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E2F1 AND TUMOR SUPPRESSION: THE ROLE OF p21, miRNAS, AND THE DNA DAMAGE RESPONSE

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**E2F1 AND TUMOR SUPPRESSION: THE ROLE OF p21, miRNAS, AND THE
DNA DAMAGE RESPONSE**

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**E2F1 AND TUMOR SUPPRESSION: THE ROLE OF p21, miRNAs, AND THE
DNA DAMAGE RESPONSE**

A DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
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in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Regina Lanell Weaks, M.S.

Houston, Texas

August 2010

Dedication

I would like to dedicate this dissertation to my family who have been the driving force behind me, always there to support me, pick me up when I fall, push me when I want to give up, and congratulate me when I finish;

To my friends who have been my voice of reason and have kept me sane throughout this journey;

And to Colby, who came into my life during this process and managed to love me despite the craziness.

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E2F1 AND TUMOR SUPPRESSION: THE ROLE OF p21, miRNAS, AND THE DNA DAMAGE RESPONSE

Publication No. _____

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E2F1 is a multi-faceted protein that has roles in a number of important cellular processes including cell cycle regulation, apoptosis, proliferation, and the DNA damage response (DDR). Moreover, E2F1 has opposing roles in tumor development, acting as either a tumor suppressor or an oncogene depending on the context. In human cancer, E2F1 is often deregulated through aberrations in the Rb-p16^{INK4a}-cyclin D1 pathway. In these studies we examined three mechanisms by which E2F1 might mediate its tumor suppressive properties: p21-induced senescence, miRNAs, and the DNA damage response. We found that E2F1 acts as a tumor suppressor in response to ras activation through a non-apoptotic mechanism requiring ARF and p53, but not p21. However, p21-loss inhibited two-stage chemical carcinogenesis in FVB mice. In response to E2F1 overexpression, we found that 22 miRNAs are differentially regulated in mouse epidermis, including let-7a, let-7c, and miR-301. Additionally, regulation of miR-301 involves binding of E2F1 to its promoter. Finally, our data indicate a role for E2F1 at sites of DNA

damage requiring E2F1's phosphorylation at serine 31 which may involve DNA repair. Further, this role in the DDR may affect tumor aggressiveness and multiplicity. In all, we have explored three mechanisms for E2F1-induced tumor suppression and identified E2F1's role in the DNA damage response as a likely contributor to this phenomenon.

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List of Abbreviations

| | |
|-------|---|
| °C | Celsius |
| μg | microgram |
| μl | microliter |
| μM | micromolar |
| 53BP1 | p53 binding protein 1 |
| A | adenine |
| AC | acetone |
| ACTR | activin receptor protein |
| Ad | adenovirus |
| Apaf1 | apoptosis associated factor 1 |
| ARF | alternative reading frame |
| ATM | ataxia telangiectasia mutated |
| ATR | ataxia telangiectasia and Rad3 related |
| Avg | average |
| B-CLL | B-cell chronic lymphocytic leukemia |
| BRCA1 | breast cancer susceptibility protein 1 |
| BRCT | C-terminal domain of breast cancer susceptibility protein |
| BrdU | bromodeoxyuridine |
| BRG1 | brahma-related gene 1 |
| BRM | brahma |
| BSA | bovine serum albumin |

| | |
|------|---|
| CBP | CREB binding protein |
| Cdc | cell division cycle |
| CDK | cyclin dependent kinase |
| cDNA | complementary DNA |
| CH72 | keratinocyte cell line derived from a squamous-cell carcinoma |
| ChIP | chromatin immunoprecipitation |
| CtBP | c-terminal binding protein |
| CtIP | CtBP-interacting protein |
| CREB | cAMP-response element binding protein |
| DAB | 3,3-Diaminobenzidine tetrahydrochloride |
| DAPI | 4',6-diamidino-2-phenylindole |
| DBD | DNA binding domain |
| DCR2 | decoy receptor 2 |
| dCTP | deoxycytidine triphosphate |
| DDR | DNA damage response |
| DEC1 | deleted in esophageal cancer 1 |
| DHFR | dihydrofolate reductase |
| DMBA | 7, 12-dimethylbenz[a]anthracene |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DNMT | DNA methyl transferase |
| DP | dimerization partner |
| DSB | double-strand break |

| | |
|-------------------------------|--|
| E2F | E2-binding factor |
| EDTA | ethylenediaminetetraacetic acid |
| ERK | extracellular signal-regulated kinase |
| FLPer | flippase recombinase expressing mouse |
| FRT | flippase recognition target |
| FVB | friend virus B-type strain |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GCN5 | general control nonderepressible 5 |
| H2AX | histone 2AX |
| H ₂ O ₂ | peroxide |
| h | hour(s) |
| H/Ha | Harvey |
| HDAC | histone deacetylase |
| HPV | human papilloma virus |
| HRP | horseradish peroxidase |
| Hy3/5 | fluorophore |
| IgG | immunoglobulin G |
| IHC | immunohistochemistry |
| INK4a | inhibitor of Cdk 4 a |
| IP | immunoprecipitation |
| IR | ionizing radiation |
| K5 | keratin 5 promoter |
| K | Kirsten |

