells, and that α6 integrin is highly expressed in metastatic 4T-1 cell lines leading to higher expression of E2F target genes (261, 262). Taken together, these studies suggest a positive-feedback loop, and add to the complexity of E2F-mediated tumor progression.

2. Regulation of tumor progression

Cancer was originally conceived to be little more than the sustained proliferation of a cell population. After decades of research, the nature and diversity of cancer is better characterized; the characteristics most frequently seen in tumors had been placed into six hallmarks: angiogenic capability, replicative immortality, invasive and metastatic capability, evasion of growth suppression, resistance to cell death, and sustainable proliferative signaling (263). In addition to the original six hallmarks, the ability to deregulate cellular energetics (presumably due to genomic instability and mutation) and avoid immune destruction (partially due to inflammation in the tumor microenvironment) was added to the list. (264). It is therefore intriguing which proteins and pathways can connect these hallmarks regulating early stage events to late stage events—we hypothesize that the Rb-E2F pathway might be a major link between these events (Figure 5). This section aims to discuss the ominous complexity of the progression of cancer.

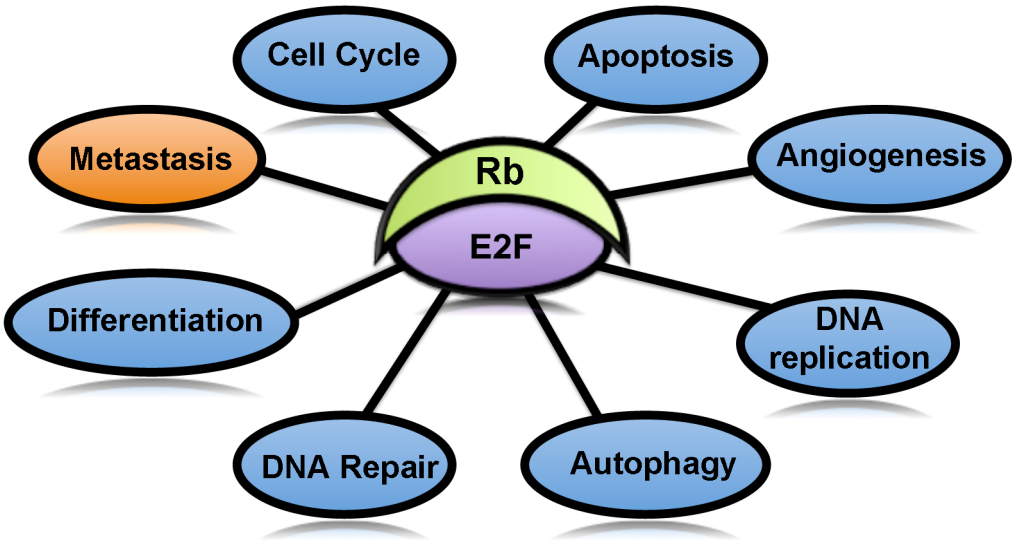

Figure 5. The Rb-E2F pathway regulates many hallmarks of cancer. The list of E2F target genes keeps growing each year. Connecting E2F to cell cycle regulation, genes that are required for S-phase entry like *cdc6, cdc25a,* and *TS* among others are direct E2F targets. Apoptotic E2F target genes include *Bok, Caspase-3, Caspase-7, PUMA,* and others. Several angiogenesis genes are regulated by E2Fs, including *FGF, FGFR-2, FLT-1, KDR, angiopoiten-2,* and *VEGF.* The MCM genes *MCM2-7* are required for DNA replication, and regulated by the Rb-E2F pathway. Data suggests that genes required for autophagic vessel formation including *LC3, ATG1, ATG5,* and *DRAM* are regulated by E2F, while DNA repair genes like *RAD51*, and *BRCA1* are also E2F-regulated. Rb and related pocket proteins tightly control the regulation of differentiation. Several studies highlight a role for the Rb-E2F pathway in metastasis as well, linking the Rb-E2F pathway to most processes in cancer.

2.1 Metastasis as a multistage process

For a tumor to progress from a primary neoplasia to metastases, multiple reprogramming events occur to promote the process (265-267). Further adding to this complexity, primary tumors will often colonize specific organs, have different rates at which metastasis emerges, and respond differently to various therapeutics (268). However, the simplified view is that metastasis is an orderly process of five steps: local invasion into stroma, intravasation into blood vessels, survival in circulation, extravasation at the distant site, and finally colonization at the distant site (266, 268, 269).

As previously described, there are various mutations that occur depending on the site of the primary tumor; these are the initiating mutations, and can include the induction of oncogenes such as *KRAS, PI3K, EGFR* and others in NSCLC (270), *B-RAF* in melanoma (271), or *HER-2* in breast cancer (272). Tumors can arise from the silencing of genes as well, including well-known examples such as *p53* in many tumor types (273), or *BRCA1* and *BRCA2* in breast and ovarian cancer (274). These mutations can be a requirement for metastasized cells as well, a theory supported by studies showing that in mouse models of breast cancer *Erbb2*-dependent breast tumors and metastatic lesions shrink when treated with ERBB2 antibody therapy (275). Conversely, in other models of cancer with tumor formation being driven by oncogene induction,

metastasis is not always inevitable (276). Further, some patients have detectable cancer cells in circulation but never develop metastasis (277).

2.2 Epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET)

The observation that primary carcinomas could progress to higher pathological stages, which had localized invasion and eventual metastasis, was coupled to the observation that epithelial cells could change shape and free themselves from other cells. This event was later molecularly characterized as the loss of *E-cadherin* either by mutation or dowregulation of the transcript (278) (279)—one of many molecular changes in the epithelial to mesenchymal transition (EMT). Signals emanating from the microenvironment such as HGF, EGF, PDGF and TGF-β appear to turn on oncofetal transcription factors including Goosecoid, FOXC2, Snail, Slug, Twist and Zeb1/2, which are known to mediate the EMT process during embryogenesis, fibrosis, and cancer (at least *in vitro* and in animal models)(280-284). Further, these transcription factors have been shown to cause metastasis when ectopically expressed (285-287), presumably through the direct *trans*-binding to the *E-cadherin* promoter (288, 289). When this key suppressor of motility is repressed, cells are free to mobilize and invade surrounding tissues. In addition, cells that lose epithelial markers such as *E-cadherin* will concomitantly gain mesenchymal markers such as *vimentin, fibronectin, desmin, FSP1, α-SMA and N-cadherin* (287). These molecular markers correlate with a spindle-shaped, or fibroblast-like morphology, making

cells more motile with higher matrix-degrading enzyme levels as well. Interestingly, cells at the tumor leading edge have been shown to undergo EMT at higher rates than cells at the core of a solid tumor (290) suggesting that EMT could be a result of signaling from cell extrinsic factors in the tumor microenvironment. This interaction is likely a part of the crosstalk between stromal cells and cancer cells (291) and leads to a small sub-population of tumor cells that can intravasate, survive in circulation, and colonize. In order for cells that have undergone EMT to colonize the secondary site, they must be able to revert back to an epithelial state. This is accompanied by genetic changes pushing cells to a more epithelial state, or an MET, and is likely also prompted by differing signals in the new microenvironment (292). Importantly, cell-intrinsic signaling pathways must also be intact to transduce these processes, utilizing proteins such as ERK, MAPK, PI3K, AKT, SMADs, and Ras (293). Although this model for cancer cell dissemination in the context of EMT has been shown for many cell lines *in vitro* and in animal models, more experimental validation is required to determine the global nature of the process, and whether this occurs in human tumors.

2.3 Mechanisms of invasion

The invasion process demonstrated by cancer cells to breach the basement membrane and initiate the metastatic process appears to be mediated through both proteolytic degradation of surrounding tissue and methods of physically moving the tumor cells through the surrounding tissue (267, 294). The

surrounding tissue, or extra-cellular matrix (ECM) is comprised of a macromolecular network of proteins, glycoproteins, and proteoglycans that aim to maintain the tissue architecture (295, 296). The precise biology of the ECM that confronts invading tumor cells can vary between the 2-D sheet like conformation, and the 3-D fibrillar meshwork of the interstitial compartment (297). In either case, the ECM composition impeding the outgrowth of cells is a significant physical barrier, and therefore cancer cells must utilize various stratagems for trafficking through the matrix.

The initial barrier in epithelial cancers is the basement membrane. It is comprised of over 50 components, mostly interwoven laminin and type IV collagen (298, 299) that contain various degrees of covalent crosslinking including disulfide and lysyl oxide–derived aldimine bridges. Studies have demonstrated that this network generates pores of approximately 50 nm, and that normal and cancer cells alike are unable to migrate through pores less than 2.0 μM in diameter (300-302). As described earlier, in order to remodel the basement membrane to allow for cancer cell migration, cells can reactivate programs used in embryonic development, the developmental EMT (287, 303). Conversely, however, it is possible that the basement membrane remodeling is the event which activates EMT in cancer cells—suggesting a paracrine loop between cells and their surrounding extra-cellular barriers (304).

Proteolytic machinery remains the crux of the process to effectively remove part of the basement membrane (305-308). This has been demonstrated *in vivo* as well, where type IV collagen networks are substantially degraded (305, 308-310). Although there are hundreds of proteases in the human degradome, numerous studies have highlighted a role for the matrix metalloproteinase family in various aspects of angiogenesis, EMT, resistance to apoptosis, and degradation of the BM and ECM. It is difficult to dissect an unambiguous role for each protease given their diverse substrates: the BM itself, chemokines and growth factors in the matrix (being able to both activate or inactivate), cell surface receptors, and adhesion molecules (311). Once passing through the BM, cells no longer maintain a differentiated state, and they continue to an environment of primarily type I collagen, or the 3-D ECM (294, 297, 311-314). Once cells are in the type I collagen networks, they utilize integrins and other cell surface markers to further promote EMT, migration, and degradation phenotypes, either moving as a single cell or a cell mass (315, 316). The goal of proteolytic systems is to provide cells a large enough diameter to pass through (317, 318). However, conflicting and complimentary reports have also suggested that protease-independent transmigratory schemes are used as a means to infiltrate type I collagen barriers (314, 319-322), including following other leading cells through the ECM (323, 324). It is logical that cells may utilize a combination of these methods in order to circumvent physical constraints and eventually intravasate into the vasculature.

2.4 Circulating tumor cells

Once cells have successfully navigated through the ECM and enter the tumor vasculature, they are once again faced with an entirely new set of challenges in the bloodstream. The bloodstream is an inhospitable environment for cancer cells; they have to undergo velocity-induced shear forces, survive without a substratum, and ward off any attempts at elimination by immune cells (267, 325). The tumor cells will often times suffer collisions with host cells, such as leukocytes and endothelial cells that line vessels. Further, cells favor growing on a surface, and without this attachment cells face the risk of cell death—also known as anoikis (326). Only the circulating tumor cells (CTCs) which are resistant to anoikis, can overcome shear forces, immunosurveillance, and then adhere to the vascular endothelium of organs will have a chance to survive, and colonize at their distant site. Only a minute fraction of CTCs survive, whereas most die or remain dormant for months, or even years, depending on the tumor type (268, 327). Beyond survival, cells also need to end their journey either by binding to coagulation factors within the vessel, or by mechanical trapping (physical occlusion) in the capillary bed. The coagulation factors are variable depending on the location the tumor cell arrests, including E-selectin, P-Selectin, I-CAM1, V-CAM1 and others on the endothelial cells, and linking to CD44, CEA, PODXL, and integrins on the tumor cell surface (325). Other studies have shown that tumor cells can metastasize early, and then complete their proliferation inside the vasculature before adhesion (328). Tumor cells of epithelial origin are

approximately 10 μM in size or greater, and small vessels or capillaries are less than 10 μM (325, 329). To add to the variability of factors that contribute to metastasis complexity, capillary beds can express different factors. For example metadherin is a protein that can home breast cancer cells to the lung rather than the skin, kidney, or other organs. Targeting metadherin with antibodies or siRNA could reduce experimental metastases, demonstrating that molecular markers could be viable anti-metastatic therapeutics (330).

2.5 Metastatic colonization

The final step of metastasis is the infiltration and colonization of distant organs by CTCs. This requires the passage through the capillary walls and survival in the new parenchyma (268). The genes required for this colonization could already be expressed, or deregulated in the primary tumor; however once metastasized, these alterations might serve a different function at the new locale. Further, the composition and structure of certain organs may be more pervasive to metastatic infiltration than other organs, which could possibly account for the organ site-specific metastasis seen in humans (268). The most typical sites for metastatic relapse of solid tumors are the bone, lungs, brain, liver, and lymph nodes (Figure 6) (268). The molecular characterizations of these different microenvironments from the major sites demonstrate some similarities to the primary location. It also shows distinct differences, calling a demand for site-directed therapeutic strategies. There is growing evidence observed in mouse xenograft studies that the microenvironment where the CTCs “home” to plays a

large role in metastastic colonization, including the presence of myeloid derived suppressor cells, endothelial, and mesenchymal lineage (331-333). Stephen Paget when describing the tumor cells and their environment as the “seed” and the “soil” initially predicted these observations in 1889 (334).

2.5.1 Metastasis to bone

One of the most well characterized sites for metastasis, the bone, is a common site for breast cancer, multiple myeloma, and prostate cancer metastasis (266-268, 335). This type of metastasis is often paired with osteoblastic (bone forming) and osteoclastic (bone degrading) activities which is prompted either by chemokines secretion from the tumor cells or microenvironment (335). The activation of osteoblasts can be accomplished by activation of transcription factor Runx-2 and osterix, usually by chemical signals such as IGF-1R, FGFRs and endothelins (336). The osteoclastic lesions develop as part of the ‘vicious cycle’ where tumor cells secrete parathyroid hormone-related protein (PTHrP), which stimulates osteoblasts to produce RANK ligand (RANKL) and osteoprotegerin (OPG). After these osteoclasts are activated, the degraded bone matrix can release embedded growth factors like IGFs and TGFβ, which can then turn tumor cells more virulent. In addition, interleukin-11, MMP-1, chemokine receptor CXCR-4, and the connective tissue growth factor, CTGF, which can be secreted by other cells in the bone microenvironment can induce PTHrP-independent osteoclastic activity and aid bone metastasis (337).

To this end, a monoclonal antibody against PTHrP is in preclinical development (338, 339).

Recent studies have characterized a bone metastasis dormancy model that shows aberrant expression of vascular cell adhesion molecule 1 (VCAM-1), in part dependent on the activity of the NF-κB pathway, promotes the transition from micrometastasis to overt metastasis (340). Further, antibodies against VCAM-1 and integrin α4 effectively inhibited bone metastasis progression and preserved bone structure (340). A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS1) and matrix metalloproteinase-1 (MMP-1) in tumor cells can also orchestrate a paracrine signaling cascade to modulate the bone microenvironment in favor of osteoclastogenesis and bone metastasis (341). Correspondingly, MMP1 and ADAMTS1 expression is associated with increased risk of bone metastasis in breast cancer patients (341). Proteolytic release of membrane-bound epidermal growth factor (EGF)-like growth factors, including Amphiregulin (AREG), heparin-binding EGF (HB-EGF), and transforming growth factor alpha (TGFα) from tumor cells suppressed the expression of osteoprotegerin (OPG) in osteoblasts. Another oncogene important for bone metastasis of breast cancer, Src, is dispensable for homing to the bones or lungs but is critical for the survival and outgrowth of these cells in the bone marrow (342).

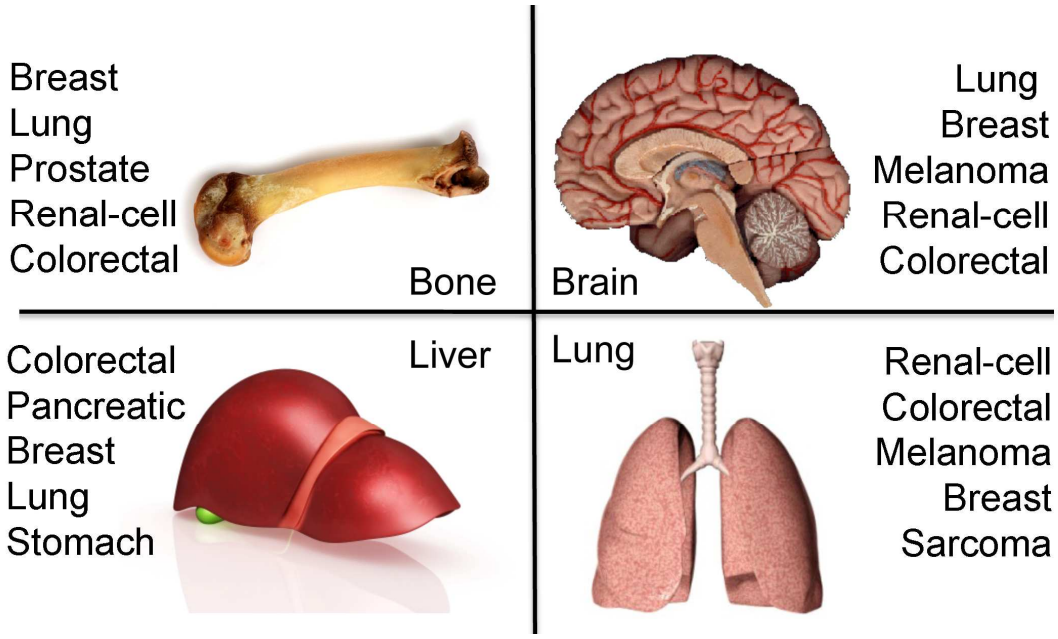

Figure 6. Common sites for metastases. The formation of bone metastasis is a common event in many types of solid tumors, particularly breast and lung cancer. Metastasis to the liver is primarily through the large portal-vein system, and seeding the blood-rich organ is also particularly common for pancreatic and colorectal cancers. Metastasis to the brain can occur after months or years of cancer dormancy, and is frequently observed in lung, breast, and melanoma patients. The lungs are also a prime location for tumor cells to become trapped by occlusion in the small lung capillaries.

2.5.2 Metastasis to lung

In animal models, the tail vein metastasis model is one of the most utilized methods to study late stage events in metastasis. Another animal model of metastasis to the lungs is the breast cancer orthotopic model—where cells are xenografted into the mammary fat pad of mice, and over time cells spontaneously metastasize to the lungs (343). Along with the lung orthotopic model, where cells are directly implanted into the lung, there are several assays used to measure metastasis in mice. Because of these experimental stratagies, studies looking for potential molecular pathways contributing to lung metastasis are numerous. An elegant study from Massagué lab demonstrated that human breast cancer cell lines that metastasized to the lung shared a gene signature different from those cells that could metastasize to the bone, although a single gene could not recapitulate the phenotype (343). In this study a 54-gene signature was established, only cells overexpressing ID1 alone were modestly more active at forming lung metastases than cells infected with vector controls (343). Other genes tested for functional validation included the epidermal-growth-factor family member *epiregulin* (344), the chemokine *GRO1/CXCL1*, the matrix metalloproteinases *MMP1* and *MMP2 (gelatinase A),* the cell adhesion molecule *SPARC*, the interleukin-13 decoy receptor *IL13Rα2*, the cell adhesion receptor *VCAM1*, and the prostaglandin-endoperoxide synthase *PTGS2/COX2*. The expression of *ID1, CXCL1, COX2, EREG* and *MMP1* increased with lung metastatic ability (343). A follow-up study showed *ID1* and its closely related

family member *ID3* were required for tumor initiating functions, both in the context of primary tumor formation and during metastatic colonization of the lung microenvironment (345). Other groups have also demonstrated a propensity for metastasis when *ID1* is upregulated or overexpressed (346-351).

Numerous other pathways have also been implicated in lung cancer metastasis, including cell survival, resistance to cell death, inflammatory pathways and antiapoptotic pathways—utilizing proteins such as ezrin, TGFβ, Bcl-2, Bcl-xL, DAP, IAP, and NF-κB (352-361). Based on clinical, functional, and molecular evidence, it seems that TGF-β in the breast tumor microenvironment also primes cancer cells for metastasis to the lungs (362). Central to this process is the induction of angiopoietin-like 4 (ANGPTL4) by TGFβ—enhancing their subsequent retention in the lungs through disruption of vascular endothelial cell-cell junctions, which increases the permeability of lung capillaries (362). In addition, the expression of tenascin C (TNC), an extracellular matrix protein of stem cell niches, was shown to be associated with survival and outgrowth of lung micrometastases (363). TNC enhanced the expression of mushashi homolog 1 (MSI1) and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)—MSI1 is a positive regulator of NOTCH signaling, whereas *LGR5* is a target gene of the WNT pathway (363). Importantly, TNC protected MSI1-dependent NOTCH signaling from inhibition by signal transducer and activator of transcription 5 (STAT5), and selectively enhanced the expression of LGR5.

2.5.3 Metastasis to brain

Brain metastasis affects an estimated 10% of cancer patients with disseminated disease (364, 365). Even small lesions can cause neurological disability, and the median survival time of patients with brain metastasis is short. Brain metastasis is the most common in people with lung and breast cancer, and also observed in melanoma (366). Metastasis from lung adenocarcinomas develops within months of diagnosis and affects several organs besides the brain (367). However the display of a brain metastasis can occur years, and even decades after the removal of a small primary malignancy, suggesting that the phenotype required to infiltrate the brain microenvironment is far more advanced than the requirements of either the lung or bone microenvironment (268, 368, 369). This difficulty to colonize the brain could be partially due to the unique nature of the blood-brain barrier, which is connected by tight junctions, filled with efflux pumps and surrounded by a basement membrane coated in astrocytes and pericytes (267, 268). Once within the brain, tumor cells are faced with astrocytes and glial cells which can stimulate the production of cytokines, chemokines, and growth factors. Further, the catecholamine neurotransmitters norepinephrine, dopamine, histamine, angiotensin, and substance P have all been reported to induce tumor cell motility (370). With the same method used to study breast cancer metastasis to the bone (337) and lung (343), studies show that breast cancer infiltration of the brain requires general mediators of extravasation, complemented by specific enhancers of cell passage through the blood-brain barrier (371). Cells from patients with advanced disease were isolated that

preferentially infiltrate the brain. Gene expression analysis of these cells and of clinical samples, coupled with functional analysis, identified cyclooxygenase *COX2*, the EGFR ligand *HBEGF*, and the α2, 6-sialyltransferase *ST6GALNAC5* as mediators of cancer cell passage through the blood–brain barrier (371). Interestingly the expression of *ST6GALNAC5,* which is normally only expressed in the brain, and therefore when expressed in breast cancer cells enhances their adhesion to brain endothelial cells and their passage through the blood–brain barrier (371, 372). Further, activation of the canonical WNT/TCF pathway was identified as a determinant of metastasis to brain and bone during lung adenocarcinoma progression. The WNT/TCF target genes *HOXB9* and *LEF1* were identified as mediators of invasion and colonization (373). Taken together, the complex interplay between various cells in the tumor microenvironment can play a large role in metastasis to various organs.

3. Matrix Metalloproteinases in cancer and tissue remodeling

Growing evidence supports the notion that the matrix metalloproteinase (MMP) family is one of the key mediators of tumor microenvironment remodeling observed in metastasis. These proteinases regulate a wide array of substrates, thereby affecting diverse biological processes and signaling events. In addition, due to the poorly understood substrate repertoire, MMP inhibitors have failed in the clinic, and new approaches to target MMP function and regulation must be developed (374).

3.1 Matrix Metalloproteinase family members

When Gross and Lapiere described the “activity” capable of degrading collagen during tadpole tail metamorphosis (375), the MMP field was born. This interstitial collagenase (now known as MMP1) was then purified after being identified in human skin and the involuting rat uterus (376-378). Additional studies have led to the discovery of a family of proteinases that are structurally similar, comprising of 23 enzymes found in man and 24 in mice, now known as the matrix metalloproteinase family (311).

MMPs are members of a larger family of proteases known as the metzincin superfamily; being zinc dependent endopeptidases with a conserved methionine reside in the active site (379). The MMP family shares a conserved domain structure, consisting of a catalytic domain and an auto-inhibitory pro-domain (Figure 7). The pro-domain harbors a cryptic cysteine residue that keeps the enzyme catalytically inactive by cooperating with the zinc-containing active site. The catalytic domain is attached to the c-terminal domain by a flexible hinge linker, which is approximately 75 amino acids long, and has no determined structure. In order for MMP-mediated cleavage to occur, the pro-domain must be removed or destabilized, allowing the catalytic site to recognize the substrate. All MMPs are synthesized with a signal peptide in a latent form (zymogen), which is then cleaved when processed through the secretory pathway. These basic features make up the minimal domain MMPs, MMP7 and MMP26. Despite

having the same minimal domain organization, MMPs are further divided into eight structural groups, five that are secreted and three which are bound in the plasma membrane, also known as the membrane-tethered MMPs (MT-MMPs) (380). Many MMP family members also contain a hemopexin domain, four-bladed β-propeller structure that serves multiple functions. The hemopexin domain has been characterized as a mediator of protein-protein interaction, substrate recognition, activation of the enzyme, protease localization, internalization and degradation (381), including MMP1, MMP3, MMP8, MMP10, MMP12, MMP13, MMP19, MMP20, MMP21, MMP27, and MMP28 (311). MMP21 is a unique member and contains a vitronectin-like insert. MMP11 is also a close structural relative, but contains a furin recognition motif between the prodomain and the catalytic motif. This allows for intracellular activation by serine proteinases. MMP2 and MMP9 are different than other MMPs having fibronectin type II repeats present between the catalytic and zinc-containing domains. This allows for gelatin substrate recognition. The membrane tethered MMPs are attached to the plasma membrane either through a transmembrane domain, including MMP14, MMP15, MMP16, and MMP24, or by a glycosylphosphatidylinositol (GPI) linkage, MMP17 and MMP25, which is attached to the hemopexin domain. All transmembrane MMPs contain a furin-like recognition element. The third type has only one member, MMP23, and is a type II transmembrane MMP, containing an N-terminal signal anchor that targets it to the cell membrane, a cysteine array and immunoglobulin (Ig)-like domain (380).

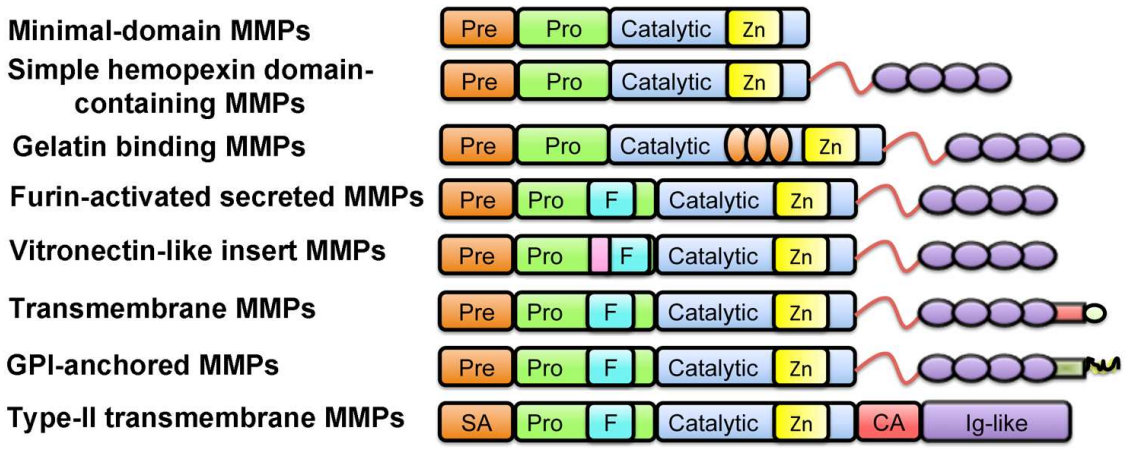

Figure 7. The protein structure of MMPs. The minimal-domain MMPs are secreted and contain an amino terminal signal sequence (Pre) which directs them to the endoplasmic reticulum, a pro-domain (Pro) which contains a thiol group, and a catalytic domain with a zinc binding site. These include MMP7 and MMP26. The MMPs that have a hemopexin domain (purple) contain a linker (red). MMP1, 3, 8, 10, 12, 13, 18,19, 20, 22, and 27 belong to this subclass. MMP2 and MMP9 contain repeats of fibronectin (orange) to mediate gelatin recognition. MMP11 and MMP28 are the furin activated secreted MMPs, and contain a furin recognition sequence for intracellular furin-mediated cleavage. MMP21 contains a vitronectin-like insert, in addition to furin. The transmembrane MMPs, MMP14, MMP15, MMP16, and MMP24 contain a transmembrane domain and a short cytoplasmic domain; MMP17 and MMP25 are anchored by a glycosylphosphatidylinosition residue. MMP23 is the only member of the type II transmembrane subclass, and contains a signal anchor in the amino terminus (SA) and a cysteine array with an Ig-like domain in the carboxy terminus.

3.2 MMP substrates and functions

Although MMP1 was initially named an interstitial collagenase due to its simple role of degrading collagen, the remainder of the MMP family seems to have varying degrees of promiscuity with biological substrates. Historically, the MMPs were named according to what component of the ECM they could degrade: collagenases, gelatinases, stromelysins, and matrilysins (380). In fact, most extracellular signaling events take place at the cell surface, where MMPs are either secreted, or tethered at the membrane to aid in pericellular proteolysis.

3.2.1 MMPs regulate signaling molecules

It is true that MMPs collectively can degrade all components of the ECM, however sometimes the cleavage can generate products with new functions. For example, the cleavage of laminin-5 and collagen type IV can result in exposure of cryptic sites that promote migration (382, 383). Similarly, cleavage of IGF-BP and basement membrane-specific heparan sulfate proteoglycan core protein (HSPG) can release IGF and FGFs, respectively (384, 385). Other cell adhesion molecules like E-cadherin, desmogleins and CD44 are cleaved by MMPs, yielding an increase in invasive behavior (386-388).

MMPs also function by activating inactive growth factors. For example, transforming growth factor α (TGFα) is activated after shedding its ecto-domain in an MMP-dependent manner (389). TGFβ can be activated by either MMP2 or

MMP9, but in a different way—it is freed from extracellular space, and then able to engage its cognate receptors (390). In drosophila, MMP1 releases the N-terminal extracellular domain of Ninjurin A (391), liberating the ectodomain, thereby promoting the loss of cell adhesion in a cell-nonautonomous manner. Further, EGFR ligands require processing by MMPs in order to function as well (392, 393) Growth factor receptors themselves can also be targeted by MMP2: FGFR1, HER2, HER4, and c-MET are all processed by an unidentified MMP or ADAM family member which is responsive to endogenous inhibitors of MMP activity, the tissue inhibitors of metalloproteinases (TIMPs) (394-396).

3.2.2 MMPs regulate apoptosis

There is also evidence that MMPs aid in evading apoptosis. Apoptosis is frequently initiated via extracellular receptors such as the Fas receptor, which can then activate the proteolytic cascade of intracellular caspases. MMPs might function by cleaving ligands, and inactivating those signals, or cleavage of extracellular receptors making them unable to engage ligands. For example, MMP7 cleaves Fas ligand in doxorubicin treated cancer cells, hindering the efficacy of the chemotherapy (397). MMP3 could induce apoptosis when overexpressed in epithelial cells (398, 399). MMP11 seems to also play a role; it can inhibit apoptosis when overexpressed in xenografts (400, 401) and MMP11 null mice have a high rate of apoptosis (402) coupled with delayed mammary tumorigenesis (403). Adding to the complexity, it seems that MMP9 and MMP11 activity might increase apoptosis rates during development, whereas they

decrease cancer cell apoptosis (404, 405). MMP8 may have a specialized role in apoptosis of the skin, since MMP8 null mice have an increased incidence of skin tumor formation (406).

3.2.3 MMPs regulate angiogenesis

The role of MMPs in normal vasculature and cancer vasculature is significant. MMP inhibitors reduce angiogenesis (407-409), although this could be attributed to less degradation of the ECM by inhibiting MMP1 cleavage of type I collagen in the interstitial parenchyma (410). MMP2 loss appears to directly down regulate angiogenesis in several *in vivo* models, including the chick chorioallantoic membrane model (CAM) (411), and in *MMP2*-deficient mice (412). MMP9 is also required for angiogenesis in animal models of skin cancer (413) and insulinoma, partially by increasing the bioavailability of VEGF (414). Further, *MMP9* and *MMP14* null mice have impaired vessels formation during development (405, 415). MMPs can also cleave plasminogen to generate angiostatin (416). Angiostatin is a potent inhibitor of endothelial cell proliferation and invasion through inhibition of MMP14 and MMP2 (417-419).

3.2.4 MMPs regulate invasion and metastasis

Collectively, the MMP family is able to degrade every component of the ECM; they are crucial proteins for the invasion and metastasis of tumor cells. This has been shown in both *in vitro* and *in vivo* metastasis assays. MMP2 (420), MMP3 (421), MMP13 (422), and MMP14 (423) promote invasion of cell lines

through collagen type I optic nerve explant models or through matrigel. MMP2 and MMP9 contribute to prostate cancer cell invasion by ADAM17-mediated shedding of TGF-α, which subsequently activates the EGFR-MEK-ERK signaling pathway (424). A potent fibrinolytic enzyme, MMP16, induced efficient invasion of cells in fibrin, a provisional matrix component frequently found at tumor-host tissue interfaces and perivascular spaces of melanoma. However, MMP16 did not demonstrate the same type I collagen degradation activity as MMP14 (425). MMP10 plays an important role in the invasion and metastasis of head and neck squamous cell carcinoma, and that invasion driven by MMP10 is partially associated with p38 MAPK inhibition (426). Further, MMP9-dependent migration, invasion, and angiogenesis of breast carcinoma cells are dependent on cholesterol levels in lipid rafts to elicit activities (427). In order for cells to migrate freely, they must detach themselves from other cells. For this purpose, E-cadherin is cleaved by MMP3 and MMP7, pushing cells towards EMT (386).

CD44 is cleaved by MMP14, and the extracellular domain is freed (387). CD44 can also tether MMP9 to the cell surface to aid in pericellular proteolysis (428). When CD44 cannot bind to MMP9, there is less invasion *in vivo* suggesting that it is not the expression level of MMPs that mediates invasion, but rather the localization. Conversely, *in vitro* overexpression studies have shown that high MMP2 levels do not confer a more invasive phenotype (420). In addition to utilizing proteolysis to degrade the ECM, cells develop specialized invasion edges known as invadopodia (429). MMP2, MMP9, and MMP14 have been

shown to localize to invadopodia, though the exact mechanisms for this localization are still unclear. Although only observed in a rodent model of prostate cancer, MMP7 expressed by osteoclasts at the tumor/bone interface triggers bone metastasis through RANKL (430).

MMP14 seems to be the climacteric protease for tumor cell invasion in most *in vitro* and *in vivo* studies (431). It has been shown to drive single-cell and subsequent collective cell migration and invasion (432). MMP14 is crucial for collagen turnover, as a collagen degradation enzyme, and through the activation of other MMPs (324, 433). Further, in the chick chorioallantoic model of invasion, when *MMP14* and *MMP15* null mouse fibroblasts are plated on top of the CAM, there is no definitive invasion (434). Later studies identified a triad of membrane-anchored proteases, MMP14, MMP15, and MMP16 as proteins responsible for invasive pseudopodia, and propagation of transmigration (435). However targeting MMP14 to inhibit metastasis is not simple, since MMP14 appears to also be the most nonredundant protease for tissue homeostasis. *MMP14* null mice have multiple organ defects that ultimately lead to death in several months (436-439). Taken together, the MMP proteins can regulate multiple processes, leading to tumor initiation and progression of cancer.

3.3 Regulation of MMP activity

The regulation of MMP activity is difficult to assess due to the multitude of mechanisms affecting bioavailability of these enzymes. The easiest way to

assess total MMP levels is by analyzing the mRNA transcript, rather than the protein or enzyme-activity level, due to complex protein regulation. In addition to the transcriptional regulation of MMPs, they are regulated at the translational level, through intracellular trafficking and secretion, by subcellular or extracellular localization, through zymogen activation, by binding to endogenous inhibitors including TIMPs and α2-macroglobulin, and finally through degradation by proteases (440). The most studied endogenous MMP inhibitors are the TIMP family, TIMP1, -2, -3, and -4, which can reversibly inhibit MMPs in a 1:1 ratio. Like the MMP family, their expression is variable between tissue types.

At the level of transcription, validated promoter analysis and bioinformatics approaches have revealed that each MMP promoter is unique (441). Many promoters have been validated to reveal a variety of functional *cis*-elements, including the first identified site in the *MMP1* promoter coding for AP-1, a member of the immediate-early response genes (442). In many promoters with AP-1 sites, there is also a PEA-3 binding site nearby, and can act in concert with AP-1. Many MMPs also have GC boxes in their proximal promoters, which can bind to Sp1, Sp3 and potentially other GC-binding proteins as well (441). Many MMPs show constitutive expression. *MMP14* has a proximal Sp1 site, and mutation of this site severely reduces the activity (443). Egr1 has also been shown to regulate *MMP14* through binding of conserved GC rich regions of the promoter (444). NFκB can also regulate many MMP genes including *MMP9* (445, 446), *MMP1* (447), and possibly many others bearing canonical NFκB binding

sites (441). Our studies indicate a novel role for E2F transcription factors in regulation of at least *MMP2, MMP9, MMP14*, and *MMP15*, though all 23 human MMP family members contain putative E2F binding sites (448). Given that aberrant E2F activity is observed in many cancers, we hypothesize that through transcriptional regulation of MMPs, E2Fs might also function to promote metastasis.

4. Summary

Rb together with E2F transcription factors is a crucial regulator of the cell cycle, and many other biological functions. Rb physically interacts with E2F, repressing its ability to both recruit transcriptional machinery, and by retaining an Rb-E2F complex on E2F responsive promoters (449). Not surprisingly, oncogenic mutations can initiate tumors by targeting the Rb-E2F pathway and this pathway is altered in many cancers, including non-hereditary tumors. Though disruption of the Rb-E2F pathway has a clear role for initiating oncogenesis, it is unclear whether this pathway contributes to cancer progression, angiogenesis and metastasis.

It is well established that cyclin-dependent kinases phosphorylate and inactivate Rb in the G1 phase of the cell cycle, which releases E2F transcription factors from Rb, facilitating transcriptional induction of proliferative promoters (121). Raf-1 directly interacts with Rb early in the cell cycle. Further, Raf-1 can phosphorylate Rb (36). Rb-Raf-1 disruptor (RRD-251), can disrupt the Rb-Raf-1

interaction, suppressing the phosphorylation of Rb. RRD-251 can affect many facets of cancer, including proliferation, cell cycle control, and angiogenesis. Importantly, it is effective at inhibiting tumor growth *in vivo*, though the exact mechanism for these effects has yet to be investigated on the molecular level (39).

In most clinical cancer cases, metastasis is associated with late-stage oncogenic events, tumor progression, and eventual death. Despite the pastiche of mutations that give rise to tumors, which varies greatly between tumor types, the steps that drive efficient tumor cell dissemination remain similar for all tumors (450). This suggests that clinically targeting proteins involved in the metastatic cascade could be an efficient mode to treat cancer from disparate genetic and tissue origin. The complex interaction between cell types in the tumor microenvironment, including tumor cells, stromal fibroblasts, tumor associated macrophages, immune cells, and bone-marrow derived stem cells, epithelial progenitor cells, and their individual contributions make studying metastasis *in vitro* quite daunting (451).

Studies showing the overexpression of MMPs in a variety of cancer types have lead to a strong effort in the development of MMP inhibitors (MMPis) since the 1980s (452-454). Due to the broad spectrum of substrates, both known and unknown that are cleaved by MMPs, including essential immune regulating chemokines and cytokines, the failure of large-scale Phase III clinical trials is not

surprising. In the following chapters, we describe studies that link the Rb-E2F pathway and upstream regulation by the Raf-1 kinase, to regulation of *MMP* gene expression and subsequent metastasis and colonization. These studies not only link early oncogenic events to late stage events in the oncogenic process, but they also highlight the Rb-Raf-1 disruptor, RRD-251, as a viable chemotherapeutic that can target multiple hallmarks of cancer.

Chapter 2: Materials and Methods

Cell Lines and Reagents

A549 NSCLC cells were cultured in F12K medium with 10% serum (Cellgro). MDA-MB-468, MDA-MB-435, T47D and MDA-MB-231 human breast cancer cell lines were cultured in Dulbecco modified Eagle Medium (DMEM; Mediatech) containing 10% FBS. H1299, H358, H1975, and H1650 human NSCLC cells were grown in RPMI with 10% serum. A549 cells stably expressing the firefly luciferase gene (A549-luc) were obtained from Caliper and grown in RPMI with neomycin (200 ng/mL)**.** ShRNA cells lines were maintained in media containing 0.5 µg/ml puromycin. For treatment with RRD-251, cells were rendered quiescent by serum starvation for 18 hours, and then grown in 10% serum-containing in F12K medium with RRD-251. The Rb-Raf disruptor, RRD-251, was prepared as described and was >99% pure as analyzed by HPLC. Nicotine (Sigma) was dosed at 1 µM concentration for all nicotine experiments. Twenty-four hours after luciferase constructs were transfected, suberoylanilide hydroxamic acid (Vorinostat, or SAHA) and trichostatin A (TSA) (Cayman Chemicals) were dosed at 50 nM in respective media for 24 hours. For treatment with (±)-Nutlin-3 (Cayman Chemicals), cells were treated with drugs for 72 hours at indicated concentration. ShRNA cell lines were made by stably transfecting

A549 cells with two different shRNA constructs that specifically targeted E2F1 or E2F3 obtained from an shRNAmir library from Open Biosystems, Huntsville, AL.

Molecular cloning and constructs

DNA was extracted from primary aortic endothelial cells using standard protocols (36). Primers spanning 2 Kb of the MMP9 and MMP15 promoter were used to PCR amplify the fragment with Hotmaster Taq (5-Prime). Primer sequences were:

5'-TACGGTGCTTGACACAGTAAATC-3' *(MMP9 forward);*

5'-CTGACTGCAGCTGCTGTTGTGG-3' *(MMP9 reverse);*

5'-GCTACTTTCCTTCACTGAACAGG-3' *(MMP15 forward);*

5'-CGAGTGAAGTGCGACAGTGCGGCC-3' *(MMP15 reverse).*

The fragments were then subcloned into pCR2.1 using TA cloning (Invitrogen). The plasmids were digested with *Kpn1* and *Xho1* and ligated into pGL3-basic luciferase vector (Promega). The *MMP14* promoter was a kind gift from Dr. Jouko Lohi at The University of Helsinski (443). The *MMP2* promoter was a gift from Dr. Etty Benveniste at The University of Alabama. For the generation of 5' deletion mutants, the following primer sequences were used to PCR amplify from the original full length *MMP2*-luc construct DNA:

5'-GACCCAAGCCGCAGAGACTTTTC-3' (*Δ1617F);*

5'-CTTCCTAGGCTGGTCCTTACTGAC-3' *(Δ1467F);*

5'-GCCATGGCACTGGTGGGTGCTTC-3' *(Δ945F);*

5'-CCATCTCTCTCTTTCCATCTCTG-3' *(Δ508F);*

5’-GTGACGAGGTCGTGCACTGAGGGT-3’ *(Δ221F)*

5’-CAGATGCGCAGCCTCCAGCCAC-3’ *(R).* The fragments were then subcloned into pCR2.1 using TA cloning (Invitrogen). The plasmids were digested with Kpn1 and Xho1 and ligated into pGL3-basic luciferase vector (Promega). The following primers were used with overlap extension PCR to incorporate a c-MYC, p53, or E2F binding site mutation into the *MMP2*-luc construct. For c-MYC:

5’-CTTCCTAGGCTGGTCCTTACTGAC-3’- *F1*

5’CAAGAATCCACCTGGCCTCTCAGG-3’-*R1-OEPCR-MYCmut*

5’-CCTGAGAGGCCAGGTGGATTCTTG-3’-*F2-OEPCR-MYCmut*

5’-CAGATGCGCAGCCTCCAGCCAC-3’ – *R2*

For p53 and E2F1:

5’-CTGCGGGGCAAGGTCCCTC-3’-*F1*

5’-GTGGGCTTCAGAAAATTTCAGGATTTTC-3’-*R1-OEPCR-p53mut*

5’-GAAAATCCTGAAATTTTCTGAAGCCCAC-3’- *F2-OEPCR-p53mut*

5’-AAGCCCCAGATGCGCAGCCTCCAGCCAC-3’-*R2*

5’-GCCGCAGAGACTTCGATAGATGTGA-*F2-3’-OEPCR-E2Fmut*

5’-TCACATCTATCGAAGTCTCTGCGGC-*R1*-3’-*OEPCR-E2Fmut.* For overlap extension PCR, 3 rounds of amplification are used; primers F1 and R1and primers F2 and R2 are used to first generate two fragments containing the mutation. This DNA is then pooled and used for a third round of amplification, where primers F1 and R2 are used, generating the full length product with the

mutation incorporated. To ensure a proper clone was developed, DNA sequencing was performed.

Transient transfections and Luciferase Assays

Eighty-five thousand cells were plated per well in six well plates, grown to 70% confluency, then were transfected with 0.5 μg of MMP-luciferase reporters along with 1 μg of E2F1, 2 μg of Rb-Large Pocket or full length, and 2 μg of Raf-1 full length expression vector using Fugene HD reagent in a ratio of 4 μl of Fugene (Roche and Promega) to 2 μg of plasmid. For the transfection of c-MYC, ID1, p53, p300, MDM2, or KAP1, 1 μg of plasmid was used unless otherwise indicated. Cotransfection with 0.5 μg of pRL construct containing *Renilla reniformis* luciferase gene was used as normalizing control. Luciferase assays were performed using Dual Luciferase Assay System (Promega). Relative luciferase activity was defined as the ratio of firefly luciferase activity to Renilla luciferase activity. Error bars represent standard deviation of three experiments.

Gelatin Zymography

Media was concentrated using 7 kD molecular weight cut off protein concentrators at 4^{o}C (455) and subjected to electrophoresis on 8% polyacrylamide gels containing 2 mg/mL bovine skin gelatin (Sigma). Gels were washed twice with 2.5% Triton-X100, and then incubated for 24 hours at 37^{o} C in Tris-HCl buffer (150 mM NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl pH7.6 and 0.05% NaN_3). Gels were stained with 0.2% Coomassie Brilliant Blue and destained

(30% methanol, 10% glacial acetic acid, and 60% H_20) until gelatinolytic bands could be detected. Gelatinolytic signals were quantified by densitometry.

Chromatin immunoprecipitation (ChIP) assays

Cells were treated with 1% formaldehyde for 10 minutes at room temperature for cross-linking the DNA to the proteins. The cells were scraped, washed in ice-cold PBS, and centrifuged at 1500 x g at 4℃ for 5 minutes. Subsequently, the pellet was resuspended in cell lysis buffer (44 mM Tris–HCl (pH 8.1), 1% SDS, 1 mM EDTA (pH 8.0)). The cells were sonicated thrice for 15 seconds each. Subsequently, the cell lysates were centrifuged at 10,000x*g* at 4℃ for 15 minutes. An aliquot of the sonicated DNA was used as the input for the ChIP assay. The remainder of the chromatin was diluted with ChIP dilution buffer (16 mM Tris–HCl (pH 8.1), 250 mM NaCl, 0.1% SDS, 1% Triton-X-100, 1.2 mM EDTA) and rotated overnight with primary antibody. Immunoprecipitations were done using polyclonal antibodies for E2F1-5 and Rb (Santa Cruz), p53, HDAC1, MDM2, monoclonal (Santa Cruz) and KAP1 polyclonal (Bethyl); a Rabbit anti-mouse secondary antibody (455) was used as the negative control. The next day, 60 µL of 1:1 protein G-Sepharose was added to the immune complexes, and the mixture was rotated at 4℃ for 2 hours. The beads were washed five times with ChIP dilution buffer and eluted with ChIP elution buffer (0.1 M sodium bicarbonate, 1% SDS, 5 mM NaCl). The cross-links were reversed by incubation at 65℃ for 4 hours. DNA was isolated by ethanol pr ecipitation. The associated proteins with the DNA were digested with 50 µg Proteinase K at 37℃ for 30

minutes. DNA was purified by phenol:chloroform extraction method followed by ethanol precipitation. Purified DNA was resuspended in 30 µL water. The differential binding between proteins and *MMP2, MMP9, MMP14, MMP15, DHFR, CDC6, CDC25a,* and *c-FOS* promoter DNA was examined by polymerase chain reaction (PCR). For ChIP assays after nicotine stimulation, quiescent A549 cells (2.5×10^7) were incubated with 1 µM nicotine at 37℃ for 48 hours. The interaction with specific promoters was detected using PCR with primer sequences from Table 1. For all ChIP assays throughout this manuscript, ChIP assays were conducted using primer sequences listed. The binding sites listed represent binding elements upstream of TSS.

Table 1. Primer Sequences used in ChIP assays.

ChIP primer sequences			
Gene Promoter	Amplification Site (upstream of TSS	Primer Sequence	Orientation
MMP9	1920-1904	5'-TACGGTGCTTGACACAGTAAATCT-3'	Forward
		5'-AGGGCCTACTATGTGCCAGG-3'	Reverse
	1201-1185	5'-ATGGAGCAGGGCTGGAGAAC-3'	Forward
		5'-ATGTCACTGCACTCCAGCCTGG-3'	Reverse
	518-502	5'-AGATGAAGCAGGGAGAGGAAGC-3'	Forward
		5'-CCTCCAGAGGTCAGCCAA-3'	Reverse
MMP14	1667-1532	5'-CCATAGGACTAGCCCAACTATGAG-3'	Forward
		5'-GAAGACTGACACCAGATGCTTGC-3'	Reverse
	941-862	5'-CTCACTGGCCAAATGCAATTCTTGG-3'	Forward
		5'-AGGGACTTACTGGACTCAGAACA-3'	Reverse
	(+)37-(+)53	5'-CAGTTCGCCGACTAAGCAGAAG-3'	Forward
		5'-GCGGTGCCGAGCGTGA-3'	Reverse
	(+)234-(+)250	5'-CACACTGCCCGGCTGAC-3'	Forward
		5'-ATAGAGGCTGTCCCCTAGGAGAC-3'	Reverse
MMP15	1624-1609	5'-GGCTGCAGTTCACACATCTCAG-3'	Forward
		5'-CTGTGACCAGATCTTGAAGCTC-3'	Reverse
	1201-1185	5'-GAGCTTCAAGATCTGGTCACAG-3'	Forward
		5'-ATGCAGTGAGCTATGATCATGCCG-3'	Reverse
	1036-1020	5'-GCAGTGGTCTTCAGACACGGAC-3'	Forward
		5'-CCAGGCTGGTCTTGAACTCCTG-3'	Reverse
	559-543	5'-GGGTCTGAAGATAAGGAACAGCTATC-3'	Forward
		5'-GAGTGAAGTGCGACAGTGC-3'	Reverse
DHFR	(+)425-(+)437	5'-GCCTGGCGAGGAGCGCGAGCCCG-3'	Forward
		5'-AGCAGCGGGAGGACCTCCGAGC-3'	Reverse
C-FOS	321-296	5'-TGTTGGCTGCAGCCCGCGAGCAGTTC-3'	Forward
		5'-GGCGCGTGTCCTAATCTCGTGAGCAT-3'	Reverse
MMP2	1616-1600	5'-TGCTGTTCAGGCTGGATTTAGC-3'	Forward
		5'-GCATTGGAGGTCACGATTA-3'	Reverse

siRNA transfections and Real-time PCR

For siRNA transfections 100 pmol of siRNAs (Santa Cruz) with Oligofectamine were added to cells. For real-time PCR, total RNA was isolated using RNeasy miniprep kit (QIAGEN) following manufacturer's protocol, followed by first-strand cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad). Data was analyzed by ΔΔCT method, where gene of interested was normalized to 18S rRNA, then compared to the non-targeting siRNA control sample. Error bars represent the standard deviation of three independent experiments.

Invasion Assays

Boyden Chamber assays were used to assess the invasive ability of A549, and MDA-MB-231 cells as described previously (37, 456). The upper surface of the 6.5 mm filters (Corning) were coated with collagen (100 µg/filter) and Matrigel (BD Bioscience) (50 µg/filter). Twenty thousand cells were plated in the upper chamber with 0.1% bovine serum albumin (Sigma). Media containing 20% fetal bovine serum was placed in the lower well as chemoattractant. The cells that invaded through the filters were quantified by counting three fields under 20× objective magnifications.

Would healing assays

One hundred thousand A549 cells were plated in a 6-well plate and grown to confluency (457). The cells were scratched with a sterile 200 µl pipette tip in

three separate places in each well, placed in serum-free media as a negative control, stimulated with 10% serum, or 10% serum with 50 μM RRD-251, and the same area was examined after 24 hours using phase contrast microscopy (41). Similar experiments were performed with H1650, MDA-MB-468, and MDA-MB-231 cells. The data is representative of three independent experiments. For experiments with Mitomycin C, cells were treated with 10 mg/mL of Mitomycin C for 5-12 hours, then placed back in 10% serum containing media. For siRNA studies, A549 cells were plated at 50% confluency (eighty thousand cells) then transfected with siRNA targeting E2F1, E2F3 or combination of both (Santa Cruz). Cells then grew for 24 hours before being scratched with a pipette tip. Migration assays were quantified using Alpha Imager analysis software.

Collagen Degradation Assays

Collagen Degradation Assays were carried out as previously described (458). First, 1 mL of type I collagen was mixed with 7 mL of 13 mM HCl, then neutralized with a buffer containing 0.2 M $NaPO_4$, 16.6 mL 5M NaCl, 80 mL 0.1 N NaOH . Seven hundred μl of this solution was added to 12-well tissue culture plates to obtain a final concentration of 1 μg/mL of collagen. Plates were incubated at 37^{o}C for 2 hours to polymerize. Twenty-five thousand CCL-210 cells were placed in a 40 μl button and were left to attach 5 hours at 37^{o}C. Complete media was added, and after 4 days, cells were trypsinized, and the remaining collagen was stained with Coomassie Brilliant Blue for 15 minutes, and then destained (30% methanol, 10% glacial acetic acid, 60% H_20). For siRNA

experiments, eighty-five thousand CCL-210 cells were plated in 60 mm plates, then transfected with 100 pmol of siRNA (Santa Cruz). Twenty-four hours later, twenty-five thousand cells were added to the collagen films. For RRD-251 studies, cells were pretreated for 18 hours prior to being placed on collagen films. For nicotine studies, cells were plated on collagen, and placed in nicotine-containing media immediately following attachment to collagen. Images were taken using Epson Perfection V700 Photo Scanner.

Proliferation Assays

Bromodeoxyuridine (BrdU) labeling kits were obtained from Roche. Cells were plated in poly-D-lysine coated chamber slides at 6,000 cells/well and serum starved for 24 hours. Cells were then stimulated with serum in the presence or absence of 10 µg/mL Mitomycin C for 3 hours, and then incubated in complete media. S-phase cells were visualized by microscopy and quantified by counting three fields of 100 in quadruplicate.

In vivo tail vein metastasis assay

Five million A549 cells stably expressing firefly luciferase (A549-Luc-C8) (Caliper) were injected into the lateral tail vein of 5-week-old female SCID-beige mice under an IACUC approved protocol. Mice were given DPBS: DMSO vehicle control or RRD-251 diluted with DPBS: DMSO once/day. For bioluminescence imaging, mice were anesthetized and 30 mg/Kg of D-luciferin in PBS was administered by intraperitoneal (i.p.) injection. Ten minutes after injection,

bioluminescence was imaged with a charge-coupled device camera (Caliper). Bioluminescence images were obtained with a 15 cm field of view, binning (resolution) factor of 8, 1/f stop, open filter, and an imaging time of 30 s to 2 min. Bioluminescence from relative optical intensity was defined manually, and data were expressed as photon flux (photons·sec^{-1}·cm^{-2}·steradian^{-1}) and were normalized to background photon flux over a mouse that was not given an injection of luciferin.

Immunoprecipitation and Immunoblotting

Cell lysates were made from exponentially growing cultures of A549, H1650, MDA-MB-231, and MCF7 cells in respective medium by adding lysis buffer (20 mM Tris–HCl (pH 7.6), 0.5% Nonidet-40 (new name IGEPAL-CA-630), 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 4 μM dithiothreitol (DTT), 5 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 25 μg/mL leupeptin, 5 μg/mL pepstatin, 5 μg/mL aprotinin, 25 μg/mL trypsin–chymotrypsin inhibitor) to 20 μL of packed cell volume. The lysate was rotated at 4℃ for 30 minutes and subsequently centrifuged at 15,000g at 4℃ for 15 minutes. The protein concentration was measured using a Bio-Rad Protein Assay Kit.

For co-immunoprecipitation, the cell lysates containing 250-1000 μg of total proteins were incubated with 1 μg of the following antibodies: Mouse anti-human c-MYC, mouse anti-human HDAC1, and rabbit anti-human KAP1. The

total reaction volume was adjusted to 100 µL with immunoprecipitation buffer (2 mM HEPES (pH 7.9), 40 mM KCl, 0.001 mM $MgCl_2$, 25 mM EGTA, 1 mM EDTA, 1% IGEPAL-CA-630, 1 mM DTT, 5 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 25 µg/mL leupeptin, 5 µg/mL pepstatin, 5 µg/mL aprotinin, 25 µg/mL trypsin–chymotrypsin inhibitor) and rotated on a nutator at 4℃ for 2 hour. After 1 hour, 50 µL of 1:1 protein G-Sepharose, or protein A-Sepharose slurry was added (GE Healthcare) and the mixture was rotated at 4℃ for another 3 hours. The beads were washed four times in immunoprecipitation buffer. Bound proteins were eluted in sodium dodecyl sulfate (SDS) sample buffer (0.06 M Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 100 mM DTT, 0.2% w/v bromophenol blue) and resolved on an 8% SDS–polyacrylamide gel. The proteins on the SDS–polyacrylamide gel were transferred to 0.45 µm nitrocellulose membranes (Bio-Rad), and the interacting proteins were detected by immunoblotting as described below. As a control for the experiment, one-third of the amount of protein was analyzed on the SDS–polyacrylamide gel for each immunoprecipitation reaction.

For immunoblotting, nitrocellulose membranes were incubated in a blocking solution containing 5% nonfat dry milk in PBS containing 0.1% Tween-20 at room temperature for 1 hour. Membranes were incubated at 4℃ overnight with indicated primary antibodies followed by incubation with horseradish peroxidase–conjugated anti-mouse IgG or anti-rabbit IgG (1:3000 dilution) at room temperature for 2 hour. Antibody–protein complexes were detected using

enhanced chemiluminescence immunoblotting detection reagent (GE Healthcare). The immunoblot signals were quantified using Alpha Imager HP gel documentation system. The experiment was performed twice with two independent sets of cell lysates and tumor lysates.