| | |
|-------|---|
| Kb | kilobase |
| KI | knockin |
| KO | knockout |
| let-7 | lethal-7 |
| LNA | locked nucleic acid |
| MAF | mouse adult fibroblast |
| MB | marked box |
| MEF | mouse embryonic fibroblast |
| MEOX2 | mesenchyme homeobox 2 |
| miRNA | microRNA |
| miR | microRNA |
| ml | milliliter |
| mm | millimeter |
| mM | millimolar |
| MMP-1 | matrix metalloproteinase-1 |
| mRNA | messenger RNA |
| Myc | myelocytomatosis oncogene |
| N | neuroblastoma |
| NaOH | sodium hydroxide |
| NCS | neocarzinostatin |
| Neo | neomycin |
| NHF | normal human fibroblast |
| NIH | National Institutes of Health-derived Swiss outbred mouse |

| | |
|------------------|---|
| nmol | nanomole |
| nt | nucleotide |
| OCT | optimum cutting temperature |
| O/N | overnight |
| PAGE | polyacrylamide gel electrophoresis |
| PAI-1 | plasminogen activator inhibitor-1 |
| PBS | phosphate buffered saline |
| PBST | phosphate buffered saline with Tween-20 |
| PCR | polymerase chain reaction |
| PGK | phosphoglycerate kinase promoter |
| PRMT5 | protein arginine N-methyltransferase 5 |
| PVDF | polyvinylidene fluoride |
| Ras | rat sarcoma |
| Rb | retinoblastoma protein |
| RIPA | radio-immunoprecipitation assay |
| RIN | RNA integrity number |
| RISC | RNA-induced silencing complex |
| RNA | ribonucleic acid |
| RT | room temperature |
| S29A | serine mutated to alanine at amino acid position 29 |
| SA- β -gal | senescence-associated β -galactosidase |
| SAHF | senescence-associated heterochromatic foci |
| SA-HRP | streptavidin-horseradish peroxidase |

| | |
|--------|--|
| SCC | squamous cell carcinoma |
| SCGE | single cell gel electrophoresis |
| SDS | sodium dodecyl sulfate |
| Ska2 | spindle and kinetochore-associated protein 2 |
| snRNA | small nuclear RNA |
| SSIN | inbred selected SENCAR mouse |
| SV40 | simian virus 40 |
| SUV39H | suppressor of variegation 3-9 homolog |
| T | thymidine |
| TBE | tris-borate-EDTA |
| Tdt | terminal deoxynucleotidyl transferase |
| TFIID | transcription factor IID |
| Tip | transactivator of transcription (Tat) interacting protein |
| TopBP1 | topoisomerase II-binding protein 1 |
| TPA | O-tetradecanoyl-phorbol-13-acetate |
| TRAPP | transport protein particle |
| TUNEL | terminal deoxynucleotidyl transferase dUTP nick end labeling |
| WT | wildtype |
| X-gal | 5-bromo-4-chloro-3-indolyl B-D-galactopyranoside |

Chapter 1: Background and Introduction

1.1 E2F1 and the E2F Family

The E2F family is a group of transcription factors consisting of E2Fs 1-8 along with three dimerization partners, DP 1, 2/3, and 4, reviewed in (1). Yet, there are nine E2F proteins in total because two forms of E2F3 are transcribed from alternate promoters, E2F3a and E2F3b (2, 3). E2F1 was first identified as a cellular component that binds to the adenoviral E2 promoter (E2-binding factor), thus the name (4, 5). Most members of the E2F family (E2F1-6) contain a DNA binding domain and a dimerization domain through which E2F proteins heterodimerize with one of three DP proteins to allow high affinity DNA binding to promoters (Figure 1.1) (6-10). E2F7 and 8 bind DNA independent of DP (11-14). As well, E2F1-3 contain a cyclin A/cdk2 binding domain important for cell cycle control, and E2F1-5 contain a pocket protein binding domain also important in cell cycle control as well as transcription. Additionally, E2Fs 1-5 contain a marked box domain important in protein-protein interactions and, in the case of E2F1, apoptosis (15, 16). In all, E2F family members are multi-faceted molecules with roles in cell cycle progression and proliferation, transcription, apoptosis, and an emerging role in the DNA damage response. Consequently, many E2Fs, including E2F1, have been identified as key players in cancer development both as tumor suppressors and as oncogenes.

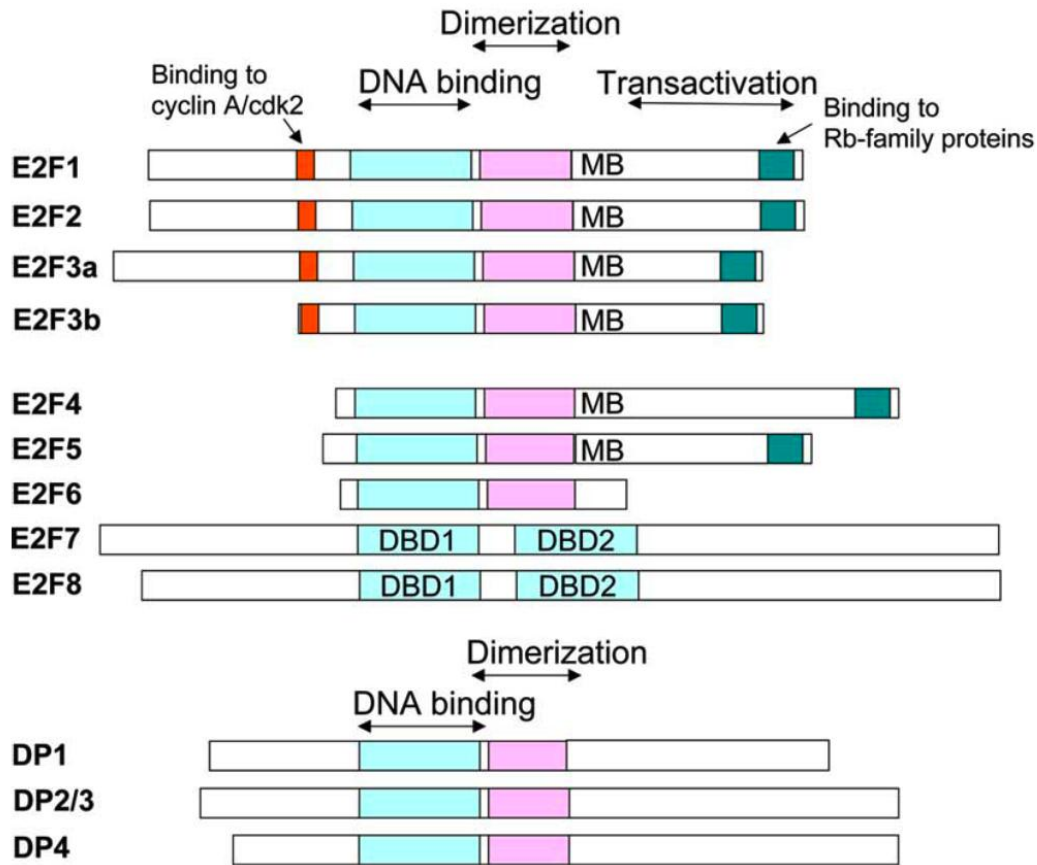


Figure 1.1. The E2F family. The DNA binding domain (DBD) is indicated in light blue, and the dimerization domain is shown in pink. The domains required for a cyclin A/cdk2 and Rb family member binding are shown in red and green, respectively. Mouse homologs of human DP-2 are referred to as DP-3; and, several differentially spliced variants of DP2/DP3 exist that are not shown. Also, there are two isoforms of E2F7 which differ in their C-terminus (not shown) (1). MB=marked box, cdk=cyclin-dependent kinase, DP=dimerization partner, Rb=retinoblastoma

1.2 E2F1 and Cell Cycle

E2F1 functions as a regulator of the cell cycle and promotes entry of cells into S phase by transcriptionally regulating target genes. E2F family members are intricately regulated through a series of protein-protein interactions, including binding by the pocket proteins Rb, p130, and p107 (17, 18). When bound by hypophosphorylated Rb, E2F1 is transcriptionally inactive (19-21). However, as Rb and other pocket proteins are phosphorylated by cyclin/CDK (cyclin-dependent kinase) complexes, E2F is released to activate genes important for cell cycle progression, such as cyclin E, dihydrofolate reductase (DHFR), and Cdc6 (cell division cycle 6) (21). Positive regulation of CDK activity occurs through the cyclic expression and degradation of cyclins, while negative regulation occurs through two classes of CDK inhibitors, the Ink4a proteins and the Cip/Kip family, which includes p21 (22).

1.3 E2F1 and Transcription

E2Fs form active transcriptional factors through dimerization with DP proteins, which then bind canonical E2F recognition sites within promoters. Yet, functionality as transcriptional repressors or activators is determined primarily by the E2F subunit. The E2Fs have classically been divided into three classes: activators (E2F1, 2, and 3a), repressors (E2F3b, 4, 5), and repressors independent of Rb family members (E2F6, 7, and 8) based on expression patterns (23) and structure.

However, it is becoming increasingly clear that this classification is over simplistic at best. Many family members have been shown to both repress and activate transcription based upon the cellular context (1). For example, besides its well-documented role as a transcriptional activator of cell cycle related genes, E2F1 has been shown to repress an equal number of genes, as well (24-28).

Regulation of transcriptional activity of E2Fs is primarily through the Rb family. Pocket proteins block the transactivational domain of activator E2Fs, which prevent the transcription factor from recruiting the basal transcriptional machinery subunit, TFIID (29). Additionally, this association prevents the recruitment of co-activators such as ACTR, GCN5, TRAPP, Tip60, and p300/CBP (30-34). E2F1, specifically, can be blocked from binding DNA through its interaction with Rb (35). As well, E2F-DP heterodimers can be converted to transcriptional repressors through the pocket protein-mediated recruitment of chromatin remodeling factors such as BRM/BRG1, HDACs, DNMT1, CtIP, CtBP, SUV39H, PRMT5 and others (1, 36-50).