Tissue Processing and Immunohistochemical Staining

Lungs were fixed in 10% neutral buffered formalin after necropsy, before processing into paraffin blocks. Paraffin sections (5-Am thick) were rehydrated and processed using hematoxylin and eosin staining with standard techniques. For other studies, paraffin sections were deparaffinized by baking at 62℃ for 1 hour and then immersed twice in 100% xylene at room temperature for 10 minutes each, followed by incubating in 100% ethyl alcohol for 10 minutes, and rehydrated with decreasing concentrations (90%, 80%, 70% and 50%; vol/vol in water) of ethyl alcohol for 5 minutes each. Sections were rinsed in dH2O, and then subjected to microwave antigen retrieval in 0.01 M sodium citrate, pH 6.0 for 20 minutes on 70% power, with a 30-minute cooling period. Sections were rinsed 3 times in dH2O, twice in PBS and then staining was done according to manufacturer's protocol (VECTASTAIN Elite ABC Kit, Vector Laboratories). The kit contained blocking serum, secondary antibody and avidin–biotin–horseradish peroxidase complex. The slide was blocked with blocking horse serum for one hour at room temperature followed by incubation with primary antibody for HDAC1 (1:3000 dilution; Ed Seto Lab) or KAP1 (1:4000; Bethyl) at 4℃, overnight. The slide was rinsed three times in PBS for 10 minutes each and

incubated with secondary antibody for one hour at room temperature. Following three rinses in PBS for 10 minutes each, the slide was incubated with avidin–biotin–horseradish peroxidase complex for 30 minutes at room temperature. To detect the bound antibody, the slide was treated with peroxidase substrate kit (Vector Laboratories), wherein 3,3′-diaminobenzidine (459) was the chromogen, and color developed within 5 minutes of adding the DAB solution. After a final rinse in distilled water, sections were lightly counterstained in hematoxylin, dehydrated by immersing in increasing concentrations of ethyl alcohol (50%, 70%, 80%, 90%, and 100%; vol/vol in water) for 3 minutes each and finally immersed in 100% xylene twice for 2 minutes each. The slides were mounted in Clarion mounting medium (Santa Cruz Biotechnology). Immunostained slides were scanned using the Aperio Automatic Scanning System from Applied Imaging (San Jose, CA). The experiment was repeated three times.

Immunofluorescence and confocal microscopy

A549, H1650, MCF-7 and MDA-MB-231 cells were plated onto poly-D-lysine-coated eight-well glass chamber slides (5,000 cells per well) for immunostaining. The cells were fixed with 10% buffered-formalin and double immunofluorescence was performed as per the protocol published previously (48). Primary antibodies used were monoclonal c-MYC (cell signaling; 1:200) monoclonal HDAC1 (Santa Cruz; 1:200) and rabbit monoclonal ID1 (Biocheck; 1:3000) or polyclonal KAP1 (Bethyl; 1:4000). Secondary antibodies were goat anti-rabbit Alexa Fluor-594 and anti-mouse Alexa Fluor-488 (Molecular Probes)

respectively. DAPI (Vector labs) was used to stain the nuclei. Cells were visualized with a DM16000 inverted Leica TCS SP5 tandem scanning confocal microscope. Images were produced with three cooled photomultiplier detectors and analyzed with the LAS AF software version 1.6.0 build 1016 (Leica Microsystems, Germany).

In vivo orthotopic metastasis assay

For orthotopic transplantation of A549-luciferase cells, 250,000 cells/100 µl were injected into the right lung of SCID-beige mice. Mice are anesthetized by gas anesthesia (3% isoflurane). Thirty gauge needles were used in an open technique where cells were implanted into the right lung. For injection, mice were placed in the left lateral decubitus position with a 15 ml conical placed under the animal to expose the thoracic rib cage. A small posterolateral incision, 5-7 mm was made at the lateral dorsal axillary line, just below the inferior border of the scapula. The needle was inserted between the 4^{th} and 5^{th} intercostals spaces and placed 5 mm into the thoracic cavity before injection of cell slurry. Skin and subcutaneous tissue is closed by staple. Mice were imaged 6 hours after surgery to monitor implantation of cells with IVIS-100 (Caliper).

For bioluminescence imaging, mice were anesthetized and 30 mg/Kg of D-luciferin in PBS was administered by intraperitoneal (i.p.) injection. Ten minutes after injection, bioluminescence was imaged with a charge-coupled device camera (Caliper). Bioluminescence images were obtained with a 15 cm

field of view, binning (resolution) factor of 8, 1/f stop, open filter, and an imaging time of 30 s to 2 min. Bioluminescence from relative optical intensity was defined manually, and data were expressed as photon flux (photons·sec^{-1} ·cm^{-2}·steradian^{-1}) and were normalized to background photon flux over a mouse that was not given an injection of luciferin.

For nicotine studies, the mice were randomized 3-7 days after injection of tumor cells. Mice were separated into two groups Vehicle (*n*=6) and Nicotine (*n*=6). Mice received nicotine by i.p. injection at a dose of 1 mg/kg three times a week. Mice were imaged for increase in bioluminescence once/week for five weeks. At the completion of the experiment, vital organs were collected, and imaged *ex vivo* by placing dissected organs in luciferin solution.

Statistical analysis

Statistical analysis was performed using one-tailed Student's *t* test. Values were considered significant when the *P* value was <0.05.

Chapter 3: Regulation of Matrix Metalloproteinase Genes by E2F transcription factors: Rb-Raf-1 interaction as a novel target for metastatic disease

Abstract

The Rb-E2F transcriptional regulatory pathway plays a major role in cell cycle regulation, but its role in invasion and metastasis is less understood. We find that many genes involved in the invasion of cancer cells, such as matrix metalloproteinases, have potential E2F binding sites in their promoters. E2F binding sites were predicted on all 23 human *MMP* gene promoters, many of which harbored multiple E2F binding sites. Studies presented here show that *MMP* genes such as *MMP9, MMP14,* and *MMP15*, which are overexpressed in non-small cell lung cancer (NSCLC) have multiple E2F binding sites and are regulated by the Rb-E2F pathway. Chromatin immunoprecipitation assays showed the association of E2F1 with the *MMP9, MMP14*, and *MMP15* promoters and transient transfection experiments showed that these promoters are E2F responsive. Correspondingly, depletion of E2F family members by RNAi techniques reduced the expression of these genes with a corresponding reduction in collagen degradation activity. Further, activating Rb by inhibiting the interaction of Raf-1 with Rb using the Rb-Raf-1 disruptor RRD-251 was sufficient to inhibit *MMP* transcription. This led to reduced invasion and migration of cancer

cells *in vitro* and metastatic foci development in a tail vein lung metastasis model in mice. These results suggest that E2F transcription factors may play a role in promoting metastasis through regulation of *MMP* genes, and that targeting the Rb-Raf-1 interaction is a promising approach for the treatment of metastatic disease.

Introduction

The retinoblastoma tumor suppressor protein, Rb, together with the E2F transcription factors is the main regulator of the mammalian cell cycle (66). Rb physically interacts with E2Fs 1-3 via their transcriptional activation domain, repressing their transcriptional activity (460). In response to mitogenic signaling, Rb is inactivated in the G1 phase of the cell cycle in multiple waves of phosphorylation by cyclin-dependent kinases 2, 4 and 6, leading to its dissociation from E2Fs 1-3. This facilitates the expression of various genes that are necessary for DNA synthesis and cell cycle progression, including *cyclin E, dihydrofolate reductase*, *DNA polymerase* α etc. Not surprisingly, oncogenic mutations target the Rb-E2F pathway to promote cell proliferation (12). The *Rb* gene itself is mutated in a variety of cancers, while mutations in signaling molecules like *K-Ras, p16INK4* and *PTEN* that affect Rb function are prevalent in almost all cancers (12, 461, 462). This indicates a major role for the Rb-E2F pathway in cell cycle progression and oncogenesis. Further, E2Fs are known to be important for proper execution of development, differentiation, apoptosis, and

DNA damage repair programs (53, 449), establishing a larger role for E2Fs in the biology of normal mammalian cells and their transformation into cancer cells.

Our earlier studies had shown that the kinase Raf-1 physically interacts with Rb early in the cell cycle, facilitating Rb phosphorylation (36). Disruption of the interaction of Raf-1 with Rb using the small molecule disruptor RRD-251 prevented Rb phosphorylation, cell cycle progression, angiogenesis, and tumor growth in mouse models (39, 463, 464). It was found that RRD-251 could inhibit the expression of E2F-regulated proliferative promoters like *Cdc25A* and *TS*. Interestingly, recent studies from our lab have demonstrated that E2F1 could induce VEGF receptors, *FLT-1* and *KDR*, indicating a role for E2F1 in tumor angiogenesis as well (253). Given this background, attempts were made to assess whether E2Fs can also affect the expression of genes involved in cell invasion and cancer metastasis. Towards this purpose, we used Genomatix MatInspector software to analyze the promoters of matrix metalloproteinase genes, which remodel the extracellular matrix and facilitate cell invasion and metastasis (374). We find that all human *MMP* promoters have multiple E2F binding sites; data presented here show that three *MMP*s that are overexpressed in NSCLC, namely *MMP9, MMP14*, and *MMP15* are in fact E2F regulated. Supporting this contention, the Rb-Raf-1 disruptor, RRD-251, which prevents Rb phosphorylation and inhibits E2F1-mediated transcription, could inhibit the transcription of *MMP* genes. In addition, RRD-251 could prevent invasion *in vitro*, and decrease colonization of the lung in an *in vivo* tail vein metastasis model.

These results suggest that the Rb-E2F pathway contributes to the expression of *MMP* genes and that targeting this pathway might be a potential avenue to combat metastatic disease.

Results

MMP9, MMP14, and MMP15 promoters recruit E2F1 and Rb

Microarray studies had suggested that *MMP* genes may be E2F responsive (122, 258) and to explore this possibility, we examined the promoter region 2 Kb upstream of the transcription start site of 23 *MMP* genes using MatInspector (Genomatix) program. Putative E2F binding sites were observed on the promoters of all 23 MMP genes examined (Table 2. Table represents number of binding sites identified, location upstream of transcription start site (TSS), nucleotide sequence identified, and matrix score.) Since *MMP9, MMP14*, and *MMP15* are overexpressed in a variety of metastatic tumors including non-small cell lung cancer (NSCLC), these promoters were studied further. *MMP9, MMP14,* and *MMP15* promoters had three, five, and four E2F binding sites respectively upstream of the TSS within the 2 Kb regions. In addition, the *MMP14* promoter had two E2F binding sites downstream of TSS (Figure 8).

ChIP assays were conducted on asynchronously growing A549 cells to assess whether E2F1 and Rb associate with these promoters. The location of primers used is shown in Figure 1A as arrows. As shown in figure 8B, there was a significant amount of E2F1 bound to *MMP9, MMP14*, and *MMP15* promoters,

and at least two E2F binding sites recruited E2F1 on each promoter. As in the case of E2F-regulated proliferative promoters, Rb could also be detected on most *MMP* promoters. E2F1 was present on three positive control promoters, *DHFR, Cdc6*, and *Cdc25a*. There was no Rb or E2F1 present on the unrelated *c-Fos* promoter, which was the negative control. There was no DNA associated with an IP done with an irrelevant antibody, further establishing the specificity of the assay. This experiment suggests that the E2F sites present on these *MMP* promoters can recruit E2F1 and Rb.

Table 2. Matinspector Analysis of Putative E2F binding sites on MMP Promoters

Promoter	# of E2F binding sites	E2F Binding Sites	Nucleotide Sequence	Matrix Sim.
MMP1	3	1541 to -1525	tttctGCGTcaaga	0.716
		1538 to -1522	tcttgACGCagaaa	0.716
		1043 to -1027	aaaaGGCTggaaa	0.899
MMP2	4	1616 to -1600	tcacagctaGAAAa	0.752
		1077 to -1060	ttcctGCGCcaagccaa	0.866
		914 to -897	aattagcgtAAAAtg	0.867
		256 to -1793	agatcGCGAgaga	0.767
MMP3	3	1610 to -1594	tgatgggggGAAAa	0.752
		1489 to -1464	ccagagctcGAAAc	0.849
		233 to -217	ggtagaggaGAAA	0.777
MMP7	3	1979 to -1963	aagatgggcCAAAaacc	0.849
		1764 to -1748	ctgaggcacGAGAa	0.951
		1548 to -1532	aaattgggtGAAAa	0.779
MMP8	1	1734 to -1718	ttcctccacGAAAat	0.753
MMP9	3	1920 to -1904	acatagctgGAAAa	0.813
		1201 to -1185	gatatggggGAAAa	0.786
		518 to -502	tcagggaggGAAA	0.786
MMP10	3	1001 to -985	ttagtgagaGAAAa	0.769
		904 to -888	ttactgcgtAAAAag	0.866
		743 to -727	agaaaggggGAAA	0.762
MMP11	2	1499 to -1483	ggtaaccccGAAAatca	0.794
		45 to -29	gggtggGGCGgaagcta	0.847
MMP12	2	1434 to 1418	caactgggtGAAAaatt	0.753
		1285 to -1269	tgttttccaGAAAattg	0.763
MMP13	2	1753 to -1737	aagaagctgGAAAacaa	0.768
		-993 to -997	agtgcccaaGAAAacta	0.765
MMP14	7	1667 to -1651	tgtgaacgtGAAAaggg	0.834
		1645 to -1629	caaaactggGAAAactg	0.782
		1548 to -1532	ccttggctaGAAAatct	0.762
		-941 to -925	cagaagcagGAAAagtg	0.802
		-878 to -862	gctgaccgtGAGAaagg	0.750
		37 to 53	aaaaaCCGGaaaagagg	0.822
		234 to 250	ttggGGCGggagacatg	0.817
MMP15	9	1625 to 1609	ccaaagcccGAAAggac	0.849
		1434 to -1418	caacagagaGAAAatca	0.754
		1036 to -1020	acctagcccCAAActtt	0.849
		559 to -543	ccctgGCGCggcggact	0.850
		317 to 333	ccaatGCGCggacccgg	0.854
		421 to 437	agcggGCGCccgacgtc	0.851
		607 to 623	gctggGCGCgcagggag	0.884
		754 to 770	agccggcggGAAAgggg	0.927
		878 to 894	tcctgGCCCggcaggcg	0.859

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Continuation of Table 2

MMP16	10	1157 to -1143	caagGGCGggagg	0.847
		1135 to -1119	gaaaGGCGagtaa	0.859
		546 to -530	ggtgGGCGggaag	0.833
		446 to -430	ccagcccgcGACAa	0.791
		282 to -266	cgcgcGCGCgcac	0.865
		270 to -254	ccttgGCGCgcgcg	0.877
		269 to -253	cgcgcGCGCgcca	0.871
		160 to -144	cagaGGCGagaga	0.837
		23 to 39	acgtgGCGCcacgt	0.877
		230 to 246	agagagggaGAAA	0.772
MMP17	7	1638 to -1622	agcgtgtgcCAAAgtca	0.849
		398 to -382	ggacCGCGggagaggga	0.794
		310 to -294	ccacgGCGCcgaggccc	0.916
		307 to -291	cctcgGCGCcgtggggg	0.903
		227 to -211	gatggGCGCggaggcgg	0.882
		216 to -200	gggacGCGCgggatggg	0.857
		91 to -75	aggcgGCGCgggccctt	0.854
MMP19	2	1560 to -1544	tgagatCGCGccactgt	0.841
		1559 to -1543	cagtgGCGCgatctcag	0.871
MMP20	4	1937 to -1921	tgatggcccCAAAgcaa	0.849
		1900 to -1884	taaaggagaGAAAaagg	0.799
		1192 to -1176	acacagagcCAAAccat	0.849
		767 to -751	atatggtggGAAAagca	0.769
MMP21	5	2012 to -1996	accctgcaaGAAAaggg	0.761
		952 to -1936	agtgacccaGAAAactg	0.770
		350 to -334	gtttgCCGCtaaatgag	0.900
		347 to -331	atttagcggCAAActtg	0.928
		337 to -321	atacTGCGggcaagttt	0.817
MMP23B	14	1852 to -1836	agctgGCGCggcgggcc	0.861
		1653 to -1637	ggacgGCGCggctcaca	0.868
		1400 to -1384	ttcagGCGCgggcacgg	0.863
		1289 to -1273	tcagaGCGCgagtcccc	0.851
		1112 to -1096	ggaaaGCGCgagaggct	0.866
		1071 to -1055	ggtcaGCGCcaagcagc	0.896
		1068 to -1052	gcttgGCGCtgaccccg	0.734
		1021 to -1005	cgccgGCGCggaaaacg	0.950
		1018 to -1002	ccggcgcggAAAAcggg	0.885
		978 to -962	gtctgGCTCggaatctc	0.900
		892 to -876	tccagGCGCgagcggct	0.864
		708 to -692	gggtagCGCGtgaggtg	0.920
		707 to -691	acctcaCGCGctaccct	0.912
		119 to -103	acggGGCGggagagggg	0.787
MMP24	3	1257 to -1241	ctttaGCGTcagaccta	0.786
		1254 to -1238	gtctgacgcTAAAgcct	0.843
		1194 to -1178	atgatgtgtGAAAatag	0.756

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Continuation of Table 2

MMP25	15	1429 to -1413	ggttcACGCcattctcc	0.716
		1426 to -1410	gaatGGCGtgaaccagg	0.887
		1389 to -1373	cagtgGCGCgatctcgg	0.871
		1388 to -1372	cgagatCGCGccactgc	0.841
		1086 to -1070	agcggGCGCgggatcgc	0.913
		878 to -862	tctcgGCGCgggaggag	0.902
		772 to -756	ccactGCGCggaagcca	0.881
		652 to -636	gggctGCGCgcggggct	0.878
		651 to -635	gccccGCGCgcagccca	0.882
		566 to -550	ggaggGCGGgccgggac	0.859
		454 to -438	gaggGGCGgatacagg	0.887
		176 to -160	taaggGCGCggcaaggg	0.915
		167 to -151	gatatGCGGtaagggcg	0.766
		111 to -95	tccggGCGCgagcggag	0.870
		28 to -12	agaggGCGCgggagcgg	0.910
MMP26	8	1199 to -1183	aacaagagcGAAActcc	0.849
		721 to -705	cgagatCGCGccactgc	0.841
		720 to -704	cagtgGCGCgatctcgg	0.871
		683 to -667	gaatGGCGtgaacccgg	0.877
		680 to -664	ggttcACGCcattctcc	0.716
		641 to -623	ggtggGCGCctgtagtc	0.869
		302 to -286	aataggggGAAAacaa	0.801
		330 to -214	ctgtgGCCCgggatgct	0.851
MMP27	2	1455 to 1439	tgaaggagcGAAAggtt	0.849
		507 to -491	aagaagggcCAAAggca	0.849
MMP28	2	1162 to -1146	ctctgtCGCCcaagctg	0.867
		1161 to -1145	agcttgGGCGacagagc	0.870

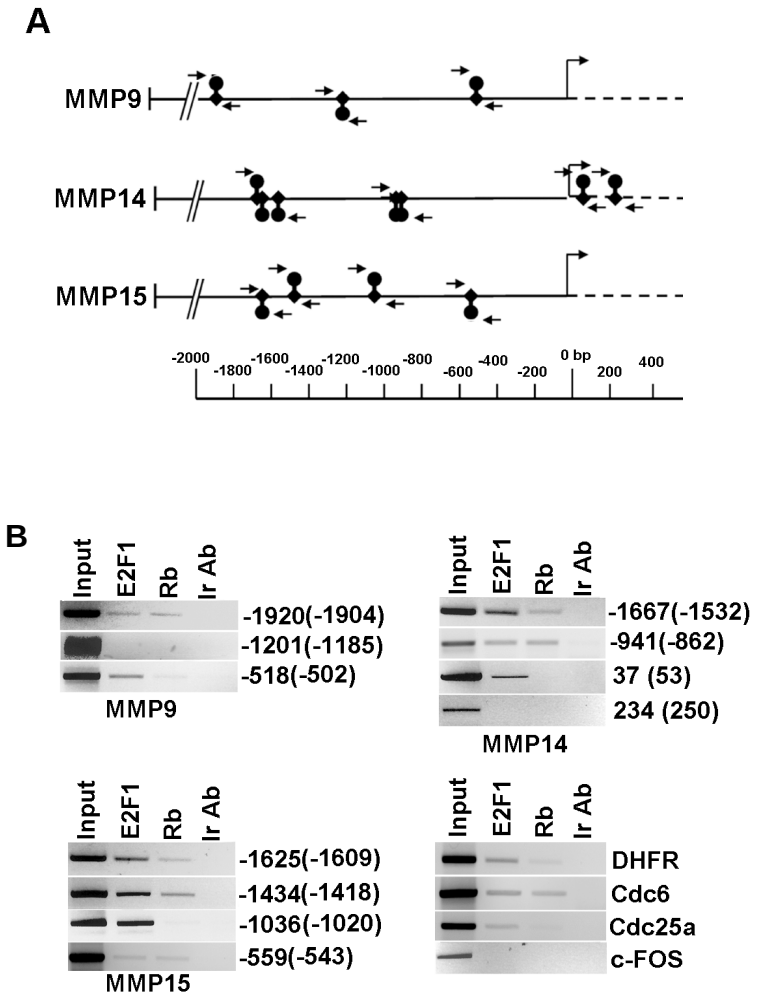

Figure 8. MMP promoters recruit E2F1 and Rb. (A) Schematic representation of *MMP9, MMP14,* and *MMP15* promoters showing potential E2F binding sites as diamond/circle symbols. The arrows represent the position of primers spanning E2F binding sites tested in ChIP assays. (B) ChIP assays conducted on asynchronously growing A549 cells using the indicated antibodies. Sonicated genomic DNA is used for input. The numbers to the right indicate the position in the promoter, in respect to TSS, where a putative E2F binding site was identified. *C-Fos* was used as a negative control, whereas *DHFR, Cdc6,* and *Cdc25a* are used as positive controls. Irrelevant antibody was used (Ir Ab) as a negative control for IP.

MMP9, MMP14, and MMP15 promoters are responsive to E2F1, Rb, and Raf-1

Experiments were done to assess whether these E2F binding sites were functional. Towards this purpose, A549 cells were transiently transfected with luciferase reporter constructs driven by *MMP9, MMP14,* and *MMP15* promoters. It was found that co-transfection of E2F1 led to a significant induction of all the three promoters (Figure 9A); further, co-transfection of the large pocket region of Rb (Rb-LP) or the full length Rb (Rb-FL) could repress the E2F1-mediated induction. Consistent with previous studies on proliferative E2F-target genes (36, 38), over-expression of Raf-1 could relieve the repression mediated by Rb. Taken together, these results suggest that the Rb-E2F pathway might regulate *MMP9, MMP14*, and *MMP15* expression.

Gelatin zymography was used to determine whether overexpression of E2F1 enhances MMP9 gelatinase activity. Consistent with the transfection data, MMP9 activity was increased 1.78 fold in A549 cells and 2.54 fold in H1650s overexpressing E2F1 (Figure 9B-C). This suggests that the endogenous MMP9 promoter is responsive to E2F1 overexpression, leading to MMP9 secretion in cell lines.

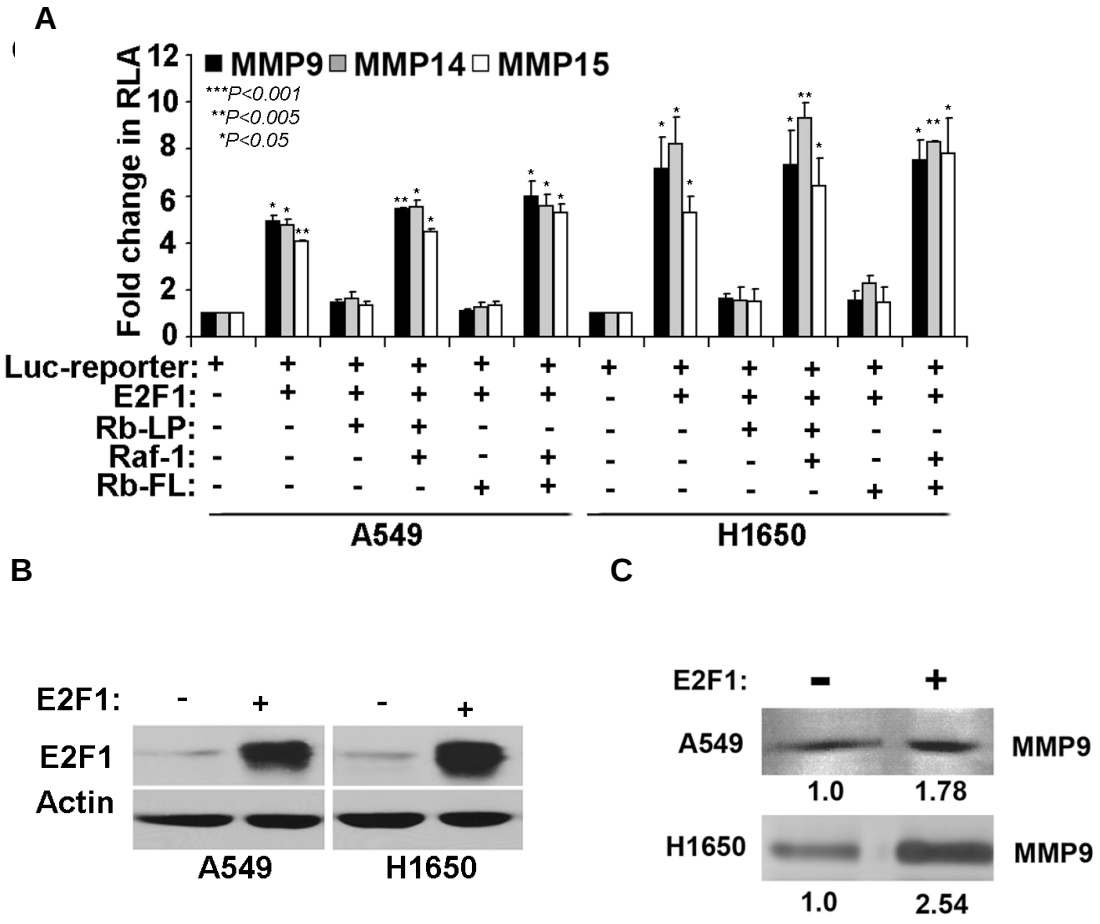

Figure 9. E2F1 induces MMP levels (A) Transient transfection experiments in A549 and H1650 cells showed that E2F1 could significantly (**, *P*<0.005; * *P*<0.05) induce MMP9, 14 and 15 promoters, and this was repressed by Rb large pocket or full length Rb; co-transfection of Raf-1 could reverse Rb-mediated repression. Control lanes include luciferase reporter and empty vector. (B). Western blot shows E2F1 overexpression in E2F1 transfected cells compared to empty vector in A549 and H1650 cells. (C) A549 and H1650 cells transiently transfected with E2F1 have increased levels of MMP9 (A549 *P*=0.019*; H1650 *P*<0.001**) gelatinase activity as seen by Coomassie stained zymography (inverted image).

MMP9, MMP14, and MMP15 are induced by E2F1-5

Previous studies had demonstrated that proliferative promoters are induced mainly by the transcriptionally active family members, E2F1-3. To determine if MMP genes are also regulated exclusively by E2F1-3, ChIP assays were performed on asynchronous A549 cells. While the proliferative dihydrofolate reductase (*DHFR*) promoter recruited only E2Fs 1 and 3, E2Fs1-5 were recruited to the promoters of both *MMP9* and *MMP15* (Figure 10A); E2Fs1-4 were recruited to the *MMP14* promoter. Consistent with the ChIP assay data, transient transfection experiments on A549 cells showed that MMP promoters are significantly induced by E2F1-5 whereas *DHFR* is significantly induced by E2F1-3 (Figure 10B-E). This data suggests that *MMPs* may be a new class of E2F target genes, which can positively respond to E2Fs 1-5.