1.4 E2F1 and Apoptosis

In addition to its ability to promote proliferation, E2F1 has unique properties when compared to most other E2F family members. When over-expressed, E2F1 has the capacity to efficiently induce apoptosis *in vitro* and *in vivo* in mice (23, 51, 52). Of the other E2F family members, E2F2, 3, and 4 can also promote apoptosis, although E2F3 may do so through E2F1 (53-58). Apoptosis is especially important

for maintaining tissue homeostasis and because it can be deregulated in many cancers. E2F1's induction of apoptosis can be either in a p53-dependent or p53-independent manner. E2F1 can upregulate p53 protein levels by transcriptionally activating the tumor suppressor ARF, but ARF is not required for p53-dependent apoptosis by E2F1 (59). Also, E2F1 overexpression can stimulate ATM transcription and autophosphorylation leading to p53 stabilization and apoptosis (60-62). In p53-independent apoptosis, E2F1 up-regulates a homolog of p53 (p73), and this mechanism appears to play an important role in apoptosis induction in human tumor cell lines that have lost p53 (63). E2F1 can also promote apoptosis through alternative routes, such as the activation of caspases and apoptosis associated factor 1 (Apaf1) (64, 65).

1.5 E2F1 in Human Cancer

As well as being able to promote apoptosis, E2F1 also distinguishes itself from most other members of the E2F family because it has both tumor suppressive and oncogenic properties. In human cancer, numerous examples exist demonstrating E2F1's dual nature. Several cancer cell lines and primary tumors have amplifications in chromosome 20q, the location of E2F1. These include prostate and leukemia cell lines, as well as the following tumor types: melanoma, cervical, esophageal, and colon cancer (66-71). Moreover, many cancers have increased levels of E2F1 expression, though this may be a consequence of Rb pathway deregulation. This includes colorectal, lung, and breast cancer (72-74).

Finally, increased E2F1 expression is correlated with a worse outcome in some cancer types (74). All of this points to E2F1 acting as an oncogene in human cancer. On the other hand, some tumor types (bladder, colon, and B-cell lymphoma) are more aggressive when E2F1 expression is low, indicating the possibility that E2F1 is acting as a tumor suppressor in these cancers (75-78).

1.6 E2F1 as an Oncogene

E2F1 has oncogenic properties both *in vitro* in cell culture, and *in vivo* in mouse models. NIH 3T3 cells and rat embryo fibroblasts were induced to form colonies with E2F1 overexpression, indicative of transformation (79, 80). As well, cells expressing an E2F1 mutant incapable of Rb inhibition formed tumors in nude mice (80). *In vivo*, post-mitotic lense fiber cells are stimulated to re-enter the cell cycle upon transgenic E2F1 expression, though p53 stimulates apoptosis of these cells (56). Further, expression of E2F1 in megakaryocytes blocks the differentiation of these cells into platelets leading to severe thrombocytopenia and accumulation of megakaryocytes (81). These examples highlight the proliferative potential associated with E2F1 overexpression. Additionally, several mouse models demonstrate the oncogenic potential of E2F1. In K5 E2F1 transgenic mice, which overexpress E2F1 in the skin and other keratin 5-expressing tissues, spontaneous tumors develop in the skin, vagina, forestomach, and odontogenic epithelium (82). In the context of p53 loss, this tumorigenesis is accelerated (23). In a transgenic model where E2F1 is expressed in the liver, all mice develop adenomas of the liver

by 10 months of age, with some developing hepatocellular carcinomas by one year (83). Finally, E2F1 expression in testes has complex consequences. Constant E2F1 expression leads to massive apoptosis and testicular atrophy, while a short exposure to E2F1 still causes apoptosis, but also dysplastic changes resembling carcinoma in situ (83, 84). Thus, E2F1 has clear oncogenic properties that seem to vary depending on cellular context.

1.7 E2F1 as a Tumor Suppressor

Besides its oncogenic potential, E2F1 has also been demonstrated to function as a tumor suppressor. In contrast to a report by Xu and colleagues mentioned previously, Lee et al. found that E2F1 inhibited the growth of NIH 3T3 cells in response to Ras (79, 85). In addition, transformation of keratinocytes by HPV E6 and E7 was inhibited by high E2F1 levels, but not lower levels (86). This again points to the importance of cellular context and perhaps oncogenic stimuli in determining the effects of E2F1. Similar results are seen in mouse models. E2F1 knockout mice have an increased incidence of thymic lymphomas and reproductive tract tumors, indicative of a role in tumor suppression by E2F1 (87, 88). Moreover, E2F1 loss in Rb heterozygous mice predisposes these mice to some tumor types, while protecting them from others (89). Additionally, K5 E2F1 overexpressing mice are resistant to two-stage skin carcinogenesis (82). Together, these models demonstrate the dual nature of E2F1 as both an oncogene and tumor suppressor, and give clues to the molecular switch involved in this process, which seems to

depend on cellular context, E2F1 expression levels, timing, and genetic environment.