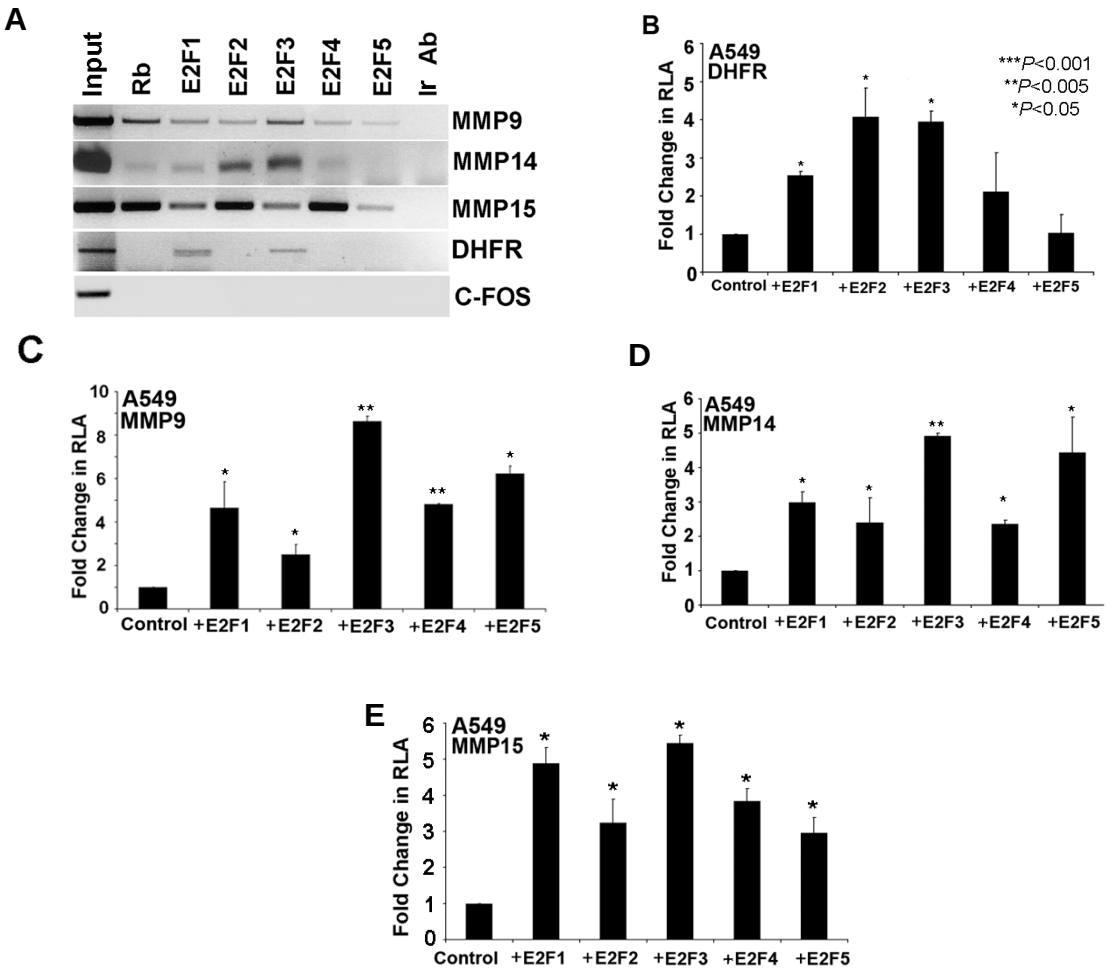

Figure 10. *MMP9, MMP14,* and *MMP15*-luc are responsive to E2F1-5. (A) ChIP assays were carried out on asynchronously growing A549 cells. One binding site in each promoter was analyzed: -1920 to -1904 in *MMP9*; -1667 to -1532 in *MMP14*; and -1625 to -1609 in *MMP15*. (B-E) Transient transfection experiments in A549 cells showed that *DHFR* is significantly induced by E2F1-3 (***, *P*<0.001; **, *P*<0.005; * *P*<0.05) though no significant difference with E2F4 (*P*=0.18) or E2F5 (*P*=0.47), whereas E2F1-5 could significantly induce all *MMP* promoters.

E2F1, E2F3, and E2F5 are required for MMP gene expression

Given that E2F1-5 could induce *MMP9, MMP14* and *MMP15* promoters in transient transfections, attempts were made to assess whether E2Fs regulate the expression of their endogenous promoters in NSCLC cells. Towards this purpose, A549 and H1650 cells were transfected with 100 pMol of siRNAs to E2F1-5, or a non-targeting control siRNA. Transfection with siRNA targeting E2F1, E2F3, or E2F5 significantly reduced the expression of *MMP9* and *MMP14* mRNA as seen by quantitative RT-PCR, whereas E2F2 and E2F4 had no effect (Figure 11A). Since we could not detect E2F5 on the *MMP14* promoter with our chip assay of site -1657, it is possible that the regulation by E2F5 is through a different E2F binding site in the promoter. Surprisingly, *MMP15* mRNA levels were not changed when E2Fs were depleted in either A549 cells or H1650 cells (Figure 11B). *DHFR* mRNA levels were significantly reduced when E2F1 or E2F3 were depleted, correlating with E2F1 and E2F3 preferentially binding to *DHFR* promoter in ChIP assays (Figure 10). This suggests that E2F1, E2F3, and E2F5 are involved in transcriptional induction of *MMP9* and *MMP14* genes in NSCLC cells, but they may play a lesser role in regulating the endogenous *MMP15* promoter.

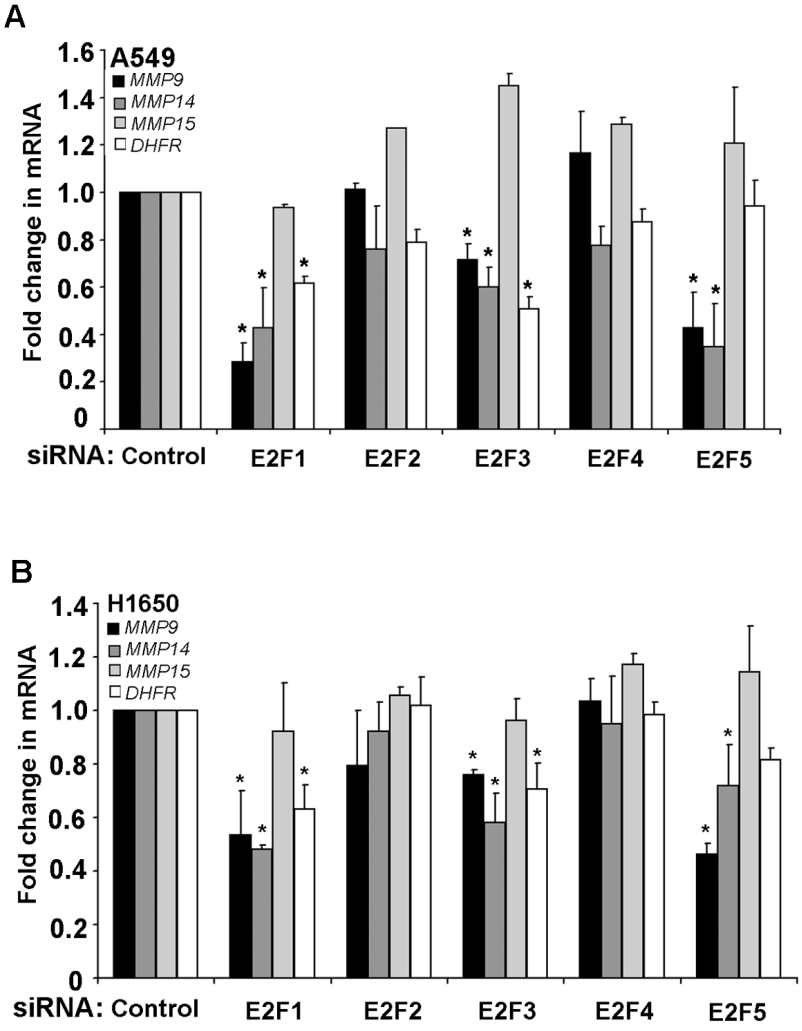

Figure 11. E2F depletion inhibits *MMP* gene transcription **(**A-B) Transiently transfecting 100 pmol of E2F1, E2F3, and E2F5 siRNA reduced the expression of *MMP9* and *MMP14* mRNA in A549 and H1650, and there was no significant difference with E2F2 or E2F4 siRNA. *DHFR* mRNA levels were significantly reduced by E2F1 or E2F3 siRNA *(P<0.05). MMP15* mRNA levels were not affected significantly in A549 or H1650 cells.

Depletion of E2Fs inhibits collagen degradation

Recent studies suggest that in certain tumor milieus, the ability of fibroblasts to actively degrade extracellular collagen is a climacteric step that allows cancer cells to escape the primary tumor site (465). Since we found that NSCLC cells depleted of E2F1, E2F3, or E2F5 had less MMP9 and MMP14, we next examined whether CCL-210 lung fibroblasts had an impaired ability to degrade type I collagen, when depleted of E2F1-5. To this end, CCL-210 cells were transfected with siRNA to E2F1-5 or a non-targeting control RNA and plated on type I collagen. After four days, CCL-210 cells with depleted E2F1-5 had less collagen degradation as indicated by Coomassie staining of the residual collagen, though the depletion of E2F1 or E2F3 had the most pronounced effect (Figure 12). To determine whether siRNA had any effect of proliferation of CCL-210 cells, cells were counted after being trypsinized off the collagen. There was no significant difference in cell number with any siRNA suggesting that CCL-210 cells grown to confluency are not dependent of proliferation for collagen degradation. Taken together, these results suggest that depletion of E2Fs in lung cells significantly diminishes MMP gene transcription, and hinders resultant biological processes such as collagen degradation.

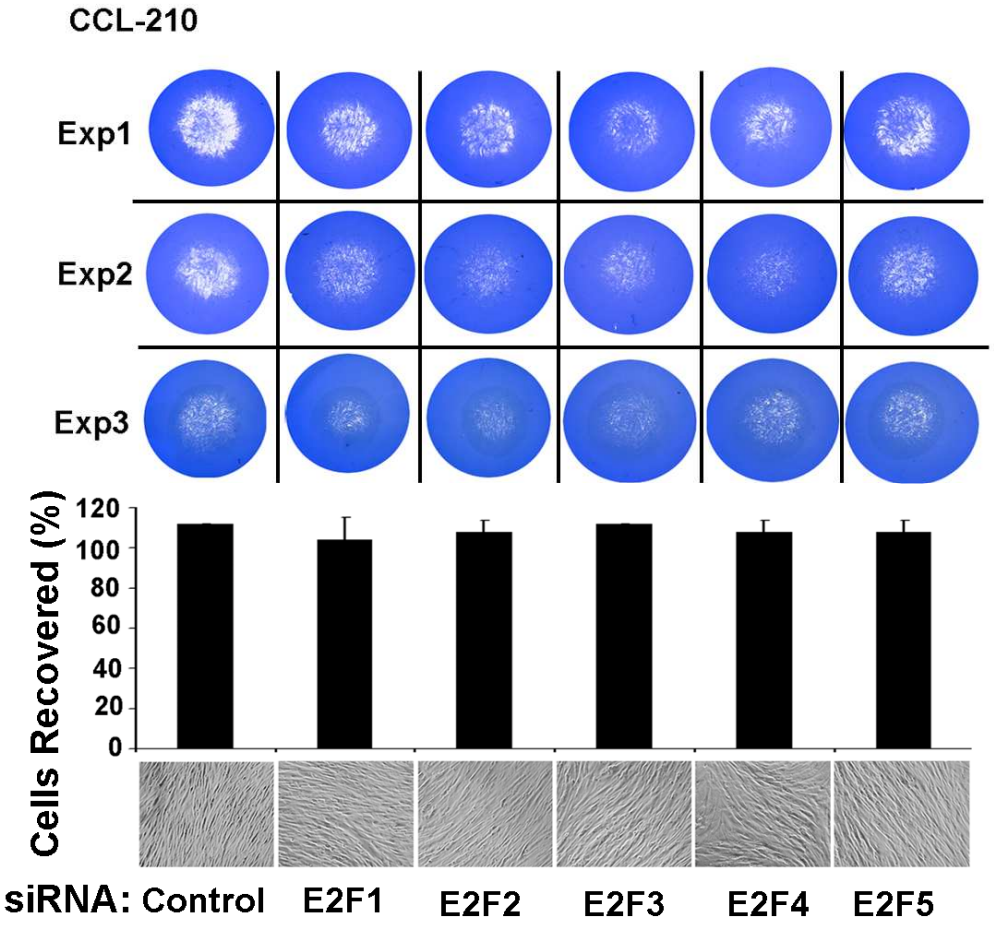

Figure 12. E2F depletion inhibits collagen degradation. CCL-210 lung fibroblast cells depleted of E2F1-5 by siRNA have less collagen degradation compared to Control siRNA. The results of three independent experiments are shown. The total cell number on top of collagen was trypsinized, and counted at the termination of the experiment. Depletion of E2F1-5 had no effect on growth of CCL-210 cells when plated as a confluent monolayer. Images are one representative field of CCL-210 cells atop collagen, taken at 100x total magnification with phase contrast microscopy.

E2F1 overexpression can rescue MMP-luciferase activity when E2F3 is depleted

To determine whether an alternate E2F family member could rescue MMP activity when E2F1 is depleted, A549 cells were transiently transfected with siRNA targeting E2F1, then transfected with *MMP*-luc constructs, with or without E2F3 expression vector. E2F1 depletion lead to reduced *MMP9* and *MMP14-luc* activity, and overexpression of E2F3 could rescue *MMP9* and *MMP14-luc*iferase activity. Similar results were obtained when E2F3 was depleted, followed by overexpression of E2F1. *MMP15-luc*iferase activity was not affected by E2F1 or E2F3 depletion (Figure 13). This suggests that E2Fs might have functional redundancy in regulating MMP promoters.

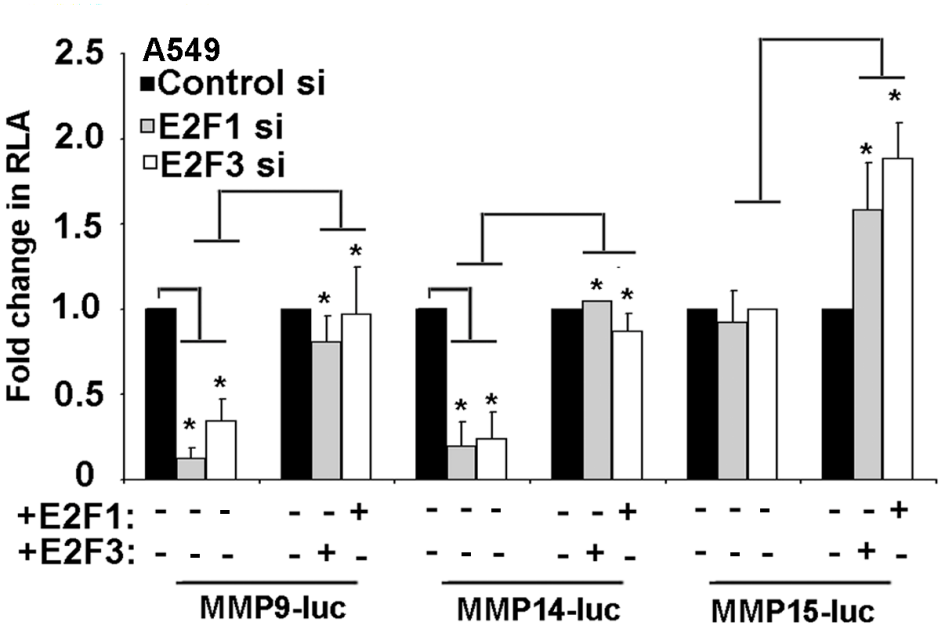

Figure 13. E2Fs can rescue the effect of depleting a different family member. *MMP9* and *MMP14* luciferase activity is reduced by transiently transfecting E2F1 or E2F3 siRNA, followed by transfection of *MMP*-luc reporters. Co-transfection with the alternate family member (E2F1 overexpression in E2F3 siRNA cells, E2F3 overexpression in E2F1 siRNA cells) could rescue *MMP*-luciferase activity. Basal levels of *MMP15* were not affected, though both E2F1 and E2F3 overexpression could induce *MMP15-luc*iferase activity *(*P<0.05).*

Depletion of E2F1 or E2F3 reduces cell migration independent of proliferation

There is evidence that cell migration is accomplished in part through cleavage of adherens junctions by MMPs (311). To determine whether E2F-mediated modulation of *MMP* genes might affect migration of A549 cells, wound-healing assays were conducted *in vitro.* To ensure that changes in migration were independent of cell proliferation, asynchronous cells were pretreated with 10 μg/mL Mitomycin C, which arrests the cell cycle (Figure 14A). There was a comparable amount of migration in Mitomycin C treated and untreated cells, indicating that the observed migration was a direct result of motility into the empty space and independent of proliferation (Figure 14B-C). Next, to determine whether E2F depletion affected migration, cells were transfected with siRNA to E2F1, E2F3, a combination thereof, or a non-targeting control siRNA. Serum induced migration of cells transfected with the control, non-targeting siRNA into the wound; but migration was significantly reduced in cells transfected with E2F1 and E2F3 siRNA (Figure 14D-E). This suggests that E2F1 and E2F3 contribute to the migration of cells. This agrees with studies showing that E2F1(-/-) mice have abnormal epidermal repair upon injury, and impaired cutaneous wound healing (466).

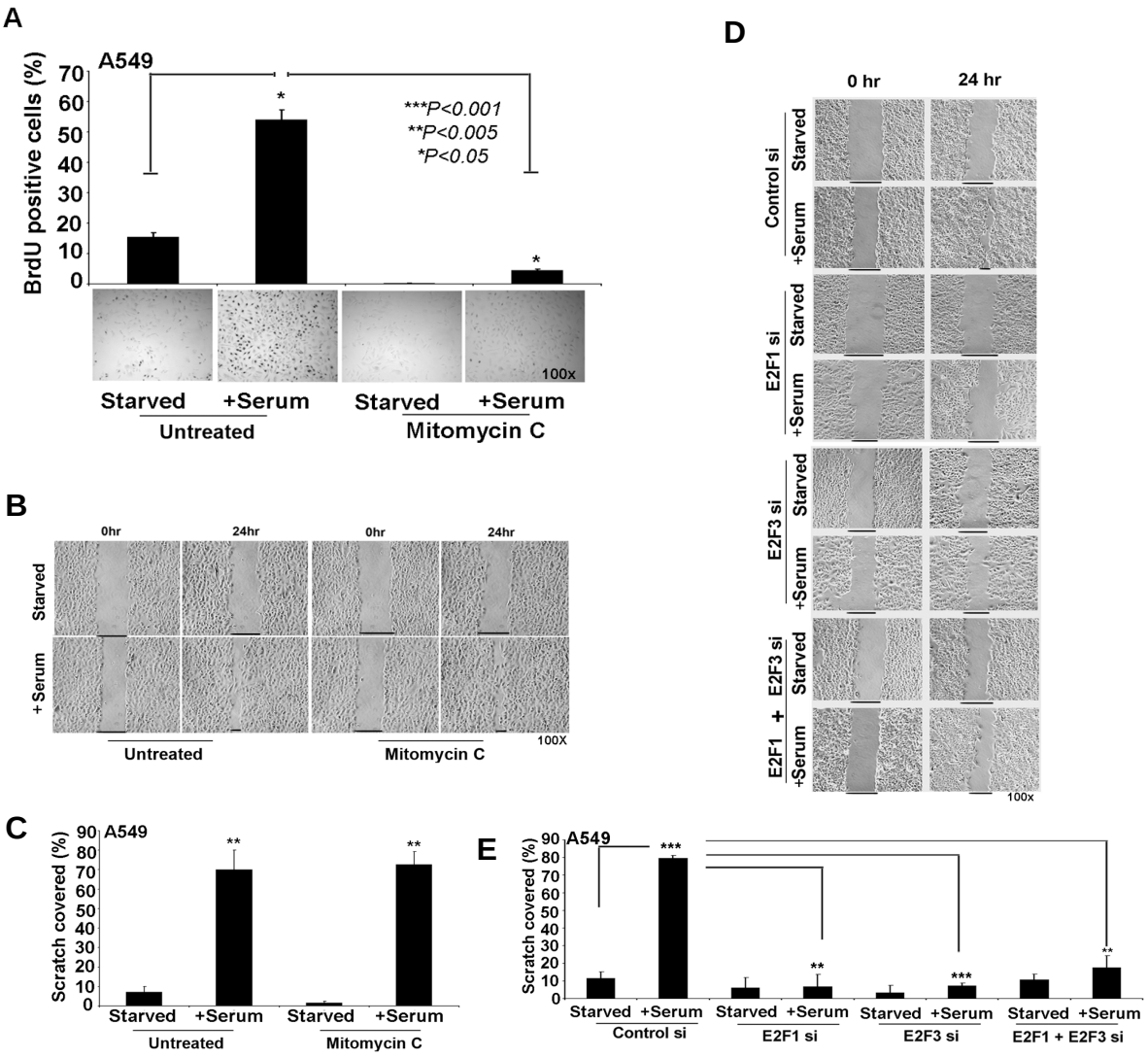

Figure 14. Depletion of E2F1 or E2F3 inhibits migration independent of proliferation (A) A549 cells that have been treated with 10 µg/mL Mitomycin C have significantly reduced BrdU incorporation compared to untreated A549 cells *(*P<0.05).* (B) Serum stimulated A549 cells treated with 10 µg/mL Mitomycin C have similar migratory capacity as untreated A549 cells *(**P<0.005)* 100x total magnification. (D) Depletion of E2F1, E2F3, or E2F1 and E2F3 combined significantly hinders the ability to A549 cells to migrate in response to serum *(**P<0.005; ***P<0.001).*

Depletion of E2F1 or E2F3 reduces cell invasion

We next examined whether invasion was affected by depletion of E2F1 or E2F3 using a Boyden Chamber assay. A549 cells were transfected with siRNA targeting E2F1, E2F3 or a combination thereof. As shown in Figure 15A, cells which were depleted of E2F1 or E2F3 had completely lost the ability to invade through collagen and matrigel coated transwell filters**,** while cells transfected with a non-targeting control siRNA showed 1.8 ± 0.4 fold invasion in serum stimulated cells. This suggests that E2F1 or E2F3 are required for degradation of the ECM components, through the modulation of genes involved in their degradation.

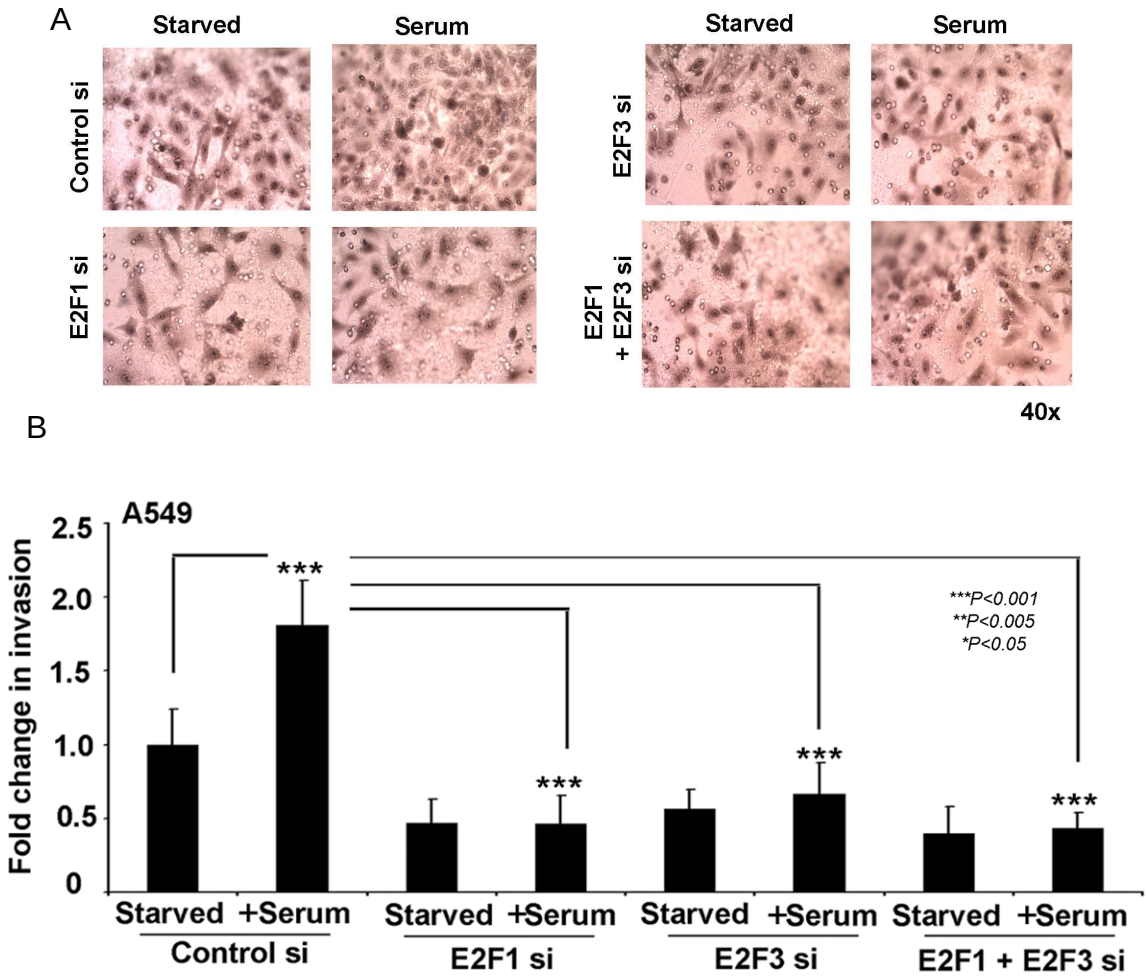

Figure 15. Depletion of E2F1 or E2F3 inhibits invasion (A) Depletion of E2Fs significantly reduced invasive properties, as seen in a Boyden Chamber assay *(***P<0.001).* Images of films removed from transwell filters. Dark spots are cells that have invaded through matrigel and collagen coated films to the other side of the filter. Images are taken at 400X total magnification. (B) Quantification of five images in three independent experiments. Data is plotted as fold change compared to serum-starved controls.

Rb-Raf-1 disruptor, RRD-251, inhibits invasion and migration in vitro

Previous work in our lab has demonstrated that the Raf-1 kinase interacts with Rb, and phosphorylates Rb early in the cell cycle (36). A small molecule Rb-Raf-1 disruptor, RRD-251, inhibited Rb phosphorylation, thereby keeping Rb associated with E2F1, preventing cell proliferation and tumor growth (38, 39). We hypothesized that RRD-251 would likely inhibit the migration of cancer cells as well, given that depletion of E2Fs inhibited migration. Wound healing assays conducted on MDA-MB-231, MDA-MB-435, A549, and H1650 cells showed that treatment with RRD-251 significantly reduced the migration of cells (Figure 16A). The ability of RRD-251 to inhibit invasion of cancer cells was also examined. Given that the depletion of E2F1, E2F3, or the combination of the two significantly inhibited invasion, we wanted to use RRD-251 to inhibit invasion *in vitro.* A549-luc-C8 cells were rendered quiescent by serum starvation for 24 hours, and then stimulated with either serum alone, or serum and RRD-251 for 18 hours and invasion was measured by a Boyden Chamber assay. It was found that RRD-251 could significantly abrogate the invasive capacity of A549-luc and MDA-MB-231 cells (Fig. 16B-C).

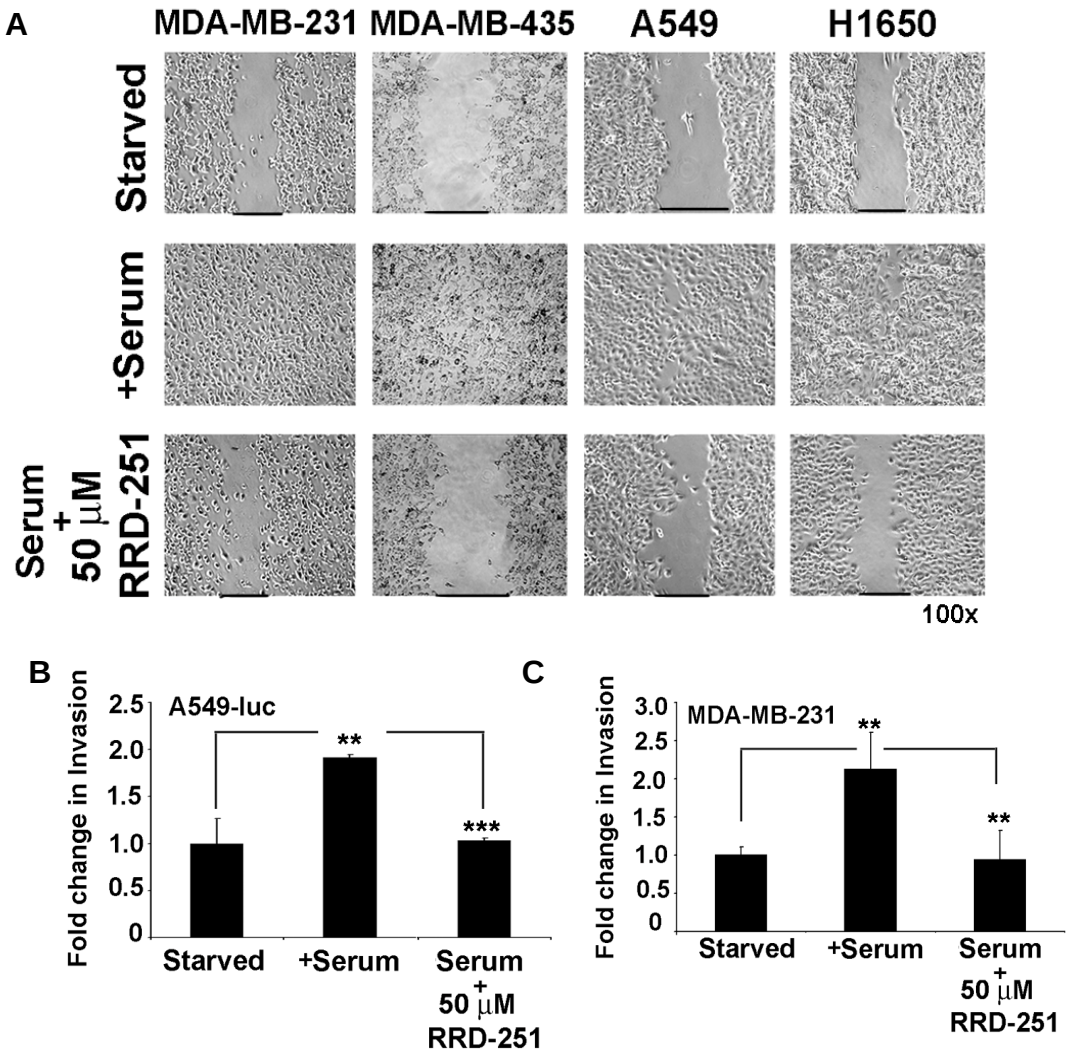

Figure 16. RRD-251 inhibits migration and invasion *in vitro* (A) MDA-MB-231, MDA-MB-435, A549, and H1650 cells treated with RRD-251 show reduced migration compared to serum. (B-C) A549-luc-C8 and MDA-MB-231 cells have significantly more invasion when stimulated with serum (**P<0.005). This effect is significantly abrogated in both cell lines when treated with RRD-251 (A549-luc, ****P<0.001;* MDA-MB-231, ***P<0.005*).

Rb-Raf-1 disruptor, RRD-251, inhibits MMP transcription and collagen degradation

Since RRD-251 was effective at inhibiting migration and invasion in a panel of invasive cancer cell lines, we next tested the ability of RRD-251 to inhibit collagen degradation. Similarly, collagen degradation was also greatly inhibited after CCL-210 cells were treated with RRD-251 (Figure 17A). Given that invasion, migration, and collagen degradation were all inhibited by RRD-251, we hypothesized that the mechanism was likely due to a down-regulation of key *MMP* gene transcription. By blocking the Rb-Raf-1 interaction, Rb remains hypophosphorylated and active, thereby inhibiting E2F-mediated transcription of target genes. To determine if RRD-251 could prevent E2F-mediated transcription of MMP genes, quiescent A549 cells were serum stimulated in the presence or absence of 20 μM RRD-251. *MMP* mRNA levels decreased significantly after treatment with RRD-251 (Figure 17B), comparable to the reduction in expression when E2Fs were depleted. Collectively, these results suggest that RRD-251 inhibits the invasion and migration of cancer cells, and this correlates with the repression of *MMP* genes. Taken with previous studies showing the efficacy of RRD-251 at inhibiting human tumor xenograft growth, cell proliferation, and angiogenesis (39), the Rb-Raf-1 interaction seems to play a crucial role in many aspects of cancer development and progression.

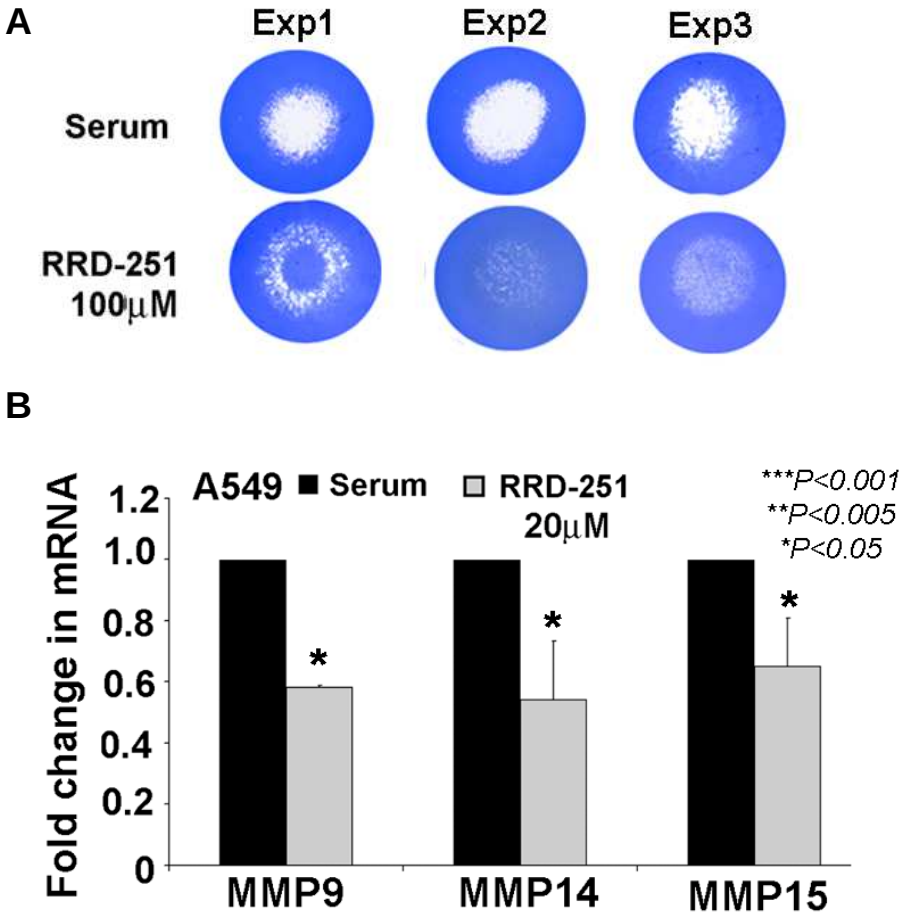

Figure 17. RRD-251 inhibits transcription of *MMP*s and collagen degradation (B) CCL-210 cells treated with RRD-251 show reduced degradation of collagen. Images show three independent experiments. (C) A549 cells treated with RRD-251 have significantly reduced *MMP9, MMP14,* and *MMP15* mRNA *(*P<0.05).*

RRD-251 inhibits metastatic lung colonization in vivo

Given that RRD-251 could modulate MMP levels and inhibit invasion and migration *in vitro*, we next investigated if RRD-251 could inhibit metastasis *in vivo*. We injected A549-luc-C8 cells ($5x10^6$) into the lateral tail vein of 5-week-old female, SCID-beige mice. Mice were then randomized into either the DMSO vehicle group, or the RRD-251 group, which received i.p. injection of 50 mg/kg everyday for four weeks. Colonization of lungs was monitored using the Caliper-IVIS 200 system after administration of luciferin. Mice treated with RRD-251 had significantly less metastasis to the lung and surrounding tissues (Figure 18A-B). Photon Flux in vehicle treated mice was 3.9 ± 0.6 fold higher than mice treated with RRD-251. To confirm these observations seen *in vivo*, lung bioluminescence was examined *ex vivo*. Mice treated with RRD-251 had 80% less lung bioluminescence (Fig 18C-D), indicating less metastasis. H&E staining indicates that few tumors were able to seed in the lungs of mice treated with RRD-251 (Fig. 18E-F). It is difficult to differentiate between anti-proliferative effects and anti-metastastic effects, however, as proliferation also contributes to the growth of metastatic lesions. Ideally, drugs that can affect invasion and proliferation would have the highest benefit as anti-tumor agents. RRD-251, by its ability to inhibit these hallmarks of cancer and others, appears to have potent therapeutic efficacy *in vivo*.

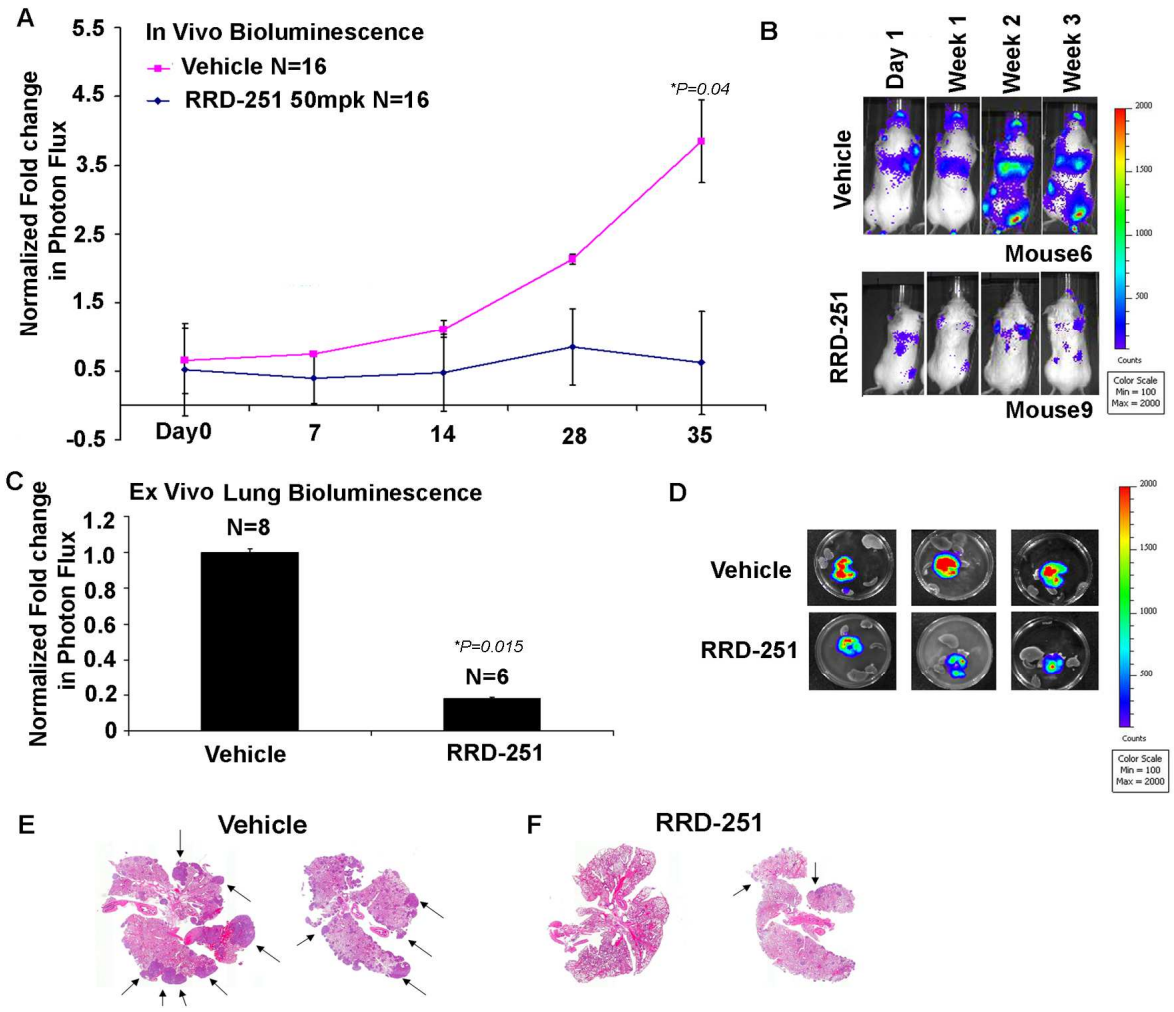

Figure 18. RRD-251 inhibits lung metastasis *in vivo* (A) A549-luc-C8 cells were injected into the lateral tail vein of SCID-beige mice, and animals were imaged once/week for 5 weeks. Daily administration of 50mg/kg RRD-251 significantly reduced lung colonization *(*P<0.05).* Representative images are shown in (B). (C and D) At the completion of the experiment, lungs were analyzed *ex vivo* and extent of colonization quantified. Mice treated with RRD-251 had significantly less tumor burden *(*P=0.015)*. (E and F) H & E staining confirms the presence of numerous distinct metastatic colony formation in the lungs of vehicle treated mice, shown by arrows, but few in mice treated with RRD-251.

Discussion

It is well established that cyclin-dependent kinases phosphorylate and inactivate Rb in the G1 phase of the cell cycle, releasing E2F transcription factors from Rb (467). It was initially believed that the predominant function of E2Fs was to activate genes required for the progression of the cell cycle through S-phase (8, 468, 469). Later studies showed that E2F transcription factors could regulate a diverse number of biological processes including cell differentiation, development, apoptosis, DNA damage repair, and more recently, angiogenesis (122, 175, 177, 470, 471). As mentioned earlier, our lab had shown that the signaling kinase Raf-1 directly interacts with Rb early in the cell cycle; further, Raf-1 could phosphorylate Rb (36). This phosphorylation of Rb by Raf-1 was necessary for the subsequent complete inactivation of Rb by cyclin-dependent kinases. Disrupting the Rb-Raf-1 interaction using RRD-251 could inhibit cell proliferation, adherence independent growth, angiogenesis and prevent the growth of lung cancer and melanomas in xenograft models (463). Studies presented here show that disrupting the Rb-Raf-1 interaction could be a fruitful way of combating metastatic colonization by cancer cells at distant organ sites; this can likely be attributed to downregulation of *MMP* transcription in addition to inhibition of proliferation. Therefore, inhibiting the Rb-Raf-1 interaction might be a valid target for metastasis of primary tumors of any size, since there is no direct correlation between the size and metastatic potential.

A considerable amount of research has been dedicated to identifying novel E2F regulated genes by gene profiling arrays and chromatin immunoprecipitation arrays (ChIP on chip) (127, 472-474). In these arrays, various proteins and enzymes involved in the metastatic spread of tumor cells were initial hits, though validation studies have been lacking. Arguably the most crucial process for cancer cell invasion is the physical degradation of the ECM (440) but a role for E2F transcription factors in this process had not been identified. It is intriguing that, at least in lung cancer cell lines, E2Fs function as transcriptional activators of *MMP9, MMP14*, and *MMP15*. Many MMP gene promoters have multiple GC boxes, which can bind to Sp1 and Sp3, including *MMP9, MMP14, MMP15,* and others (441). It is well established that Sp1 proteins can work coordinately with E2F transcription factors to regulate gene expression (475).

Cells utilize enzymes including serine-, thiol-, proteinases, heparanases, and metalloproteinases to free them from the primary tumor locale. Though the activity of matrix metalloproteinases and other metzincin family proteins are important for metastasis, the most prognostically valuable are the Matrix Metalloproteinase family (MMPs) (476). A relevant MMP signature is MMP2, MMP9, and MMP14, which have been shown to correlate with advanced stage breast cancer morbidity and late relapse in breast cancer patients (477, 478). MMP14 and MMP2 have also been detected at high levels in NSCLC patient

samples, whereas *MMP14* and *MMP15* RNA levels have been shown to correlate with human glioma grade (479, 480). Therefore, it is a possibility that E2Fs might indirectly regulate tumor metastasis as a consequence of transcriptionally activating MMPs.

In addition to the crucial role for MMPs in degrading the extracellular matrix during invasion, they also play a role in angiogenesis. Pro-angiogenic factors like VEGF and bFGF are normally localized to the matrix, and cannot engage receptors until freed through MMP9 cleavage (434, 481, 482). Since we have previously shown that VEGF receptors, *FLT-1* and *KDR*, are also E2F regulated genes, it is likely that the role E2F has in angiogenesis is multi-faceted.

These observations raise the possibility that mutations that initiate the oncogenic process by activating the E2F transcriptional regulatory pathway might also contribute to subsequent steps of tumor progression and metastasis. There is evidence that the Rb-E2F pathway might affect EMT as well, and this requires additional investigation (483). Taken together, these studies link the Rb-E2F cell cycle regulatory pathway to advanced stages of cancer development and metastasis. The finding that disrupting the Rb-Raf-1 interaction could prevent cell proliferation, angiogenesis, tumor growth and now metastatic colonization of organs suggest that targeting the Rb-R2F pathway might be a fruitful avenue to combat metastatic disease.

Chapter 4: Differential regulation of MMP2 by E2F1 involves a p53-KAP1-HDAC1-dependent mechanism

Abstract

Recent studies have shown a role for the Rb-E2F pathway in regulation of certain matrix metalloproteinase genes involved in tumor invasion and metastasis. Another MMP implicated in migration and invasion, *MMP2*, has multiple E2F binding sites in its gene promoter and is differentially regulated by the Rb-E2F pathway in lung cancer and breast cancer cell lines. Chromatin immunoprecipitation assays showed the association of Rb and E2F1-5 with the *MMP2* promoter, and transient transfection experiments showed that *MMP2*-luciferase construct is repressed by E2F1-5, and further by Rb, in NSCLC cells. In contrast the *MMP2*-luc construct was induced by E2F1-5 in breast cancer cells. Correspondingly, depletion of E2F family members by siRNA transfection induced *MMP2* transcription in NSCLC cell lines. Using a candidate approach, we screened a panel of siRNAs targeting known transcriptional repressors and cooperative transcription factors that had putative binding sites in the *MMP2* promoter. This identified c-MYC and ID1 as potential mediators of E2F-mediated repression, though there was no effect when Rb, BRG1, or other known repressors were depleted. Deletion mutants of the *MMP2* promoter revealed a 49-base pair region (-1649/-1600) required for E2F-mediated repression in

NSCLC cells. This region contains a consensus p53-binding site that is known to regulate *MMP2* expression, in addition to an E2F binding site. Mutation analysis showed that the p53 binding site and the E2F binding site are required for E2F-mediated repression of *MMP2,* and the c-MYC binding element was dispensable for E2F-mediated repression, suggesting that the effects of c-MYC and ID1 are through an indirect mechanism. Further, KAP1 and HDAC1 could significantly inhibit p53-mediated transactivation of *MMP2*, and depletion of KAP1 prevented E2F1-mediated repression in NSCLC cells. Overexpression of p300 histone acetyltransferase could rescue E2F-mediated repression, suggesting that deacetylation of E2F1 or histones in the promoter region contribute to repression. In breast cancer cells, the mechanism for E2F inducing MMP2 activity is due to a mutation in p53, or low levels of HDAC-KAP1 binding as seen by immunoprecipitation immunoblot, or double immunofluorescence studies. Taken together, E2F1 represses *MMP2* expression in NSCLC cells through p53-KAP1-HDAC1-mediated deacetylation event, whereas c-MYC and ID1 regulate *MMP2* through an unknown, indirect mechanism.

Introduction

The retinoblastoma tumor suppressor protein, Rb, together with the E2F transcription factors are critical regulators of the cell cycle, apoptosis cascades, differentiation, and DNA damage repair for normal cells and cancer cells alike (53, 66, 449). In cancer, the Rb-E2F pathway is frequently altered (12). While *Rb* is mutated in retinoblastoma, breast cancers, lung cancers, osteosarcomas and

other cancers, numerous aberrations including overexpression, amplification, and mutations of the E2F family members themselves are being discovered (12, 66, 461, 462). Recent studies found that the Raf-1-Rb-E2F signaling cascade could directly regulate the expression of key angiogenesis and metastasis genes such as *FLT-1*, *KDR, Angiopoiten-2, MMP9, MMP14*, and to a lesser extent *MMP15*, linking early stage oncogenic events elicited by this pathway to later stage events in cancer, namely metastasis (253, 448). In addition, the Rb-Raf-1 disruptor, RRD-251, which prevents Rb phosphorylation and inhibits E2F1-mediated transcription, inhibited metastasis in an *in vivo* experimental metastasis model, and down-regulated the transcription of crucial *MMP* genes. RRD-251 inhibited invasion, migration, and collagen degradation in a proliferation-independent manner *in vitro* (448). However, the precise mechanism for the emergence of disseminated metastases in patients is still poorly understood—despite being the primary cause of mortality (266, 327).

The matrix metalloproteinases, or MMPs, are a family of 23 enzymes in humans that can collectively degrade all components of the extracellular matrix (374). A hallmark of invading tumor cells is their ability to degrade the surrounding ECM, leading to tissue pore development that can aid in the escape from the primary tumor. With most cancers, invasion is associated with an increase in MMP activity (476). Most tumors and subsequently derived cell lines express the type IV collagenases MMP2 and MMP9 at normal to high levels (431). In gliomas *MMP2* and *MMP9* had highest expression in high-grade

gliomas compared for low-grade astroglimoas and normal brain (484). Increased expression of *MMP2* in non-small cell lung cancer was associated with increased tumor recurrence *(p=0.001),* decreased overall survival *(p=0.0004),* and advanced stage disease *(p=0.001)* (485). Immunohistochemical analysis of human non-small cell lung cancer samples also revealed that MMP2 levels correlated with advanced tumor stage and the presence of distant metastasis ($p<0.05$) (486). Another study showed that strong staining of MMP2 and MMP9 in the stromal cell compartment of NSCLC, particularly in fibroblasts could also correlate with an increase in microvessel density, angiogenesis, and poor prognosis (487). *MMP2* was identified as part of a gene signature that imparted breast cancer cells the ability to metastasize to the lung, (343). MMP2 was also involved in the assembly of new tumor blood vessels, the release of tumor cells into circulation, and tumor cell seeding into the pulmonary vasculature when overexpressed with *MMP1, COX2,* and *epiregulin* (488).

The activity of MMP2 is regulated at a variety of levels: these include transcriptional, proenzyme activation by tissue inhibitor of metalloproteinase-2 and membrane-type metalloproteinase complexes, and inhibition of catalytic activity by TIMPs (311, 489). A search for promoter regions responsible for the upregulation of *MMP2* in cancer has yielded a well-characterized promoter with a number of potential regulatory *cis* elements, including those responsible for the constitutive activation of *MMP2*—AP-1, Ets-1, C/EBP, CREB, PEA3, Sp1, and AP-2 (489). Other studies have suggested that AP-2 can bind to a non-canonical

enhancer region at -1635 (490), while other groups have shown that p53 can bind to a 20 base pair sequence from -1649 to -1630, also in the enhancer region, and directly regulate the expression of *MMP2* (491). The nature of constitutive expression of *MMP2* versus expression during metastasis is unclear. Given that E2Fs are deregulated or overexpressed in various cancers, and they are responsible for regulation of other *MMP* genes, we performed studies to determine if *MMP2* was also regulated by E2Fs in both NSCLC cell lines, and breast cancer cell lines. Surprisingly, the *MMP2* promoter is repressed by E2Fs in NSCLC cell lines by cooperative activity with the adjacent p53 binding site. Together, E2F and p53 can tether a KAP1-HDAC1 complex to the promoter, effectively shutting down transcription. The *MMP2* promoter can also be repressed by c-MYC in the same region, though independent of c-MYC binding; this repression is relieved by ID1, possibly through direct de-repression by the helix-loop-helix protein. In breast cancer cells, however, E2F1 is an activator of the *MMP2* promoter, where the mechanism for activation is either from a lack of KAP1-HDAC interaction (MCF7 cells) or mutant p53 status (MDA-MB-231 cells). In this scenario, E2F1 binding does not recruit a repressive complex, as not all components are available. Taken together, the MMP2 gene is also a transcriptional target of the Rb-E2F pathway, and can be either activated or repressed depending on additional aberrations in the genetic landscape of the tumor.

Results

MMP2 promoter is transcriptionally regulated by E2F transcription factors

MMP2 is upregulated in a variety of metastatic cancers, and *MMP9*, *MMP14*, and *MMP15* are E2F target genes (448). To determine if *MMP2* is also an E2F target gene, we examined the promoter region 2 Kb upstream of the transcriptional start site using the Matinspector (Genomatix) program. In this region there were four putative E2F binding sites at -1616, -1077, -914, and -256. To determine if these binding sites were functional, a ChIP assay was conducted on asynchronously growing A549 cells. Antibodies against Rb, and E2F1-5 showed a significant amount of protein binding to chromatin (Figure 19A-B). There was no DNA associated with an IP done with an irrelevant antibody, further establishing the specificity of the assay. This suggested that there is at least one functional element in the promoter that can actively recruit E2F family members and Rb.

Since E2Fs were recruited to the *MMP2* promoter, we next examined if E2Fs could regulate the expression of the endogenous gene. Towards this purpose, A549, H1650, H1975, and CCL-210 cells, all derived from the lung, were transfected with 100 pmol of siRNA targeting E2F1, E2F3, or a non-targeting control siRNA. Surprisingly, in all four cell lines, depletion of E2F1 or E2F3 induced the *MMP2* mRNA levels as indicated by qRT-PCR (Figure 19C). This suggests that E2Fs might be acting as repressors of *MMP2* expression in lung cancer cell lines.

To further study regulation of the *MMP2* promoter by E2Fs, we utilized an exogenous *MMP2*-luciferase reporter construct. When cotransfected with E2F1 or E2F3, the *MMP2* promoter was indeed repressed (Figure 19D). Further, cotransfection with the Rb construct could repress the *MMP2* promoter even more. We next asked if E2Fs 1-5 behaved similarly when overexpressed along with the *MMP2*-luciferase construct. All E2F family members significantly repress *MMP2* In H1650 and A549 cells. Contrastingly, when MCF7 or MDA-MB-231 breast cancer cells are cotransfected with the *MMP2*-luciferase construct and E2F 1-5, *MMP2*-luc is induced (Figure 19E-F). This is intriguing given that *MMP2* levels were dramatically increased in a breast cancer to lung metastasis model (343) and that E2F activity can also be increased in breast cancer through mutation of Rb.

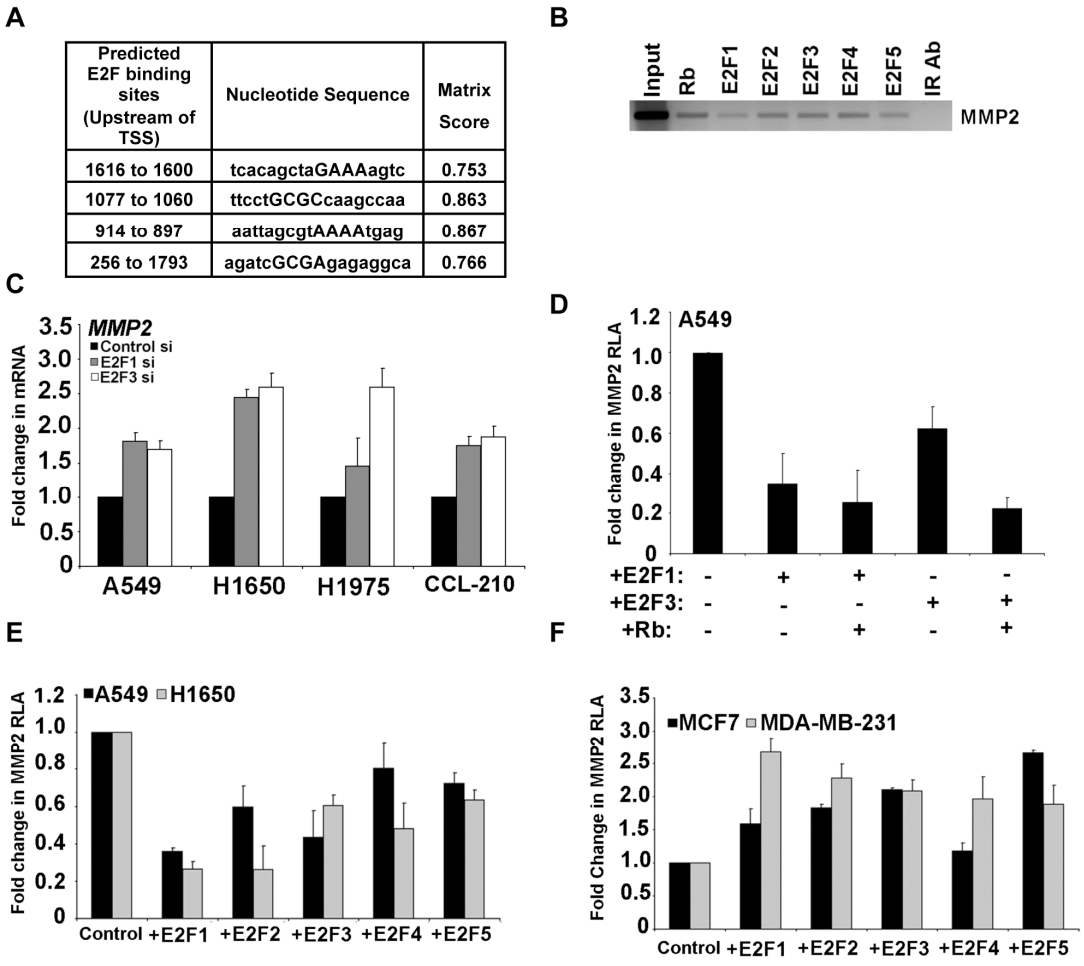

Figure 19. *MMP2* is transcriptionally regulated by the Rb-E2F pathway (A) Table of putative E2F binding elements identified by Matinspector (Genomatix). (B) ChIP assay conducted on asynchronously growing A549 cells using the indicated antibodies. Sonicated genomic DNA was used for input. Primers span the -1616 to -1600 site. (C) Transiently transfecting 100 pmol of E2F1 or E2F3 siRNA increased the expression of *MMP2* in four lung cancer cell lines. (D) Transient tranfections in A549 cells showed that E2F1 and E2F3 could repress *MMP2*-luciferase activity, and further repression was observed with the addition of Rb. (E-F) In A549 and H1650 cells, E2F1-E2F5 could repress the *MMP2*-promoter, whereas E2F1-E2F5 activated the *MMP2* construct in breast cancer cell lines.

E2F represses MMP2 independent of Rb, BRG1 or other known corepressors

Different types of E2F complexes are capable of inhibiting transcription of target genes. For example, in quiescent cells, E2F4 and E2F5 preferentially bind to Rb family members p107 and p130, and recruit a variety of chromatin modifiers to efficiently shut down transcription (8). Further, the E2F activators have been observed repressing promoters directly *in vivo* as well through an unknown mechanism (177). Since we also observed E2F1-5 could repress exogenous *MMP2* promoter when transfected into lung cancer cells, we first explored the role of Rb. To this end, we transfected *MMP2*-luc alone, or *MMP2*-luc with E2F1 or E2F3 into cells that were stably transfected with shRNA targeting Rb, or a non-targeting control (39), (Figure 20A). In both cases, *MMP2*-luc was repressed by the E2F transcription factors. This shows that although Rb could repress *MMP2*-luc when overexpressed (Figure 19D), it was not essential for repression. We next asked whether BRG1, a known repressor of E2F proliferative genes (39), would also repress the *MMP2* promoter through E2F. We found that BRG1 overexpression is slightly activating, and had no affect on E2F-mediated repression (Figure 20B). We next tested a panel of known E2F transcriptional repressors by first transfecting siRNA targeting HP1γ (492), β-arrestin1 (48), LSD-1, Prohibitin-1 (PHB-1) (492), YY1 (493), BRG1 (39), or Sin3a (494), followed by transfection with the *MMP2-luc* construct alone, or with E2F1 (Figure 20C). In all cases, E2F1 represses *MMP2*-luc compared to *MMP2*-

luc control. Interestingly, BRG1 and Sin3a depletion results in less *MMP2* luciferase activity, suggesting they are required for basal expression of *MMP2*.