1.8 Multi-Stage Carcinogenesis Assay

The mouse two-stage carcinogenesis assay, or multi-stage carcinogenesis assay, consists of three well-defined stages that encompass consistent changes leading to carcinogenesis. This assay is often used to model epithelial-type tumors. The three steps of this multi-stage model include: initiation, promotion, and progression. The first step involves application of a single dose of a genotoxic agent, typically the polycyclic aromatic hydrocarbon 7, 12-dimethylbenz[a]anthracene (DMBA), to the shaved dorsal skin of mice. After metabolism to its ultimate carcinogen, covalent binding of the electrophile to DNA results in an A \Rightarrow T transversion in codon 61 of the Ha-ras gene, leading to a constitutively active protein (90, 91). Promotion involves repeated application of a non-mutagenic, inflammatory agent such as TPA (O-tetradecanoyl-phorbol-13-acetate) to the skin. Addition of TPA to mouse skin results in synchronized proliferation of basal keratinocytes with concomitant gene expression characteristic of S phase (92). This typically results in hyperplasia and may be accompanied by epigenetic changes that allow the clonal expansion of initiated cells and results in the formation of benign exophytic lesions called papillomas. This step is required, as a single application of DMBA alone does not result in skin carcinogenesis. Moreover, promotion is reversible and papillomas will often regress. Finally, the last

stage, progression, involves the accumulation of genetic changes leading to the conversion of a subset of papillomas into malignant squamous cell carcinomas characterized by invasion of the basement membrane and metastasis (93).

1.9 K5 E2F1 Transgenic Model

Since the Rb-p16^{INK4a}-cyclin D1 pathway is the most mutated pathway in human cancer, and this pathway controls activity of the E2F family members, it was reasoned that an *in vivo* model for deregulation of this pathway could be made by overexpressing E2F1. Though some might argue that a transgenic mouse that overexpresses a protein at a very high level may not be physiological, there is much evidence to the contrary. This evidence includes data from the mouse as well as human studies. During the two-stage carcinogenesis assay in the mouse, E2Fs 1-5, have all been shown to be upregulated in tumors as compared to normal skin (94, 95). In addition, high levels of E2F1 have been found in many human tumors including those from breast, pancreas, thyroid, esophagus, lung, colon, and bladder (73, 96-102); and, these levels tend to correlate with increased tumorigenicity and decreased disease-free survival. Additionally, the E2F1 gene is amplified in a subset of gastric and colorectal carcinomas (73). Though these high levels of E2F1 protein may be only a consequence of tumorigenesis and not causative, the issue warrants further study.

Thus, a transgenic mouse was generated using a construct containing the bovine (K5) keratin 5 promoter, human E2F1 cDNA, the rabbit β -globin intron and

the SV40 polyadenylation signal (52). This construct directs expression of E2F1 to the basal layer of the epidermis as well as other epithelial tissues. Two lines were generated, line 1.0 and line 1.1, both with different expression levels. At the RNA level, the two lines differ by 3-4 fold, with line 1.0 being the highest expressing line; the protein levels were estimated to be 80 and 50- fold above wildtype level by Western blot for line 1.0 and line 1.1 respectively (52). The lower expressing line, 1.1, will be used for all experiments.

Phenotypic characteristics of the K5 E2F1 transgenic mouse include epidermal hyperplasia, hair loss, and increased epidermal apoptosis as compared to wildtype mice (52). These mice also develop spontaneous tumors of the skin, vagina, forestomach, and odontogenic epithelium (82). Moreover, K5 E2F1 overexpression cooperates with a v-Ha-ras transgene to induce papilloma formation, as does loss of p53 (23, 52). However, K5 E2F1 mice are resistant to a classic chemical carcinogenesis protocol driven by ras activation (82).

1.10 Physiological Relevance of Tumor Studies in the K5 E2F1 Transgenic Model

Several experiments have been completed to date that shed light on E2F1's role in tumor development in the mouse skin. As mentioned previously, using the K5 E2F1 transgenic model, our lab has shown that E2F1 is tumor suppressive when mice were subjected to a two-stage carcinogenesis protocol including DMBA and TPA (103). Though the physiological relevance of this model has been called into question, recent studies offer support for the validity and study of E2F1

overexpression in the skin. In a report using a skin-specific conditional knockout of Rb, Rb^{-/-} mice developed less tumors than Rb^{+/+} mice when subjected to a two-stage protocol. Furthermore, when E2F1 was analyzed by Western blot, its levels were increased in the tumors arising from Rb^{-/-} mice as compared to the wildtype controls (104). Thus, these experiments suggest that Rb loss and E2F1 overexpression in the skin may be acting similarly to result in tumor suppression in both models in response to activated H-ras. Moreover *in vitro* studies using mouse cells and human cancer cell lines demonstrate that Rb is required for transformation by H-ras (105). In fact, tumors that activate the ras pathway almost never contain a mutation in the Rb gene. This lends additional support to the idea that E2F1 overexpression, a consequence of Rb inactivation, can be tumor suppressive in the context of an activated H-ras protein and is physiologically relevant.