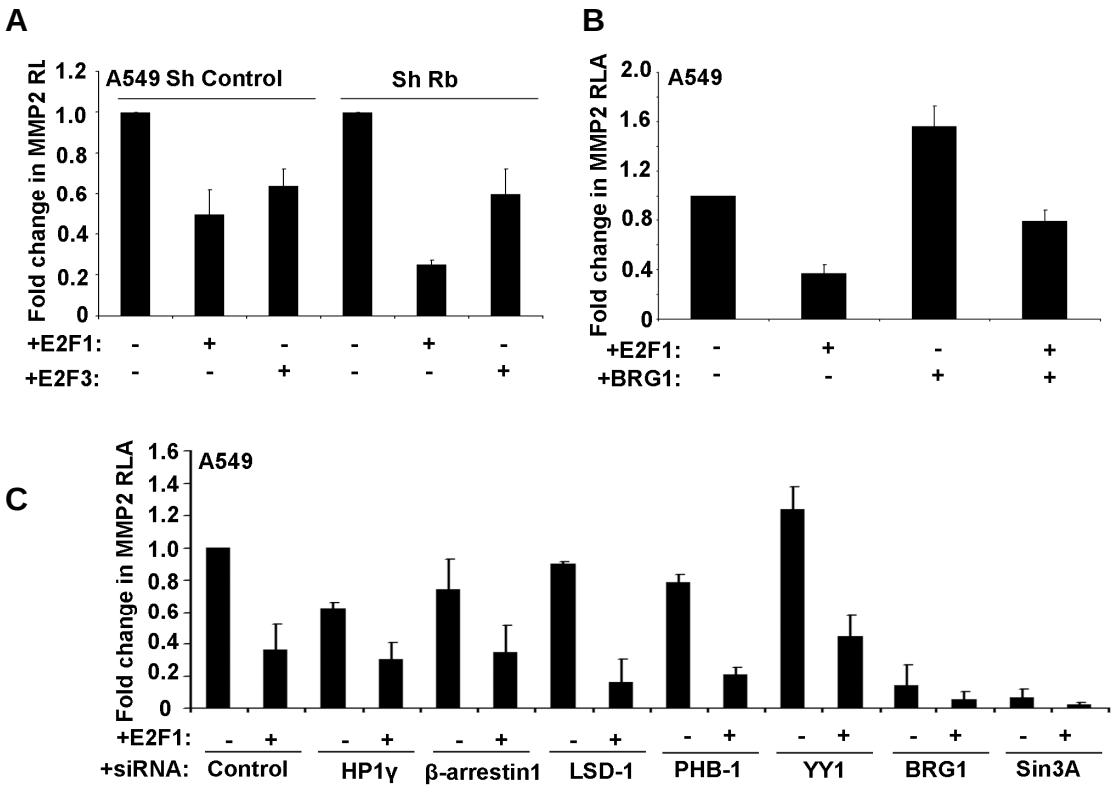

Figure 20. E2F1 represses *MMP2* independent of Rb, BRG1, or other known corepressors (A) A549 cells under puromycin selection to retain the shRNA vector targeting Rb, or a non-targeting control vector were transiently transfected with *MMP2*-luc alone, or with E2F1 or E2F3. Rb was not required for E2F-mediated repression. (B) A549 cells were transfected with *MMP2*-luc alone, or with E2F1, BRG1, or a combination of both. Although BRG1 activated the *MMP2* promoter slightly, E2F still repressed *MMP2*-luc. (C) A549 cells were first transfected with 100 pmol of siRNA targeting indicated transcripts, followed by transfection with *MMP2*-luc alone, or with E2F1. The loss of each corepressor had no effect on E2F-mediated repression of *MMP2.*

c-MYC and ID1 depletion enhances MMP2-luciferase activity

Since the depletion of various corepressor proteins had no effect on E2F-mediated repression of *MMP2,* we next hypothesized that E2F could be repressing the *MMP2* promoter cooperatively with another transcription factor. Previous studies had already identified several regulatory elements in the *MMP2* promoter, including AP-1, AP-2α, Sp1, PEA3, Ets-1, and CREB (489). We also chose to examine the effects of ID1 and c-MYC. Both proteins can act as either activators (346, 495) or repressors (496, 497) depending on promoter context, and both have been shown to play a role in regulating metastasis (346, 498). Firstly, A549 cells were transfected with an siRNA targeting either AP-2α, ID1, Sp1, c-MYC, ETS1 or a non-targeting control siRNA, followed by the *MMP2*-luc construct with or without E2F1. When Sp1 was depleted, there was slightly less basal promoter activity, consistent with previous reports done on gliomas cells (489). However when AP-2α, ID1, c-MYC or ETS1 were depleted, the basal levels of *MMP2*-luc were increased to varying degrees (Figure 21). Depletion of ID1 induced *MMP2*-luc around 3-fold. Further, with E2F1 was cotransfected, though still repressing *MMP2*-luc, the repression was reduced. This suggests that ID1 might contribute to E2F-mediated repression. Similar results were observed in the case of c-MYC. Depletion of c-MYC increases *MMP2*-luc levels as well, and the effects of E2F1 are much less pronounced.

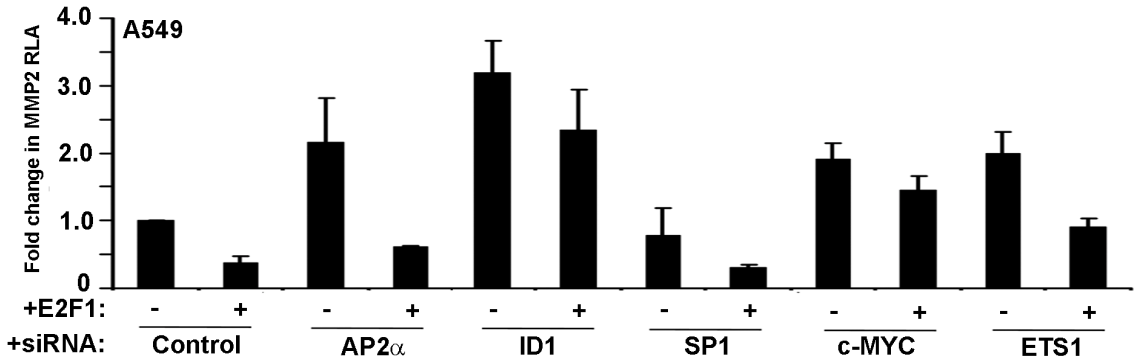

Figure 21. ID1 and c-MYC depletion inhibits E2F-mediated repression of *MMP2*-luciferase. A549 cells were first transfected with 100 pmol of siRNA targeting indicated transcripts, followed by transfection with *MMP2*-luc alone, or with E2F1. The loss of Sp1 resulted in a slightly reduced basal *MMP2*-luc activity, though the addition of E2F1 could repress even further. Although AP2α, ID1, c-MYC, and ETS1 depletion increased the basal level of *MMP2*-luc activity, only the loss of ID1 and c-MYC had any affect on E2F-mediated repression.

c-MYC directly binds and colocalizes with ID1

It has previously been reported that c-Myc can induce *ID1, ID2,* and *ID3* (499-501). However it is also possible that since both ID proteins and c-MYC are helix-loop helix proteins, they could also function through direct physical binding. In this scenario, ID1 would bind to c-MYC, and free c-MYC from DNA. To determine whether ID1 and c-MYC can colocalize in the cell, we utilized double immunofluorescence experiments. Asynchronous A549, H1650, and MDA-MB-231 cells were immunostained with ID1 and c-MYC to observe a direct interaction. In both lung cancer cell lines, A549 and H1650, there is a strong colocalization between ID1 and c-MYC (Figure 22A), whereas in MDA-MB-231 cells there is less colocalization. Upon quantification of the immunofluorescence of both ID1 and c-MYC, it was confirmed that MDA-MB-231 cells had the lowest levels of ID1-associated immunofluorescence (Figure 22B). To confirm these observations, we performed immunoprecipitation and immunoblot analysis on the three cell lines. In all cell lines, there was detectable interaction between endogenous c-MYC and ID1 (Figure 22C). This binding should result in less c-MYC associated with the *MMP2*-promoter, and if c-MYC is in fact a repressor, this release would yield more *MMP2*-luc activity. Together these results suggest that in addition to c-MYC transcriptionally regulating *ID1*, there might be a negative feedback loop, at least at the exogenous *MMP2*-luc promoter, where ID1 can bind to c-MYC.

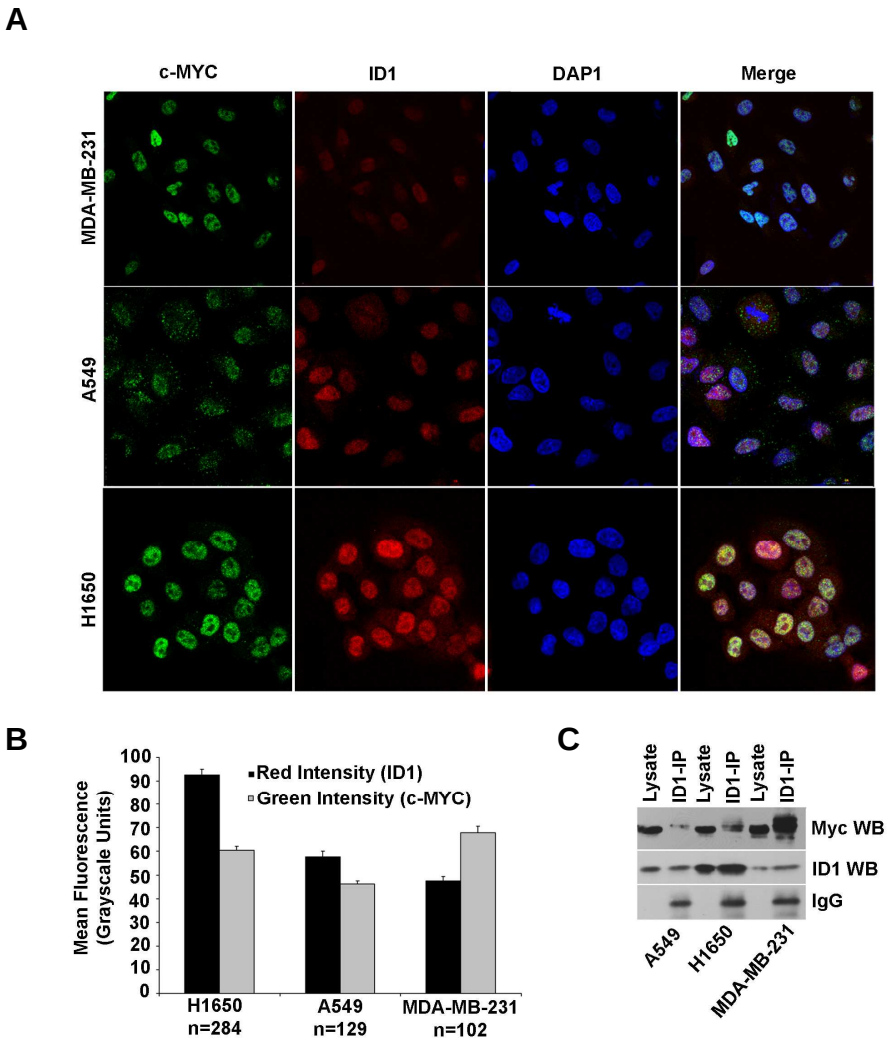

Figure 22. c-MYC directly binds to ID1 in lung cancer cells. (A) Serum-starved A549 and localization of c-MYC and ID1 was analyzed by double-immunofluorescence staining followed by confocal microscopy. The cells were counterstained with the nuclear marker, 4′6-diamidino-2-phenylindole (DAPI). Overlay of the images show yellow spots indicating colocalization in the far right panel. (B) Both channels of immunofluorescence were quantified. (C) The physical interaction of endogenous c-MYC and ID1 was confirmed by immunoprecipitation of ID1 followed by immunoblot analysis.

Identification of the promoter region responsible for E2F and c-MYC mediated repression of MMP2-luciferase

Given that Matinspector can generate hundreds of possible regulatory elements for any given DNA sequence, experiments were done to first determine the region of DNA responsible for E2F-mediated repression in the *MMP2* promoter. We generated 5' deletion mutants from the original wild type -1659 base pair *MMP2*-reporter construct (489, 491) including -1467, -945, -508, -221. Each deletion was created to eliminate one putative E2F binding site per mutation (Figure 23A). Each of these constructs was transiently transfected alone, or with E2F1 in both A549 and H1650 cell lines. All data was normalized to the full-length -1659 base pair promoter alone. The relative luciferase activity from the first deletion mutant (-1467) was increased compared to the full length (-1659) and E2F1 no longer could repress the transcription of *MMP2*-luc, but was activating instead (Figure 23A). The RLA of the remaining luciferase constructs was comparable to the first deletion construct (-1467). Interestingly, the sole c-MYC binding element identified in Matinspector was not in the DNA region required for E2F-mediated repression, although this region does have the previously characterized p53 binding site (491). To further study this region of the promoter, we transiently transfected A549 cells with the -1659 or -1467 promoter along with E2F1, c-MYC, p53, or the combination of E2F1 with p53, or c-MYC with p53 (Figure 23B). In agreement with prior studies (491), p53 was transcriptionally activating the *MMP2* promoter when transfected alone, although cotransfection with either c-MYC or E2F1 resulted in repression of the -1659

MMP2 promoter. This suggests that both E2F and c-MYC could repress p53-mediated transactivation of the *MMP2*-promoter. In the shorter fragment, E2F1, c-MYC and p53 were all slightly activating, and there was no effect when p53 was cotransfected with either E2F1 or c-MYC. It is possible that E2F1 and c-MYC can bind additional putative sites, and the effects seen with p53 in the smaller fragment could be indirect; p53 may be upregulating an unknown gene that results in upregulation of *MMP2*-luciferase.

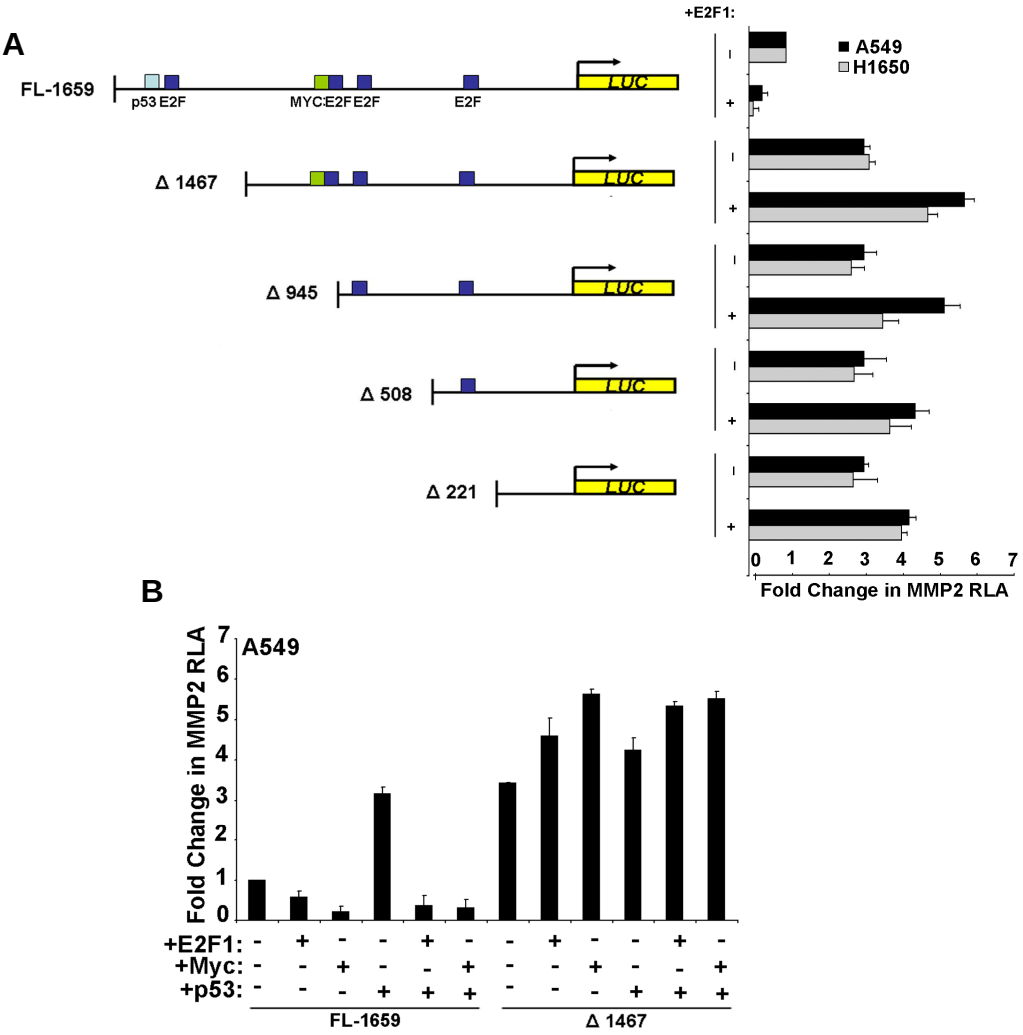

Figure 23. Identification of a repressor region in the *MMP2* promoter (A) A549 and H1650 cells were transfected with deletion mutants of *MMP2*-luc alone, or with E2F1. The first deletion mutation increased RLA, and completely abolished E2F1-mediated repression. (B) A549 cells were transfected with the full-length -1659-luc construct or the -1467-luc construct along with E2F1, c-MYC, p53, or indicated combinations. Both E2F1 and c-MYC inhibited the p53-mediated activation of *MMP2*-luc in the full-length construct, but not in the mutant construct.

Contribution of the p53, E2F, and c-MYC binding sites for the activity of the MMP2 promoter

Given that the *MMP2* promoter was repressed by E2F1 through the region between -1659 and -1467, and that *MMP2* RLA was induced by p53, we next tested the requirement of the individual binding sites to mediate these effects. We created promoter constructs with either a mutated p53, E2F or c-MYC binding site using overlap-extension PCR, as described in materials and methods (Figure 24 and 25A). We also created an additional deletion mutation yielding a -1617 construct that lacks the p53 binding element, but still has the E2F binding element intact. When transfected into A549 cells, the *MMP2*-promoter construct harboring a mutation in the p53 binding site was no longer activated by p53 (Figure 24B; lane 3 and lane 9). This mutation also resulted in E2F1 activating *MMP2*-luc, rather than repressing *MMP2*-luc. Similar results were observed when the -1617 deletion mutant was transfected (Figure 25B). Together, these data suggest that E2F1 requires the p53-binding site to repress the *MMP2* promoter. Next, we tested the requirement of the E2F binding site between -1616 and -1600. Using the -1659 *MMP2*-luc construct with a mutant E2F binding site, E2F1 was no longer able to repress *MMP2*-luc (lane 2 with lane 11). Similar results were seen when transfections were done in MCF7 cells, however E2F1 activated the full length promoter (Figure 25C), consistent with the observation that lung cancer and breast cancer cells are differentially regulated. To further investigate the contribution of c-MYC, an *MMP2-luc* construct with a mutation in the c-MYC binding site was used. When transfected in H1650 cells, with two different

promoter clones, the mutation had no effect on c-MYC-mediated repression (Figure 25D); further suggesting that c-MYC plays an indirect role in regulation of the *MMP2* promoter, or utilizes other cryptic sites on the promoter to repress *MMP2.*

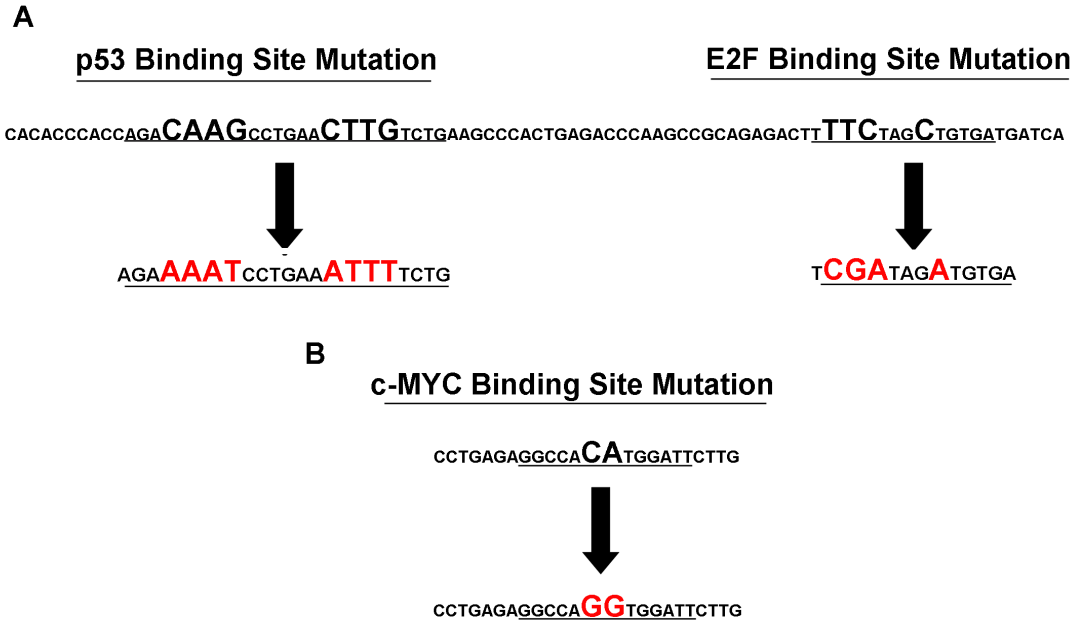

Figure 24. Design of transcription factor binding site mutants. (A) The location of the predicted p53-binding site is underlined, and the core binding sequences are shown in a larger font. The nucleotides that were changed are highlighted with red font. This was confirmed by DNA sequencing. The E2F binding site uses the TTT sequence, and the conserved cytosine reside to mediate E2F binding. The indicated changes are in red. (B) The CA residues in the center of the c-MYC binding element were changed to GG. The indicated changes are in red font.

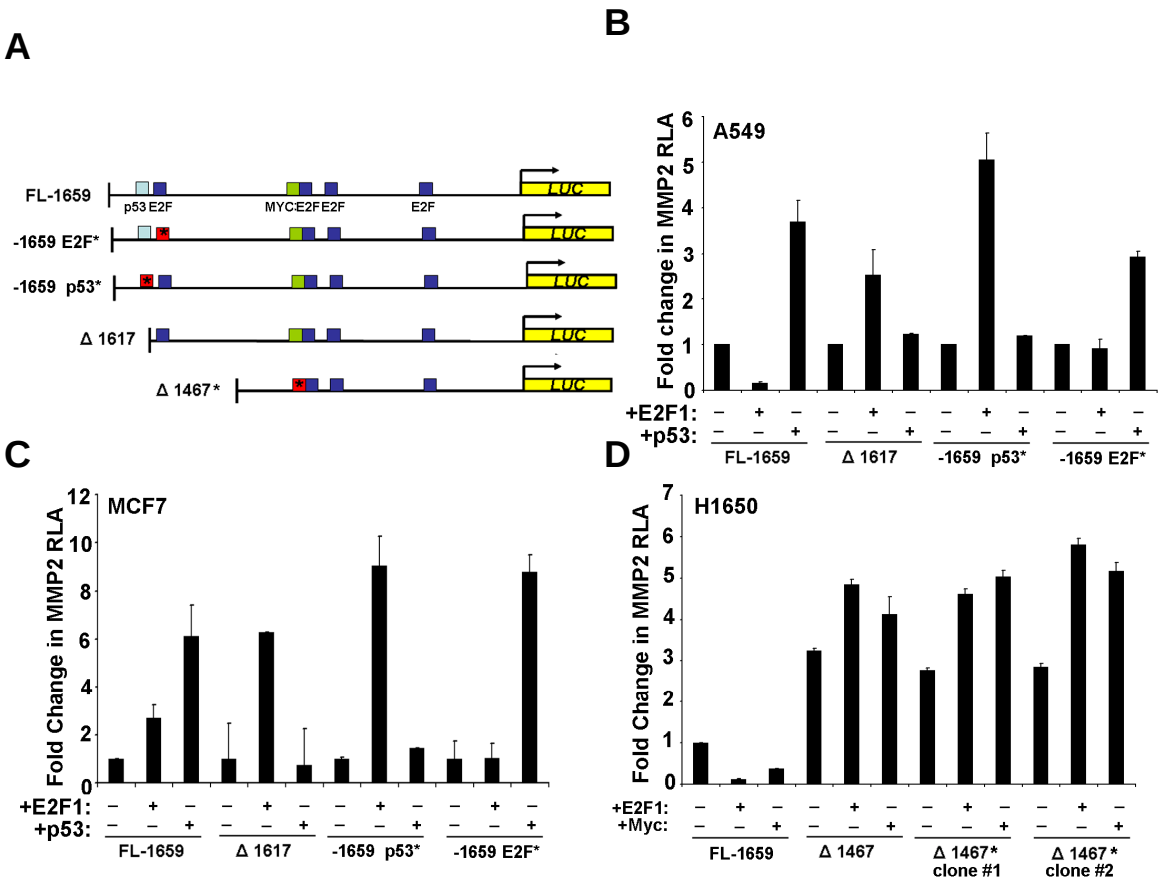

Figure 25. p53 and E2F1 binding sites contribute to *MMP2* promoter activity. (A) Schematic representation of promoter constructs made. Red box with a star represents where mutations were created. (B-C) A549 and MCF7 cells were cotransfected with *MMP2*-luc constructs with indicated mutations or deletions, along with E2F1 or p53. Both the p53 binding site and the E2F binding site were required for E2F-mediated repression of *MMP2*-luc in A549 cells, and the E2F site was essential for activation in MCF7 cells (D) H1650 cells were transfected with *MMP2*-luc constructs with a mutated c-MYC binding element. There was no effect when the c-MYC site was mutated.

A corepressor complex containing p53-KAP1-HDAC1 is responsible for E2F-mediated repression of MMP2

We next wanted to identify the mechanism that E2F employs to shut down p53-mediated activation. Previous studies have shown that nuclear corepressor KAP1 could repress p53 by interacting with MDM2, and recruiting HDAC1 to p53 target genes. This results in the deacetylation of p53 protein (502). Another study demonstrated that KAP1 can also tether HDAC1 to E2F1, and similarly deacetylate the E2F1 protein (503). Since both of these proteins were cooperating to repress *MMP2*, we sought to determine whether KAP1 plays a role in repression. A549 cells were first transfected with siRNA targeting HDAC1, KAP1, or p53, followed by transfection with the -1659 *MMP2*-luc construct alone, or with E2F1. In all cases, depletion of these components resulted in less repression by E2F1 (Figure 26A). To further characterize the contribution of KAP1 in the repression of *MMP2*, we transfected A549 and H1650 cells with the *MMP2-luc* construct, along with E2F1, KAP1, p53, or combinations of the three. In A549 cells and H1650 cells, KAP1 inhibits p53-mediated activation when cotransfected (Figure 26B). To determine if the repression of transcription is due to KAP1-HDAC complex, we performed rescue experiments in both A549 and H1650 cells. In both cell lines, the histone acetyltransferase p300 could rescue E2F-mediated repression of *MMP2* (Figure 26C). This suggests that the repressive role of KAP1 is probably through deacetylation.

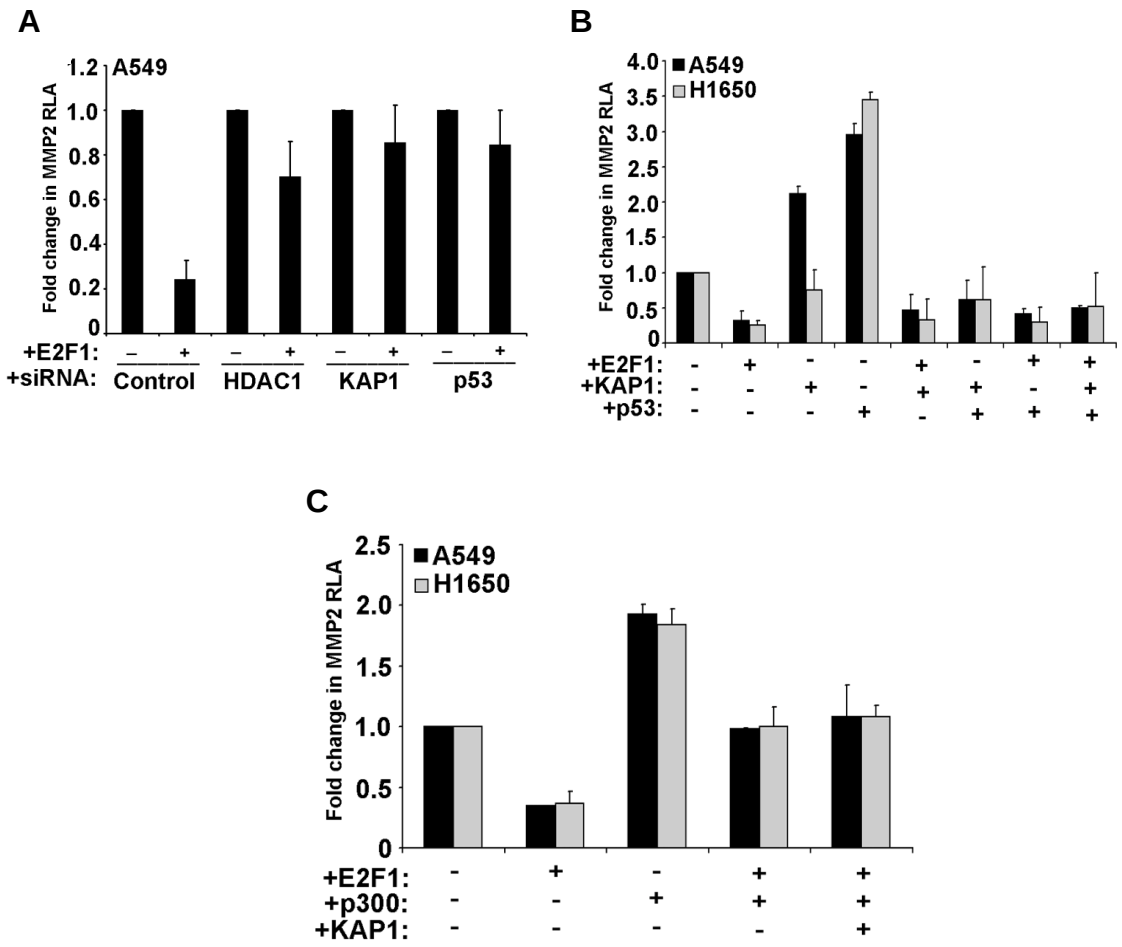

Figure 26. A corepressor complex containing p53-E2F1-KAP1-HDAC1 is responsible for repression of MMP2. (A) A549 cells were transfected with 100 pmol of siRNA targeting HDAC1, KAP1, p53, or a non-targeting control, followed by transfection with *MMP2*-luc alone or with E2F1. Depletion of these transcripts abrogated E2F-mediated repression. (B) A549 and H1650 cells were transfected with *MMP2*-luc along with E2F1, KAP1, p53, or combinations. In both cell lines, KAP1 completely inhibited p53 activation of MMP2 RLA. (C) A549 and H1650 cells were transfected with *MMP2*-luc along with E2F1, p300, KAP1, or combinations. p300 overexpression could rescue the E2F-mediated repression.

KAP1 binding to HDAC1 is required for repression of MMP2

E2F-mediated repression of *MMP2*-luc activity was only observed in lung cancer cells, whereas in breast cancer cell lines E2F was activating. MDA-MB-231 cells express high levels of mutant p53 (504, 505), and mutant p53 can interfere with wild type p53 through several mechanisms, including acting as a dominant negative protein (506). Further, although mutant p53 is more stable than wild type p53 (507, 508), it can be recruited to different promoters than wild type, indicating that the DNA binding specificity might change (509, 510). This could explain why MDA-MB-231 can use E2F1 to activate *MMP2*-luc, since p53 binding upstream is required for repression. Interestingly, MCF7 cells have wild type p53, though E2F1 can still activate the *MMP2* promoter in this cell line. To try to understand the mechanism for activation in these breast cancer cell lines, ChIP assays were conducted with antibodies against Rb, E2F1, HDAC1, p53, MDM2, KAP1, and irrelevant antibody was used as a negative IP control. A549 and H1650 cells recruited each protein to the *MMP2* promoter (Figure 27A). As predicted, in MDA-MB-231 cells, there is no p53 recruitment. MCF7 cells recruited each protein except HDAC1. Since HDAC1 activity is required for repression of *MMP2* by E2F1, we next performed immunoprecipitation immunoblots to determine the level of HDAC1-KAP1 binding in these four cell lines. A549, H1650, and MDA-MB-231 cells showed an interaction of endogenous HDAC1 with KAP1, while MCF7 cells showed no detectable interaction (Figure 27B-C). To confirm these observations, we utilized double immunofluorescence experiments to further visualize the endogenous interaction

between HDAC1 and KAP1 in the four cell lines. Asynchronous A549, H1650, MCF7 and MDA-MB-231 cells were immunostained with HDAC1 and KAP1 to observe a direct interaction, then subjected to confocal microscopy. Corresponding with the immunoprecipitation experiments, A549, H1650, and MDA-MB-231 cells show a strong colocalization between HDAC1 and KAP1 (Figure 27D), whereas in MCF7 cells, there were high levels of KAP1, but low levels of colocalization with HDAC1. Therefore, two separate mechanisms can account for the activation of the *MMP2*-luciferase construct in breast cancer cells—MDA-MB-231 cells, which have mutant p53, cannot recruit the repressor complex, and MCF7 cells have insufficient KAP1 binding to HDAC1, which also results in activation of *MMP2*-luc.

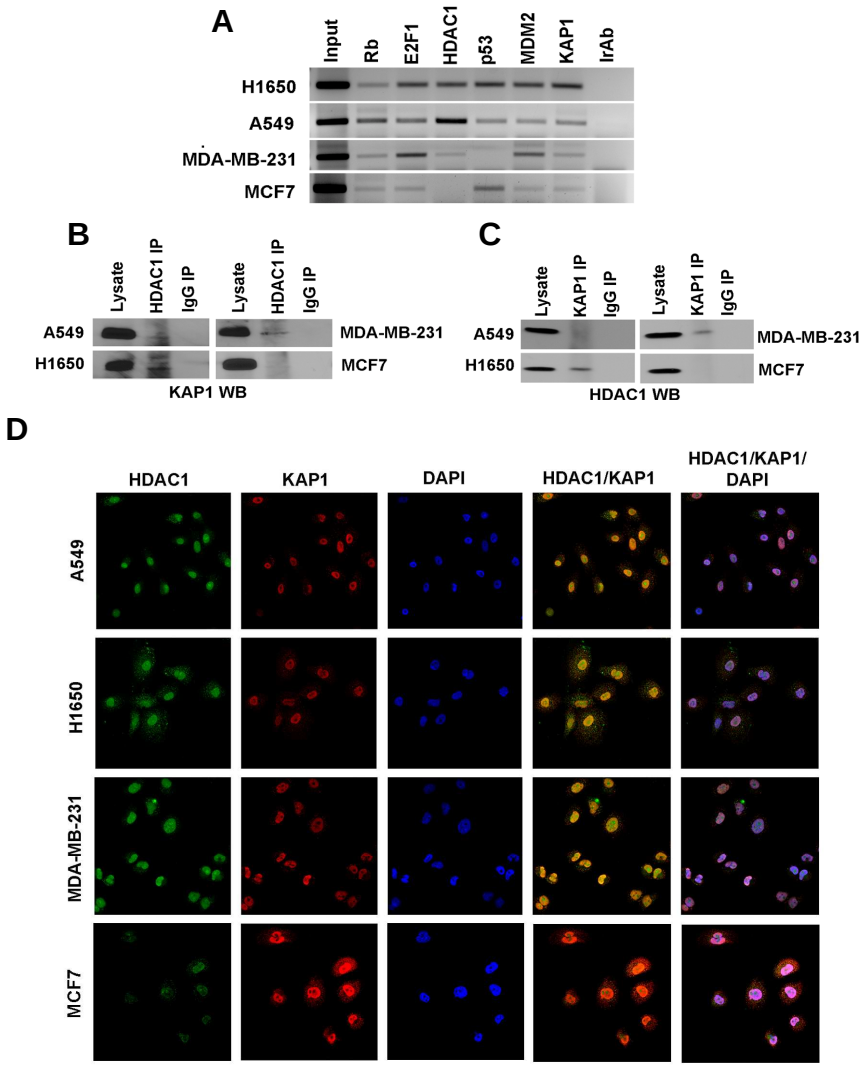

Figure 27. KAP1-HDAC1 interaction is required for repression of MMP2. (A) ChIP assays were performed on A549, H1650, MDA-MB-231, and MCF7 cell lines with indicated antibodies. There is no binding of p53 in MDA-MB-231 cells, and no binding of HDAC1 in MCF7 cells. (B-C) Immunoprecipitation of HDAC1 (B) or KAP1 (C) followed by immunoblotting with the other protein shows interaction of the endogenous proteins in A549, H1650, and MDA-MB-231 cells, but not MCF7 cells. (D) Double immunofluorescence confocal microscopy on A549, H1650, MDA-MB-231, and MCF7 cells reveals that KAP1 does not colocalize with HDAC1 in MCF7 cells.

(±)-Nutlin-3, the p53-MDM2 disruptor, activates the MMP2 promoter

(±)-Nutlin-3 is a small molecule inhibitor of the MDM2-p53 interaction, which leads to p53 stabilization, activation of cell cycle arrest and apoptosis (511). There are a number of clinical trials that employ the concept that selective p53 activation by (±)-Nutlin-3 might represent an alternative to current cytotoxic chemotherapy, in particular for pediatric tumors and hematological malignancies, which retain a high percentage of wild type p53 (512). Prior studies have also shown that MDM2 was required for KAP1 to recruit HDAC1 to p53, and mediate deacetylation (502). To determine the effects of (±)-Nutlin-3 on *MMP2*-luciferase activity, A549 and H1650 cells were transfected with the *MMP2*-luc construct, then treated with increasing amounts of (±)-Nutlin-3 for 24 hours. By blocking the p53-MDM2 interaction, and consequently inhibiting KAP1 recruitment of corepressor HDAC1, the *MMP2*-luc activity was de-repressed in a dose-dependent manner (Figure 28A). To determine if these same effects could be observed on the endogenous *MMP2* promoter, A549 cells were treated with (±)-Nutlin-3 for 24 hours, then total RNA was collected and p53 target gene levels were analyzed by qRT-PCR. As predicted, *p21* levels were increased and *survivin* levels were reduced, both representative of p53-dependent cell cycle arrest. *MMP2* levels were also induced by treatment with (±)-Nutlin-3 (Figure 28B), suggesting that blocking the MDM2-p53 interaction might result in a more metastatic phenotype for cancer cells, despite stabilization of p53.

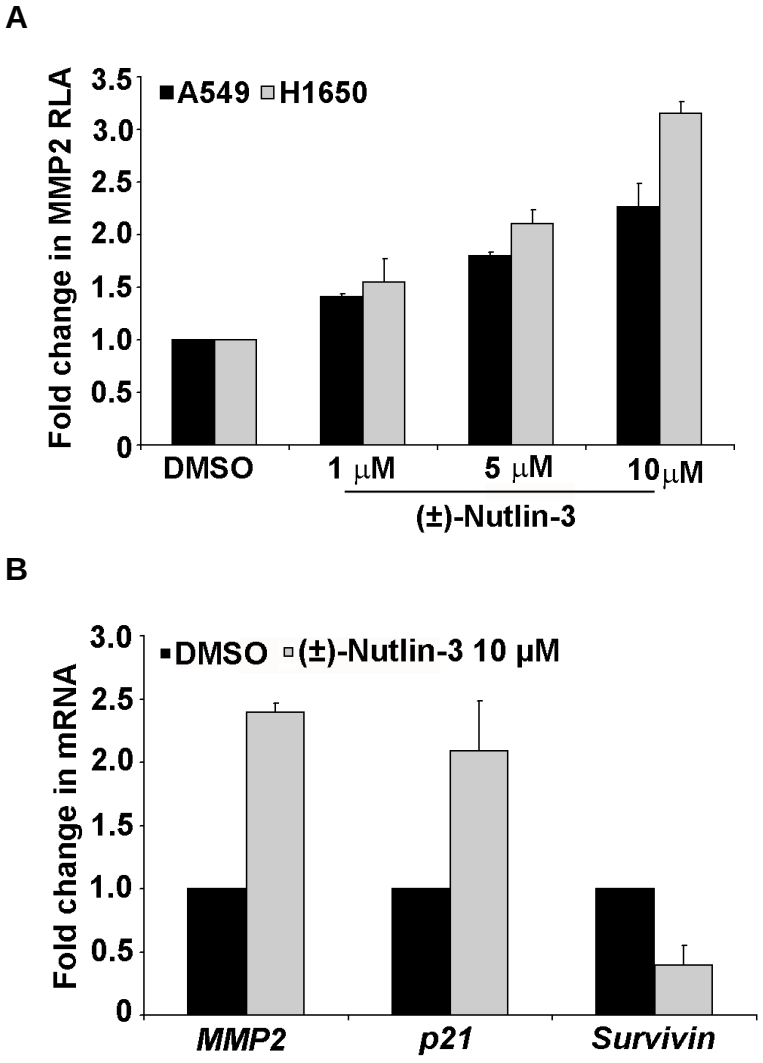

Figure 28. The p53-MDM2 disruptor, (±)-Nutlin 3, activates the *MMP2* promoter (A) A549 and H1650 cells treated with increasing concentrations of (±)-Nutlin 3 have increased *MMP2* RLA. (B) Total RNA was collected from A549 cells treated with 10 μM (±)-Nutlin 3 for 24 hours. qRT-PCR data shows that (±)-Nutlin 3 treated cells had increased levels of *MMP2* and *p21*, and decreased levels of *survivin*.

Discussion

The type IV collagenase, MMP2 or 72-kDa gelatinase A is one of the well-studied family members in the large family of matrix metalloproteinases. The enzyme is secreted as an inactive zymogen, and it requires further processing for activation (513). This is usually achieved through proteolytic degradation. The main substrates for MMP2 include type IV collagen and fibronectins, both main components of the basement membrane, and it is overexpressed in a variety of pathological conditions, particularly in cancer (374, 514-516). MMP2 regulation is complex, being regulated on the transcriptional level, at level of zymogen processing, and binding to tissue inhibitors of metalloproteinases (TIMPs). In fact, the activation of MMP2 is largely dependent on TIMP2 tethering to the cell surface associated MMP14 (517-520). Further, MMP2 activity can be upregulated by a number of stimuli, including TGF-β1 (521), interferon, transfection of Ras (522) or nicotine stimulation (523).

When the *MMP2* 5' upstream promoter region was cloned, it was demonstrated that wild type p53 was a transcriptional activator in HT1080 cells (491); however other groups found that in astroglioma cells the p53 response element was not required for constitutive activation of *MMP2*. This suggests that the regulatory mechanism of *MMP2* is cell type dependent. Further, studies have demonstrated a role for ATF3 in antagonizing p53-dependent trans-activation of the *MMP2* promoter through the DNA element of -1659 to -1622, the same

region that we have identified as important for E2F-mediated repression. In lung cancer cell lines, although ATF3 could antagonize p53, it was not required by E2F to mediate repression of *MMP2* (data not shown). Prior studies have utilized Matinspector (Genomatix) to identify potential mechanisms for the constitutive expression of *MMP2,* and found a number of *cis*-elements including AP1, ETS-1, C/EBP, CREB, PEA3, SP1, AP2, and a number of GC box regions assumed to be for SP transcription factors (489). Given that *MMP2* can be induced by a number of stimuli in normal physiological processes or under pathological conditions, efforts were made to identify additional promoter response elements in the *MMP2* promoter. Further sequence analysis has revealed that NFκB (441) and E2F transcription factors were putative regulatory elements in the *MMP2* promoter (448).

MMP2 deficient mice have reduced rates of tumor progression, highlighting a role for MMP2 in the advancement of cancer (412). Importantly there have been multiple studies aimed at using *MMP2* as a biomarker for the advancement of disease. In non-small cell lung cancer, MMP2 expression in the stromal fibroblasts was shown to correlate with enhanced angiogenesis and poor prognosis (487) however other studies show that MMP2 expression has a limited informative value for NSCLC prognosis (524). Further, although *MMP2* is transcriptionally silenced through an unknown mechanism or posttranscriptional inhibited in most breast cancer cells lines, multiple groups have demonstrated high levels of *MMP2* RNA and protein in more advanced stages of breast cancer

(525-528). Animal models have also shown that *MMP2* is an essential protease for metastasis of breast cancer to the lung (343) and brain (529). These observations compliment studies showing that E2F1 is overexpressed in human breast cancer (228).

Our studies show that by using 5'-deletion mutants of the *MMP2* promoter that the p53 element contributes to activation of *MMP2* in both breast and lung cancer cells. We show that in cells containing mutant *p53*, the *MMP2* promoter is activated by E2F1, likely due to the mutant p53 protein being unable to bind the MMP2 promoter and aid in E2F-mediated repression. Other studies have also demonstrated that mutant p53 could not induce the *MMP2* promoter, which is also the case with MDA-MB-231 cells (489, 491). In addition, we show that E2F1-5 can physically bind, and inhibit the transactivation of *MMP2* by p53 through a corepressor complex with KAP1 and HDAC1. Given that the depletion of HDAC1 could only partially inhibit the repressive effects of E2F1, it is possible that other HDAC family members might be compensating for the loss of HDAC1. Further, KAP1 has been shown to physically recruit the Mi-2α subunit of the NuRD chromatin-remodeling complex (530). The NuRD complex is comprised of six core subunits, including HDAC1 and HDAC2 (531). Although we present additional data showing that c-MYC and ID1 can also regulate *MMP2* through the same region, these results appear to be indirect. It is possible that part of the actions observed when E2F is overexpressed could be due to upregulation of *c-MYC* transcription, a known E2F target gene (532-534). Taken together, these

studies further our understanding of the link between the Rb-E2F cell cycle regulatory pathway to advanced stages of cancer development and metastasis.

Chapter 5: Nicotine stimulates the nAChR-Rb-E2F pathway to induce MMP transcription and metastasis in an orthotopic mouse model

Abstract

Cigarette smoking is strongly correlated with the onset of non-small cell lung cancer (NSCLC). Nicotine, the addictive component of cigarettes, has been found to induce proliferation, confer resistance to apoptosis, and induce EMT like changes in breast, pancreatic, and lung cancer cell lines. In addition, nicotine also induces morphological changes characteristic of a migratory, mesenchymal phenotype in NSCLCs. At the same time, the exact molecular mechanism for this acquired invasiveness in NSCLCs has not been fully elucidated. To determine whether this increased invasion is due to up-regulation of matrix metalloproteinases, we examined the mRNA levels of matrix metalloproteinase genes after exposure to nicotine. *MMP2, MMP9, MMP14,* and *MMP15* mRNA levels were significantly induced by treatment with nicotine when compared to serum starved A549 lung adenocarcinoma cells. Transient transfection experiments in H1650, A549, and MDA-MB-231 cell lines also showed that *MMP*-promoter luciferase reporters are all induced by treatment with nicotine. Further, nicotine could induce a stronger binding of E2F1 along with less Rb as seen in ChIP assays. Cells treated with nicotine and lacking E2F1 or E2F3 showed a lower capacity for invasion compared to control. CCL-210 lung

fibroblasts had an increase in collagen degradation ability when treated with nicotine, and this was decreased when cells were treated with nAChR subunit inhibitors. In an orthotopic model of lung cancer metastasis, A549-luciferase cells lacking either E2F1 or E2F3 had less primary tumor growth and a decreased capacity for metastasis to the brain or liver after treatment with nicotine. Taken together, *MMP* transcription is induced by nicotine treatment in lung cancer cell lines through the Rb-E2F pathway, and this pathway is required for lung cancer metastasis in an orthotopic mouse model.

Introduction

Lung cancer is the leading cause of cancer in both men and women worldwide, and it is the most preventable type of cancer—the majority of lung cancers are associated with smoking (535, 536). Despite growing evidence showing a role for smoking with the initiation and progression of cancer, 30% of smokers diagnosed with lung cancer continue to smoke. Tobacco smoke contains multiple carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN), which can cause DNA adduct formation, mutation, and eventually oncogenesis (537). While nicotine is the addictive component in cigarette smoke, it can't initiate oncogenesis in humans or most rodent models—it only initiates tumors in hamsters (538). On the molecular level, it is becoming increasingly clear that signaling events associated with smoking are contributing to the growth, progression and metastasis of a variety of cancers.

Nicotine exerts its cellular functions through nicotinic acetylcholine receptors (nAChRs), which are present on both neurons and a variety of non-neuronal cells as well (539). nAChRs are pentameric proteins consisting of nine α subunits (α2–α10) and three β subunits (β2–β4) in neuronal cells (540) and form two groups including a heteromeric pentamer of α2–α6 and β2–β4, and the other being a homomeric pentamer of α7–α9 (541, 542). A third type, the non-neuronal muscle type receptors are composed of either α1, β1, δ and γ subunits in the embryonic form, or as α1, β1, δ or ε subunits in the adult form (543). The finding that nAChRs are present on non-neuronal cells gave way to studies showing that nicotine could induce the proliferation of endothelial cells (42) and lung cancer cell lines (45). Nicotine can also act as a paracrine or autocrine growth regulator through the secretion of growth factors such as bFGF, TGF-α, VEGF, and PDGF (544).

The accepted dogma is that nicotine can exert its functions by initiating cell signaling cascades through binding to a subunit of the nAChR pentamer, particularly α7 (40). When nicotine binds to nAChRs, this leads to the recruitment of β-arrestin-1 and Src to the nicotinic receptors, followed by the activation of downstream signaling pathways such as the MAPK cascade and the Raf-1-Rb pathway (37). Later studies identified the key players mediating the mitogenic effects of nicotine to include β-arrestin-1 and the Src kinase, where both components are indispensable for nicotine-mediated activation of cell proliferation (48). This signaling event causes the recruitment of E2F1, Raf-1 and

Rb on E2F responsive proliferative promoters including *cdc6, cdc25a, TS*, and *survivin*.

While numerous studies suggest a role for nAChRs in tumor growth and angiogenesis, there are fewer studies linking nAChRs to later stage tumor events such as EMT and metastasis. We had previously shown that in a panel of cell lines, nicotine exposure could induce EMT, invasion and migration *in vitro* (41). Further, nicotine could induce the Src-c-MYC-ID1-ZPB89 pathway to induce *vimentin* and *fibronectin,* conferring a more metastatic phenotype in lung cancer cell lines (346). Nicotine could also promote the metastic growth and tumor recurrence in an immunocompetent mouse model of lung cancer (51). Other studies had linked the proangiogenic activity of nicotine to the upregulation of *MMP2* and *MMP9* in retinal models of angiogenesis (523). These studies were aimed to further explore the biology of nicotine-induced metastasis. Nicotine could induce the expression of key invasion promoting genes, *MMP2, MMP9, MMP14,* and *MMP15.* We found that the Rb-E2F pathway regulated these genes. Further, E2F and subunits of nAChRs were required for nicotine induced collagen degradation, invasion, and metastasis to the brain and liver in an orthotopic model of NSCLC.

Results

Nicotine Induces MMP gene transcription in cell lines

To determine the effects of nicotine exposure on the expression of *MMP* gene transcription, H1650, A549, and MDA-MB-231 cell lines were

transfected with the exogenous *MMP9*-luciferase reporter along with the pRL construct and empty vector for normalization of transfection. Twelve hours after transfection, cells were treated with 1 μM nicotine for 24 - 48 hours, or placed in serum-free media as control. In all three cell lines, *MMP9*-luc was induced by treatment of nicotine alone (Figure 29A). To determine if the endogenous promoters are also induced by nicotine, A549 cells were serum starved for 18 hours, and then kept in serum-free media, or in the presence of 1 μM nicotine for 24 to 48 hours before total RNA was collected from cells. qRT-PCR was performed to analyze the effects of nicotine on the expression of *MMP* genes required for metastatic events. *MMP2, MMP9, MMP14,* and *MMP15* RNA levels were all induced by treatment with nicotine (Figure 29B).

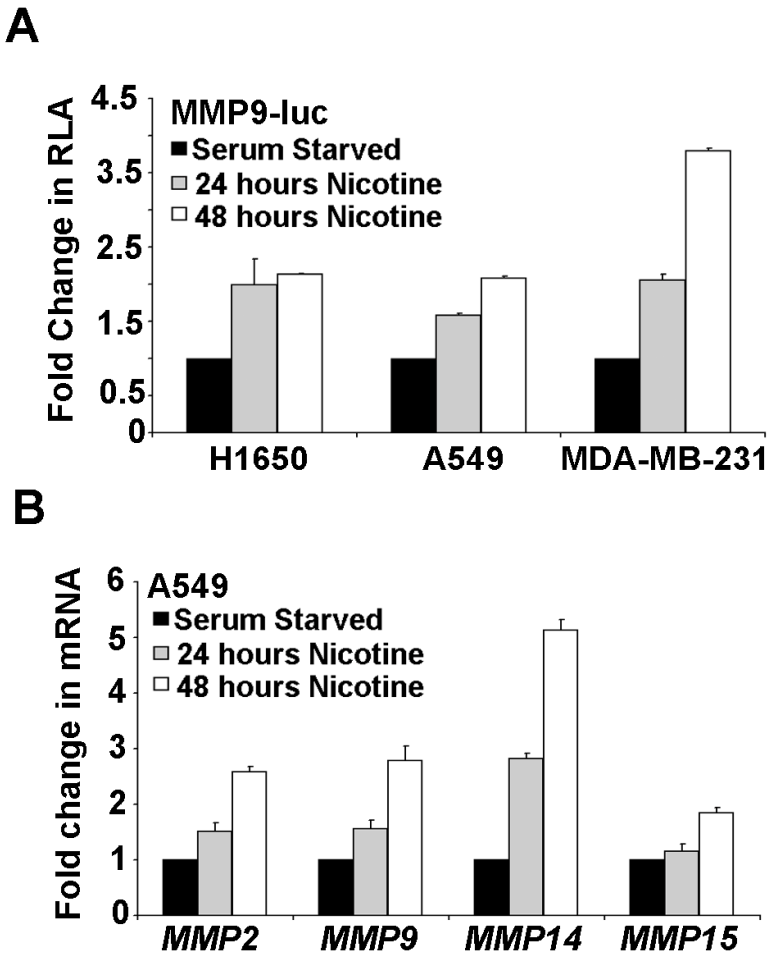

Figure 29. *MMP* genes are induced by nicotine in multiple cell lines. (A) H1650, A549, and MDA-MB-231 cells were transfected with *MMP9*-luciferase containing the *MMP9* 5' promoter DNA, in addition to pRL containing the luciferase gene from *Renilla reniformis* for quantification of relative luciferase. In all cases, the *MMP9*-luciferase construct was induced by nicotine at 24 hours, but further by 48 hours. (B) A549 cells were made quiescent by serum starvation for 18 hours, followed by treatment with nicotine for 24 or 48 hours. The mRNA levels of *MMP2, MMP9, MMP14* and *MMP15* were all induced at both time points after treatment with nicotine.

Nicotine induces MMP-luc activity and collagen degradation via nAChRs

We had previously shown that the α7 subunit of nAChRs were required for proliferation in a panel of lung cancer cell lines and to mediate the induction of *ID1* (37, 346). To further understand the biological mechanism mediating the induction of *MMP*-promoters, we wanted to examine the effects of inhibiting various components of nAChRs. A549 cells were first transfected with *MMP2, MMP9,* or *MMP14* luciferase constructs along with pRL, and then treated with either nicotine alone, or nicotine in the presence of indicated inhibitors. The general nAChR antagonist hexamethonium bromide (HBR) and the α7 specific inhibitor, alpha bungarotoxin (α-BT), inhibited the nicotine-mediated induction of all three promoter constructs (Figure 30A). Contrasting to observations seen with proliferation assays, using the α3β2 and α4β2-subunit inhibitor dihydro β-erythoidine (DHβE) and the muscarinic subunit inhibitor, atropine, could also inhibit the induction of *MMP*-luciferase activity. Given that these inhibitors could inhibit *MMP* RLA, we next performed collagen degradation assays to test if the biological functions of MMPs were also affected. Using any of the subunit inhibitors was effective at inhibiting nicotine-mediated collagen degradation (Figure 30B). This suggests that *MMP* transcription and collagen degradation can be induced by nicotine through multiple nAChR subunits, and might also be induced through the closely related muscarinic acetylcholine receptors as well.

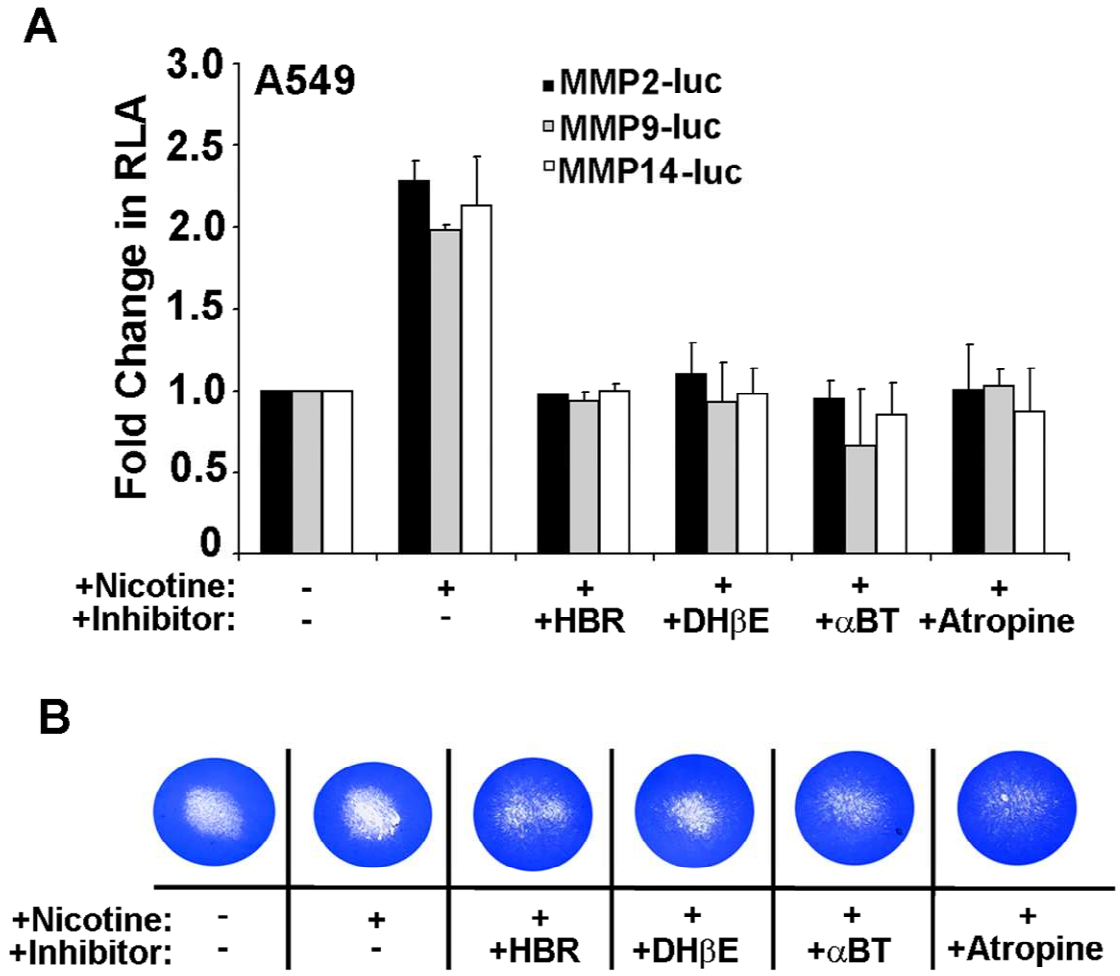

Figure 30. Nicotine induces *MMP*-luc activity and collagen degradation via nAChRs. (A) A549 cells were transfected with *MMP2*-luc, *MMP9*-luc, and *MMP14*-luc constructs, each containing the 5' promoter DNA sequence for their respective genes. Eighteen hours after transfection, cells were treated with nicotine alone, or nicotine along with the indicated nAChR inhibitors. Using each inhibitor was able to repress *MMP* RLA. (B) Collagen degradation assays were conducted on CCL-210 normal lung fibroblast cells. The use of any nAChR inhibitor could abrogate collagen degradation.