1.11 K5 Myc Transgenic Model

The transcription factors Myc and E2F1 have many similarities. Both proteins regulate genes important for cell cycle progression; and both can induce apoptosis, principally dependent on p53. Additionally, Myc and E2F1 can transform rodent cells that have been immortalized or in cooperation with *ras*. And finally, deregulated expression of either protein can lead to spontaneous tumorigenesis (106). To further explore these similarities and possible differences with E2F1, we generated the K5 Myc transgenic mouse.

The K5 Myc transgenic mouse model was generated in much the same way as the K5 E2F1 transgenic mouse. Briefly, a genomic murine *c-myc* fragment containing the entire Myc coding region was cloned into a vector under the control of the bovine K5 promoter with the rabbit β -globin intron 2 and the SV40 polyadenylation signal. A total of three K5 Myc transgenic lines were generated, MM1, MM3, and MM5. The level of *c-myc* overexpression for each line is 3.7-fold, 6.0-fold, and 2.8-fold, respectively, by northern blot (106). The lowest expressing line, MM5, will be used for all experiments.

The K5 Myc transgenic mouse has similar phenotypic characteristics to the K5 E2F1 mouse. These mice display epidermal hyperplasia, increased proliferation and apoptosis in the skin, as well as hair loss. Spontaneous lesions in the K5 Myc mouse include: ameloblastomas, squamous cell carcinomas, papillomas, and odontogenic tumors (106). Inactivation of *E2f1* or p53 enhances spontaneous tumorigenesis in response to K5 Myc (106, 107). Conversely, loss of cyclin-dependent kinase 4 (*Cdk4*) inhibits K5 Myc-induced tumors, pointing to a role for proliferation in spontaneous tumor formation driven by K5 Myc (108). In contrast to K5 E2F1 mice, the K5 Myc mice are not resistant to two-stage chemical carcinogenesis, suggesting key differences between the functions of these two transcription factors.

Chapter 2: The Role of p21 and Senescence in Tumor Suppression by the E2F1 Transcription Factor

2.1 Abstract

The transcription factor E2F1 has opposing roles in tumor development, acting as either a tumor suppressor or an oncogene depending on the context. In human cancer the importance of E2F1 is demonstrated by the fact that it is targeted for deregulation in many cancer types through aberrations in the Rb-p16^{INK4a}-cyclin D1 pathway. In order to study the role of E2F1 in tumorigenesis, our lab generated a transgenic mouse overexpressing E2F1 in the skin under the control of the keratin 5 promoter (K5 E2F1). We have since demonstrated that this mouse model is resistant to a classical chemical carcinogenesis protocol. Further, additional tumor studies have indicated a requirement for both ARF and p53 in E2F1-mediated tumor suppression. Yet, the exact mechanism of E2F1's tumor suppressive ability remains to be determined. Subsequently, we have found that mice lacking ARF and overexpressing E2F1 have no significant difference in apoptosis when compared with mice on a wild type *Arf* background when both are treated with chemical carcinogens. Additionally, untreated skin from mice overexpressing E2F1 and lacking ARF exhibits epidermal hyperplasia and reduced levels of p21 expression. Yet, when we crossed the K5 E2F1 transgenic mice onto a p21-null background, we found that loss of p21 in the skin enhances proliferation and apoptosis, but not hyperplasia. Further, a two-stage carcinogenesis experiment shows that mice null for p21 are resistant to tumor development independent of the K5 E2F1 transgene. Additionally, data from our lab shows that K5 E2F1 mice lacking either ARF or p53 have a decreased number of senescent cells in the epidermis when treated with a

short-term DMBA/TPA protocol; but no difference was seen in senescence-associated β -galactosidase (SA- β -gal) staining with or without p21. In conclusion, we hypothesize that E2F1 acts as a tumor suppressor in response to Ras activation through a non-apoptotic mechanism requiring ARF and p53, but not p21.

2.2 Introduction

To study the role of E2F1 in epithelial tumor development, our lab has previously generated a transgenic mouse expressing human E2F1 under the control of the keratin 5 promoter (K5 E2F1) (52). In this model, we have found that E2F1 can act as both a tumor suppressor and as an oncogene. Overexpression of E2F1 was found to increase hyperplasia, proliferation, and apoptosis in the epidermis, but did not block terminal differentiation (52). K5 E2F1 activity alone induces spontaneous tumorigenesis in 50% of mice greater than 1 year of age with tumors arising in the skin, mouth, forestomach, vagina, and other K5-expressing tissues (82). Additionally, E2F1 overexpression cooperates with the v-Ha-*ras* transgene to promote spontaneous papilloma development (52). As well, loss of one or both copies of p53 enhances E2F1-induced spontaneous tumorigenesis in the skin, resulting in the formation of carcinomas (23). In contrast to these findings, K5 E2F1 mice are resistant to two-stage skin carcinogenesis, initiated by an activating *ras* mutation, and this is independent of the promotion agent used (82, 109).