Nicotine induces MMP transcription and invasion through E2Fs

Previous studies had shown that nicotine could induce migration, invasion and EMT *in vitro* (37, 41). The MMP family is responsible for the degradation of the surrounding extracellular matrix, a crucial process for several stages of metastasis. Further, we had demonstrated that the MMPs responsible for these processes, *MMP2, MMP9, MMP14,* and *MMP15* are E2F target genes (Chapter 4 and 448). We created A549 cells that stably express an shRNA targeting E2F1, E2F3, or a non-targeting control ShRNA. Using these cells, we performed invasion assays with 20% serum as chemoattractant. Cells that were depleted of either E2F1 or E2F3 could not respond to nicotine for the induction of invasion *in vitro* (Figure 31A). Next, we conducted ChIP assays on A549 cells that were either serum starved or treated with nicotine for 48 hours (Figure 31B). Using antibodies against Rb and E2F1, we observed that when A549 cells were treated with nicotine, Rb was recruited to *MMP9, MMP14,* or *MMP15* to a much lower level than serum starved control cells. This suggests that Rb was phosphorylated and inactivated upon nicotine stimulation, corresponding with previous studies on proliferative promoters (37). On the *MMP14* promoter, the loss of Rb was also correlated with a stronger recruitment of E2F1, suggesting that this promoter is also more active upon nicotine stimulation. The *MMP2* promoter appeared to have no change in Rb recruitment; however E2F levels were slightly decreased (Figure 31B). Given that E2F1 appeared to be a repressor of *MMP2*, it is logical that nicotine treatment would result in the dissociation of a repressor to yield an activation of transcription.

Since nicotine could not induce invasion in the absence of either E2F1, or E2F3, and nicotine modulates Rb and E2F recruitment to *MMP* promoters, we next examined the effects of nicotine treatment on transcription of *MMP*s when E2Fs were depleted. Using two separate clones of A549 cells stably expressing shRNA targeting E2F3 or a non-targeting control vector, cells were made quiescent by serum starvation, then either kept in serum-free media or treated with 1 μM nicotine for 24 or 48 hours before RNA was collected. qRT-PCR results showed that when E2F3 was depleted, basal *MMP2* mRNA levels were induced (lane 1 compared to lane 4 and 7) (Figure 31C). In addition, compared to the basal levels, there was no further induction of *MMP2* mRNA after treatment with nicotine (lane 4 compared to lane 5 and 6; lane 7 compared to 8 and 9). This suggests that E2F3 was required for induction of *MMP2* by nicotine. In A549 cells expressing the non-targeting shRNA*, MMP9, MMP14*, and *MMP15* levels were all induced by nicotine as predicted (Figure 31D). Correlating with previous data showing that E2F3 depletion by siRNA decreased mRNA levels of *MMP9*, *MMP14,* and *MMP15* (448), the A549 cells stably transfected with shRNA targeting E2F3 also had less basal expression of *MMP9, MMP14, and MMP15* (lane 1 compared to lane 4 and 7)*.* In addition, compared to the basal levels, there was no further induction of *MMP9, MMP14, or MMP15* mRNA after treatment with nicotine (lane 4 compared to lane 5 and 6; lane 7 compared to 8 and 9) (Figure 31D). This suggests that E2F3 was required for induction of *MMP9, MMP14, and MMP15* by nicotine as well.

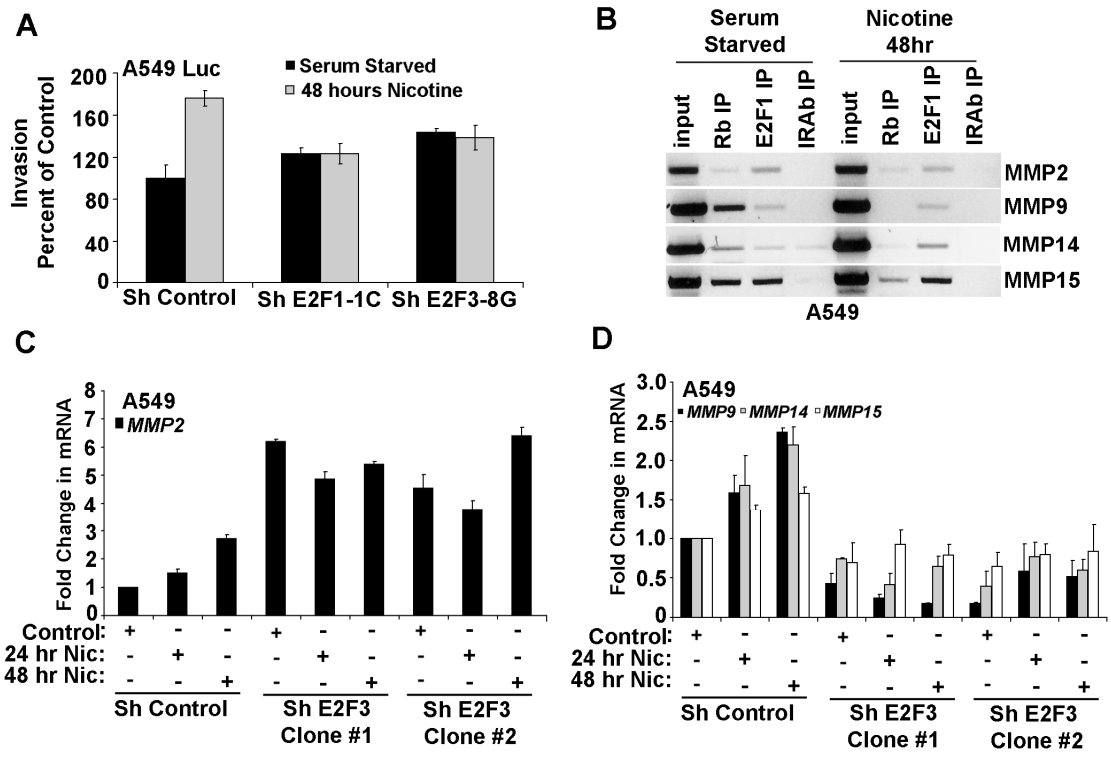

Figure 31. Nicotine induces *MMP* transcription and invasion through E2Fs. (A) A549 cells stably expressing shRNA targeting E2F1, E2F3 or a non-targeting control were used for invasion assays. Cells lacking E2F1 or E2F3 did not respond to nicotine, whereas control cells had 2-fold more invasion. (B) A549 cells were either serum starved, or treated with nicotine for 48 hours, and then ChIP assays were preformed. Nicotine promoted the dissociation of Rb from *MMP9, MMP14,* and *MMP15* promoters, with a slight enhanced binding of E2F1 on the *MMP14* promoter, whereas the *MMP2* promoter had less E2F1 binding after nicotine treatment. (C-D) qRT-PCR was used to examine *MMP2, MMP9, MMP14*, and *MMP15* RNA levels after treatment with nicotine. E2F3 was required for nicotine-mediated induction of MMP gene transcription.

Nicotine promotes growth of A549-luc cells in an E2F-dependent manner

Previous studies had shown that nicotine could promote growth, metastasis, and tumor recurrence in an immunocompetent mouse model of lung cancer (51). Given that the induction of MMP gene transcription, invasion, and collagen degradation mediated by nicotine required E2Fs, we next studied the effect of depleting E2F1 and E2F3 in an orthotopic model of lung cancer. SCID-beige mice (Charles River Laboratory) were surgically implanted with 250,000 A549 cells stably expressing the firefly luciferase gene (A549-luc) (Caliper) directly into the right lung. Six hours following surgery, animals were randomized, and then implantation of the tumor in the lungs was monitored using the Caliper-IVIS 200 system after administration of luciferin. Mice were imaged once per week, and treated with 1 mg/Kg nicotine in 100 μL PBS, or the PBS control thrice weekly. At the end of the experiment, animals underwent extensive necropsy and vital organs were separately imaged to identify nodes of metastasis (Figure 32) Nicotine treatment significantly enhanced the lung bioluminescence (Figure 33B). Further, cells depleted of E2F1 or E2F3 had less bioluminescence than the controls and did not respond to nicotine treatment (Figure 33A-B). To confirm these observations seen *in vivo*, lung bioluminescence was examined *ex vivo*. Mice treated with nicotine had more lung bioluminescence (Figure 33C-D), indicating that the injection was localized to the lungs, and not disseminated into the pleura. In addition, cells depleted of E2F1 and E2F3 had less bioluminescence *ex vivo* as well.

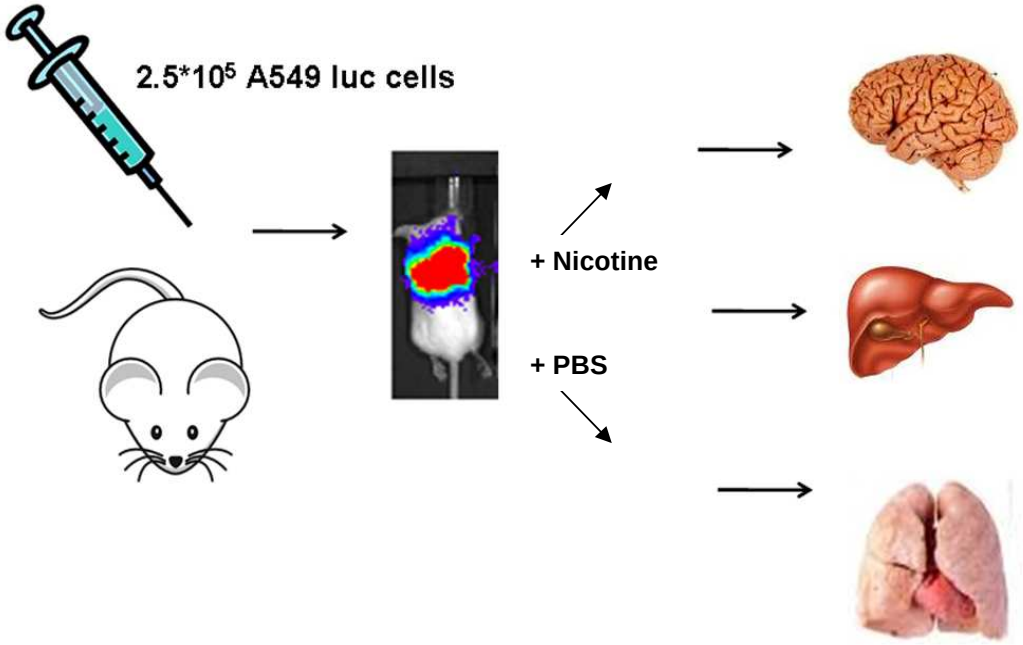

Figure 32. Schematic representation of an orthotopic model of lung cancer metastasis. SCID-beige mice are given a 100 μL injection of 250,000 cells directly into the right lung. Mice are randomized, then treated thrice weekly for five weeks either with PBS or 1 mg/Kg nicotine by intraperitoneal injection. At the completion of the study, mice were anesthetized, and the brain, liver, and lungs were imaged *ex vivo* for the identification of smaller regions of bioluminescence, being indicative of micrometastases. These signals were quenched *in vivo* due to the bright primary tumor signal in the lung.

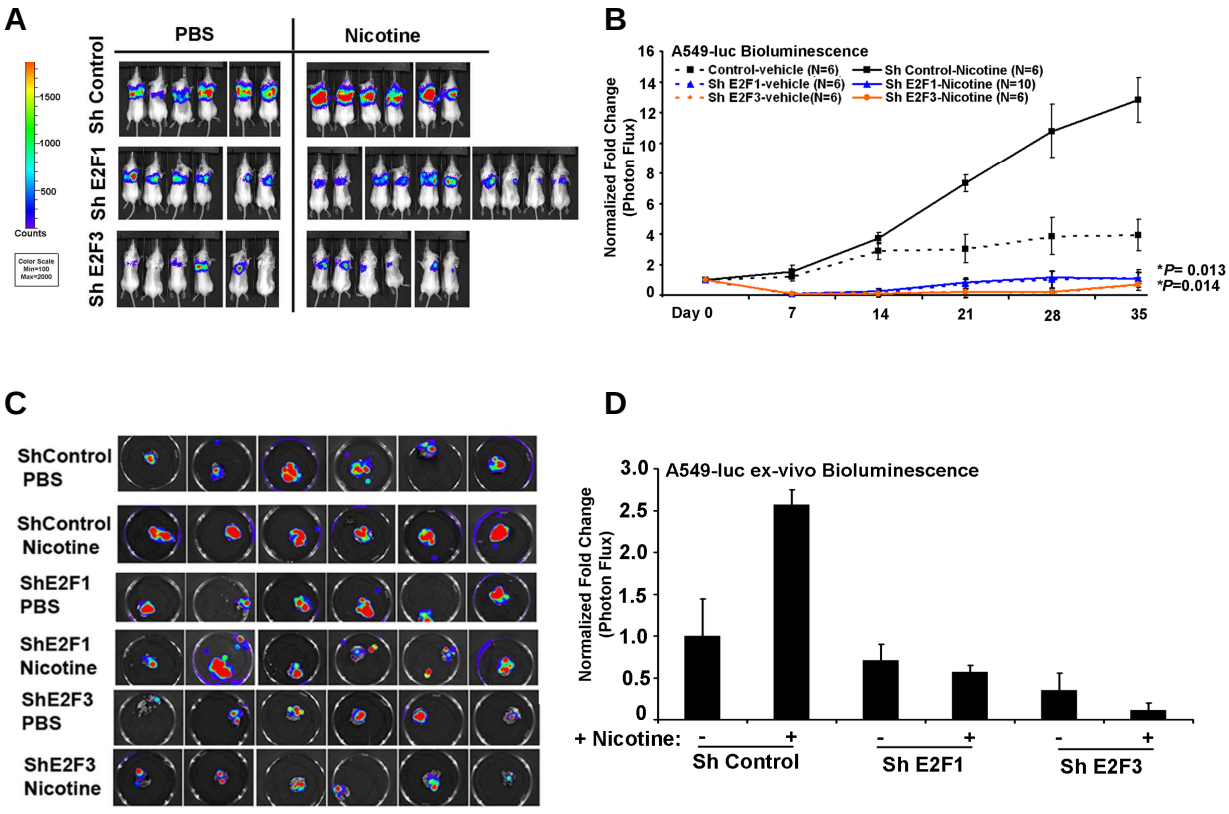

Figure 33. Nicotine promotes growth of A549-luc cells in an E2F-dependent manner *in vivo.* (A-B) A549-luc cells were implanted into the lung of SCID-beige mice, and mice were imaged once per week for five weeks. Image of total lung bioluminescence at day 35. Data were quantified and *P*-values were calculated for Sh E2F1 (**P=0.013*) and Sh E2F3 (**P=0.014*) groups treated with nicotine compared to Sh Control group treated with nicotine. (C-D) Lungs were removed from animals during routine necropsy and imaged *ex vivo* to ensure that observed signal in the mouse was due to efficient implantation of cells into the lung rather than the pleural cavity. Mice treated with nicotine had an increase in bioluminescence compared to the control. Cells lacking E2F1 or E2F3 could not respond to nicotine.

Nicotine promotes metastasis of A549-luc cells in an E2F-dependent manner

To determine if nicotine promoted metastasis to other organs, we performed routine necropsy of mice at the termination of the experiment. The brain, liver and lungs of each mouse were imaged separately within fifteen minutes post mortem using the Caliper-IVIS system. Organs were placed in 30 mg/Kg luciferin in a 60 mm tissue culture dish, and then images were taken at 1 second exposure. Animals implanted with the A549-luc cells with Sh control vector and treated with nicotine had the highest number of overall metastases, having 5/6 mice with brain metastasis, and 6/6 mice with liver metastasis. The PBS treated control mice had significantly less brain metastasis, 1/6, but still a fair amount of liver metastasis at 50% (Figure 34A-B). This is interesting due to the temporal regulation of metastasis in humans as well, where brain metastasis occurs after long periods of latency (545). Further, the Sh E2F1 group that was treated with PBS had more liver (4/6) and brain metastasis (2/6) than controls, however the nicotine treated Sh E2F1 group did not have the induction of metastasis observed in the nicotine treated Sh control group (5/10 liver; 1/10 brain). Mice implanted with Sh E2F3 cells did not have any observable metastasis in the brain in both groups, and the least amount of liver metastasis out of the three groups (1/6 liver mets in PBS group; 2/6 liver mets in nicotine group). These data suggest that E2Fs are required for the metastatic dissemination of lung cancer cells in response to nicotine, and that E2F3 might play a major role in growth and metastasis.

A

Number of Mice with Metastases

		Liver Mets	Brain Mets
Sh Control	Vehicle (N=6)	3	1
	Nicotine (N=6)	6	5
Sh E2F1	Vehicle (N=6)	4	2
	Nicotine (N=10)	5	1
Sh E2F3	Vehicle (N=6)	1	0
	Nicotine (N=6)	2	0

B

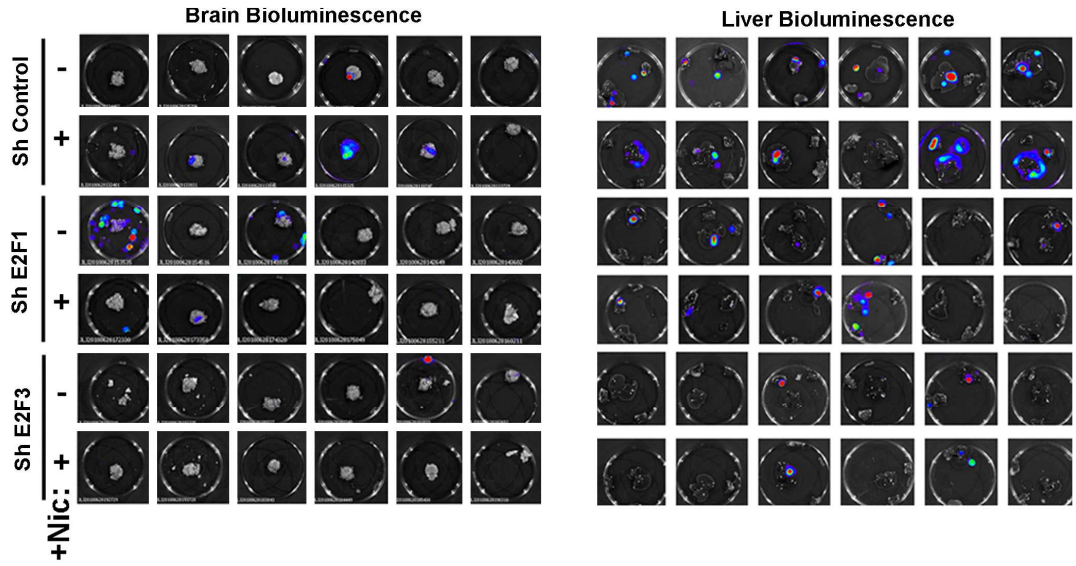

Figure 34. Nicotine promotes metastasis of A549-luc cells in an E2F-dependent manner. (A-B) Mice implanted with either Sh Control, Sh E2F1, or Sh E2F3 cells were subjected to routine necropsy, then brains and livers were imaged *ex vivo.* Mice with Sh Control tumors when treated with nicotine had many more brain metastases than the PBS group. Further, mice lacking either E2F1 or E2F3 did not respond to nicotine, and had far less brain metastasis than controls.

Discussion

Given the significant role that MMPs play in pathological conditions, the exact biological substrates for MMPs in the metastatic process are still poorly understood. MMP2 and MMP9 are the traditional gelatinases, being able to degrade gelatin and type IV collagen (314). These MMPs are both secreted into the pericellular space, and can also function through the activation of latent growth signals (546). This would further explain how nicotine could enhance proliferation of cells orthotopically implanted, as well as affecting the metastatic dissemination. Further, MMP14 is a type I collagenase, and is tethered to the cell surface. Numerous studies have demonstrated that MMP14 is the most crucial protease for metastasis (294, 324, 434). The biological functions of MMP15 are still less clear, however it is structurally similar to MMP14, and had been shown to impart an invasive phenotype in certain tumor types (547-549). MMP15 could also make cells resistant to apoptosis, which indirectly promoted cells escaping from the primary tumor and traveling to the distant site (550).

Prior work demonstrated that proliferative signaling via the nAChRs induced the dissociation of Rb from E2F1 (37). The Rb-E2F pathway is deregulated in about 90% of lung cancers, suggesting a critical role for these proteins in maintaining a normal cell phenotype (551). Further, inactivation of Rb by gene mutation or hyperphosphorylation caused it to dissociate from E2Fs. The free E2Fs could then bind to specific E2F-binding sites on the promoters of proliferative genes like *cdc6*, *cdc25a*, *TS*, and others, thereby stimulating

transcription leading to S-phase entry (552). In addition, there is evidence that E2F family members themselves have gene amplification, increased expression, or mutation in a variety of cancers, including NSCLC (553-555). However a correlation between E2F protein levels and advanced staged cancers has yet to be made.

These studies show that cancer cells activate metastasis genes such as *MMP2, MMP9, MMP14*, and *MMP15* by utilizing similar mechanisms that control proliferative gene expression in response to nicotine. Further, since multiple E2F family members are known to regulate the basal expression of MMP genes (448), it is possible that multiple E2F family members can also mediate the effects of nicotine. Depletion of E2F1 or E2F3 had a significant effect on the growth of primary tumors implanted into the lungs of mice, but it also completely abrogated the effects of nicotine-induced metastasis. We had previously demonstrated that β-arrestin-1 is required to mediate the effects of nicotine at proliferative promoters. In that study, β-arrestin-1 facilitated the acetylation of histones in NSCLCs in response to nicotine, however the acetylation of key transcription factors has not yet been tested. E2F1 can be acetylated, and the acetylation of E2F1 can make it bind to DNA more strongly (556). It seems likely that β-arrestin-1 might also regulate the expression of *MMP* genes in response to nicotine, and might also regulate the acetylation of E2F1 at these promoters. Src was also required for mediating the effects of the nicotine-β-arrestin-1 signaling cascade. In addition, Src had been shown to elicit an NNK mediated protein

kinase cascade as well, resulting in increased migration and invasion of human lung cancer cells (557). The signaling events between nicotine binding to nAChRs, and then E2Fs regulating *MMP* genes remain unclear, but may also involve Src.

It is possible that E2F family members are regulating other genes involved in metastasis. Previous studies in mouse models demonstrated that tumors harvested from nicotine treated mice had lower levels of E-cadherin and ZO-1 throughout the tumor (51). Further, we had demonstrated *in vitro* that treatment with nicotine can induce EMT changes such as a gain of vimentin expression and loss of E-cadherin, invasion and migration in a panel of cell lines (41). Further, it had been reported that the expression pattern of nAChR subunits are different in tumors from smokers and non-smokers (558). Given the ability of nicotine to promote *MMP* transcription and collagen degradation through multiple subunits of nAChRs, it is possible that general antagonists of nAChR signaling might prove beneficial in controlling metastasis of lung cancers as well.

Summary and Conclusions

Nearly two decades after the discovery of a link between E2F activity and cell cycle control, there is new evidence linking the Rb-E2F pathway to nearly all hallmarks of tumorigenesis (264). It is believed that the strongest mechanism that Rb utilizes to regulate the cell cycle is though its inhibitory interaction with E2F transcription factors. As a cell moves through the cell cycle from G1 to S phase, Rb is hyperphosphorylated by a variety of kinases, particularly cyclin D and cyclin E-associated kinases, inactivating the Rb protein, and it is subsequently released from E2F transcription factors. Rb does not work alone, however, and also utilizes its pocket protein family members, p107 and p130, to stringently regulate gene expression (8). The E2F family encodes ten transcription factors, and of these members only E2F4 can interact with all the pocket proteins. Indeed, it is the interaction between inhibitory E2F4 and E2F5 with p107 and p130 that keeps cells in a quiescent state until mitogenic stimulation initiates the cell cycle.

Not surprisingly, the Rb-E2F pathway is a critical target in a variety of cancer types (124, 449). At the level of growth factors and growth factor receptors, a variety of mutations are observed in both solid tumors and hematapoietic malignancies, including *HER2* amplification in breast cancer and the famous Philadelphia chromosome, *BCR-ABL* in chronic mylegenous

leukemia (559, 560). At the front of the intracellular portion of growth factor receptors, key kinases, such as K-Ras and Src are tethered into the hydrophobic lipid bilayer and act as mediators of extracellular signals (561, 562). It is well-known that these genes can also be mutated in cancers. Other downstream molecules such as cyclins and Cdks are also mutated in cancer; famously, in breast cancer cyclin D is overexpressed (563). All these mutations impact the readout of the Rb-E2F pathway. Further, mutations of Rb are found in a variety of cancers, and this has also been correlated with the overexpression of several E2F family members in certain cancers; E2F transcription factors are overexpressed in a portion of cancers with wild type Rb as well (66).

We had previously demonstrated that Rb can also be inactivated by an additional, non cyclin-dependent kinases, Raf-1, or c-RAF (36). When this interaction was inhibited in lung cancer, there was less proliferation, angiogenesis, and tumor growth. We now show that the Rb-Raf-1 interaction is also required for lung colonization in an *in vivo* model of metastasis. Further, when E2Fs were depleted, collagen degradation, invasion, and migration were all abrogated; MMPs are the essential regulators of all of these processes. These processes were also inhibited when the Rb-Raf-1 interaction was inhibited with RRD-251. Nicotine can stimulate the interaction of Raf-1 with Rb, leading to eventual inactivation of Rb, and ungoverned E2F activity at target gene promoters (38). Our findings show that the downstream effects of Rb and E2F at *MMP* promoters require multiple subunits of nAChRs and multiple E2F family

members. Future *in vivo* experiments could reveal whether blocking the Rb-Raf-1 interaction is also effective at inhibiting lung metastasis. These studies along with other *in vivo* models could open the door to developing novel therapeutics for treatment of NSCLC.

In the multi-step model of cancer, the development and progression of the disease is similar to Darwinian evolution. Cancer cells require advantages genotypes to promote survival and proliferation: where rare precursor cells (or cancer stem cells) will eventually, after many clonal selections, give rise to a virulent tumor cell population. In this model of selection, the propensity to metastasize is a rare trait that is acquired later, possibly after the tumor has reached critical mass. Given that metastasis is indeed a rare event, and that even cells which have entered circulation do not have a high likelihood to survive, this model begs the question: How can metastasis ever proceed?

Studies presented here suggest a differing model of metastasis, where the same proteins that conferred a proliferative advantage in early stages of disease also contribute to metastatic dissemination—the genetic abnormalities required for early stages promote the later dissemination and growth. The decision for cells to metastasize is not made until additional mutations are present or signals are received that would aid cells in their journey to a new site. This suggests that genes that are involved in oncogenesis might have overlapping functions in promoting metastasis, making it difficult to compartmentalize genes as sole

regulators of initiation or metastasis. Thus, growth promoting gene families, such as the E2Fs, might be master regulators of every stage of progression. The E2F family is known to regulate proliferation, angiogenesis, apoptosis, DNA damage repair, differentiation, and now metastasis. This could also explain how small cell populations can metastasize; for example small primary breast cancers can display detectable cells in the bone marrow (564). Further, although elegant microarray studies have identified genes that are different in metastatic populations, what is lacking from these discussions is that aside from the handful of genes identified, the remainder of the genotype is nearly identical between primary tumors and metastatic tumors. In addition to the E2F family, other key oncogenes with known roles in proliferation have also been shown to enable metastatic spread, including *cyclin D1, c-MYC* and *RAS* (498, 565-571). Genes which are required for normal cell division, and not considered oncogenes, have also been implicated in metastasis such as *EGR1* and *Sp1* (572-574). Taken together, although a large effort has been made to understand the differences between primary tumors and metastasized tumors, targeting the similarities might prove to be a more efficient strategy for treating cancer patients irrespective of the stage of their disease. Further, we believe that a significant amount of additional studies on the pathways that govern tumor initiation, progression and metastasis would be necessary to develop therapeutic strategies that will combat cancer effectively.

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