E2F1's ability to suppress tumor formation in the K5 E2F1 model has subsequently been further explored. Experiments from our laboratory indicate a requirement for both ARF (alternative reading frame) and p53 in this system as K5 E2F1 transgenic mice lacking either gene are sensitive to a two-stage skin carcinogenesis protocol (109). Moreover, K5 E2F1 mice lacking either ARF or p53 have decreased numbers of senescent cells in the epidermis when treated with a short term DMBA/TPA protocol (Russell, unpublished data). These findings,

together, point to senescence as a plausible mechanism for E2F1's anti-tumor activity.

Senescence is an irreversible exit from the cell cycle that may be induced by stresses such as oncogenes, oxygen radicals, telomere dysfunction, or other DNA damaging agents (110). As well, senescence may be initiated through the activity of tumor suppressors or by chromatin remodeling (111). Senescence was first identified almost 50 years ago as a phenomenon known as the "Hayflick Limit" which served as a barrier to infinite doubling of human fibroblasts in culture (112, 113). Two types or sub-classes of senescence exist, premature/cellular and replicative senescence. Premature senescence differs from so-called replicative senescence because it does not involve the shortening of telomeres or misfunction of telomeric proteins (111, 114).

Characteristic features of the senescence program include large, flat cells that are resistant to apoptosis and have altered gene expression patterns. In particular, a common marker for senescent cells is the expression of senescence-associated β -galactosidase (SA- β -gal) activity when stained with X-gal (5-bromo-4-chloro-3-indolyl B-D-galactopyranoside) at pH 6.0 (115). SA- β -galactosidase activity has since been correlated with an increase in classic acid lysosomal β -galactosidase enzyme activity due to an increase in the number of lysosomes found in senescent cells (116). Other markers used to detect senescence include: p16, p15, p53, ARF, p21, SAHFs (senescence-associated heterochromatic foci), DEC1 (deleted in esophageal cancer 1), and DCR2 (decoy receptor 2) (117).

Several genes have been reported to induce senescent-like phenotypes. Of particular interest to us, the p19^{ARF}/p53/p21^{Cip1} pathway has been implicated in senescence in many systems. ARF is sufficient to induce senescence in both mouse and human fibroblasts (118, 119). The tumor suppressor p53 can cause permanent cell cycle arrest possibly through increased DNA binding and transcriptional activity (111, 120, 121). As well, p21, a downstream target of p53, has been implicated to play a major role in this phenomenon. Levels of p21 are increased in senescent human fibroblasts and mouse keratinocytes, while p21-null mice have reduced age-related senescence and are less responsive to ras-induced premature senescence (122-124). Moreover, overexpression of E2F1 in normal human fibroblasts induces senescence as evidenced by morphology, senescence associated β -galactosidase (SA- β -gal) staining, and upregulation of the senescence associated genes, MMP-1 (matrix metalloproteinase-1), stromelysin, and PAI-1 (plasminogen activator inhibitor-1) (119). Furthermore, E2F1-induced senescence requires both ARF and p53 in this system (118, 119).

Finally, this research is focused on exploring the possibility that E2F1-induced senescence can suppress tumorigenesis in the mouse skin model. We also want to discern the involvement of p21 in E2F1-mediated tumor suppression, given the fact that it is a major downstream target of p53; and, it has also been implicated in senescence. Thus, it is our hypothesis that E2F1 suppresses tumor formation by inducing senescence through a mechanism involving the cell cycle regulator, p21.

2.3 Materials and Methods

2.3.1 Mice: Previously described K5 E2F1 transgenic mice generated by our lab (52) were maintained in the FVB line, $\geq 95\%$ purity. K5 E2F1 mice were crossed to p21^{-/-} mice on an FVB background to generate K5 E2F1 mice that were heterozygous for p21. F1 crosses of these mice were used to generate transgenic and non-transgenic littermates who were either wildtype or null for p21. Genotyping was performed using sequence specific PCR primers.

2.3.2 SA- β -gal Assay: The β -galactosidase assay was performed on dorsal mouse skin sections snap frozen in OCT (optimum cutting temperature) tissue matrix (Electron Microscopy Sciences, Hatfield, PA), sectioned, and mounted on glass slides. SA- β -gal activity was detected using the Senescence Detection Kit (Biovision Research Products, Mountain View, CA). Briefly, sections were washed with 1X phosphate buffered saline (PBS) and fixed for 15 minutes at room temperature with 0.5 ml of fixative solution. After two 1X PBS washes, sections were stained in a solution containing 470 μ l of staining solution, 5 μ l of staining supplement, and 25 μ l of 20 mg/ml 5-bromo-4-chloro-3-indolyl B-D-galactopyranoside (X-gal), in dimethyl sulfoxide (DMSO) for 48 hours at 37°C. After the development of blue color, sections were counterstained with nuclear fast red for 5 minutes at room temperature and coverslipped with Permount (Biomed, Foster City, CA). The number of senescent cells per 10 mm of epidermis was counted.

2.3.3 BrdU Incorporation: Mice were injected with 170 μ l of 20 mM bromodeoxyuridine (BrdU) and sacrificed 20 minutes later. Dorsal skin was collected, fixed in formalin, paraffin-embedded, and sectioned. Skin sections were next de-paraffinized and hydrated, followed by blocking with 3% H₂O₂ (peroxide) in water for 10 minutes. After washing, antigen was retrieved with 10 mM citrate buffer, pH 6.0, for 10 minutes in a microwave oven followed by a 20 minute cool down. Slides were again washed and blocked using Biocare Blocking Reagent (BS966M, Biocare, Concord, CA) for 10 minutes. After an additional wash, slides were incubated with primary BrdU antibody (347580 (1:500), Becton Dickinson, Franklin Lakes, NJ) for 1 hour and 30 minutes at RT (room temperature). Following two washes, slides were incubated with biotinylated rabbit-anti-mouse Fab' (AXL5230M, Accurate Chemical and Scientific Corporation, Westbury, NY) for 15 minutes at room temperature. Slides were then washed 2x in buffer, and incubated with SA-HRP (streptavidin-horseradish peroxidase) (BioGenex Laboratories, Inc., San Ramon, CA) for 30 minutes at RT. Buffer wash was repeated and slides were incubated with DAB (3,3-Diaminobenzidine tetrahydrochloride) (Sigma-Aldrich, St. Louis, MO) while monitoring. Slides were again washed, and then counterstained, dehydrated, cleared, and coverslipped. The number of BrdU-positive cells per 10 mm of epidermis was counted.

2.3.4 Caspase-3 Assay: Dorsal skin was collected, fixed in formalin, paraffin-embedded, and sectioned. After de-paraffinization and hydration, endogenous

peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes. After washing and antigen retrieval in 10 mM citrate buffer, pH 6.0, for 10 minutes, sections were cooled for 20 minutes, washed, and incubated with a primary caspase-3 specific antibody (AF835, R&D Systems, Minneapolis, MN) for 30 minutes at RT. After washing, slides were incubated with a biotinylated goat-anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a 1:500 dilution for 30 minutes at RT. Following two 5 minute washes, slides were incubated with SA-HRP (BioGenex Laboratories, Inc., San Ramon, CA) for 30 minutes at RT. Again, slides were washed twice for 5 minutes each time, and stained with DAB (BioGenex Laboratories, Inc., San Ramon, CA) for 2-3 minutes. Finally, slides were washed, counterstained, dehydrated, cleared, and coverslipped for viewing. The number of caspase-3-positive cells per 10 mm of epidermis was counted.

2.3.5 p53 Immunohistochemistry: Skin sections were collected, fixed in formalin, paraffin-embedded, and sectioned. After de-paraffinization and hydration, sections were blocked with 3% H₂O₂ in water for 10 minutes and then washed. Upon antigen retrieval in 10mM citrate buffer, pH 6.0, for 15 minutes in a microwave and 20 minute cool down, slides were blocked with Biocare Blocking Reagent (BS966M, Biocare, Concord, CA) for 10 minutes. Slides were then incubated with primary antibody (NCL-p53-CM5p (1:500), Novocastra, St. Louis, MO) for one hour at RT followed by two buffer washes. Incubation with Envision labeled polymer, anti-rabbit-HRP (horseradish peroxidase) (Dako, Carpinteria, CA) for 30 minutes at RT was performed followed by 5 washes. Following a five minute incubation with DAB,

slides were washed, counterstained, dehydrated, cleared, and coverslipped. The percentage of p53-positive cells was determined microscopically per 1000 basal cells.

2.3.6 p21 Immunohistochemistry: Dorsal skin was collected, fixed in formalin, paraffin-embedded, and sectioned. After de-paraffinization and hydration, sections were blocked with 3% H₂O₂ in water for 10 minutes. After washing, antigen was retrieved with 1.0 mM EDTA (ethylenediaminetetraacetic acid), pH 8.0, for 10 minutes in a microwave and cooled for 20 minutes. Slides were blocked with Biocare Blocking Reagent for 10 minutes (BS966M, Biocare, Concord, CA). Slides were then incubated with p21 primary antibody (sc-6246, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:50 dilution O/N (overnight) at 4°C. Following two washes, slides were incubated with biotinylated rabbit-anti-mouse Fab' (AXL5230M, Accurate Chemical and Scientific Corporation, Westbury, NY) for 15 minutes at room temperature. Slides were then washed 5x in buffer, and incubated with SA-HRP (BioGenex Laboratories, Inc., San Ramon, CA) for 30 minutes at RT. Buffer wash was repeated and slides were incubated with DAB (Sigma-Aldrich, St. Louis, MO) while monitoring. Slides were again washed, and then counterstained, dehydrated, cleared, and coverslipped. The percentage of p21-positive cells per 1000 basal cells was determined microscopically.

2.3.7 Two-stage Mouse Skin Carcinogenesis Assay: Six- to eight-week old male and female mice were shaved 1-2 days prior to experiments. Mice were initiated

with 50 nmol of 9,10-dimethyl-1,2-benzathracene (DMBA) in 200 μ l of acetone applied topically to the dorsal skin. For long-term studies, tumors were promoted by the twice-weekly application of 12-O-tetradecanylphorbol-13-acetate (TPA), 2 μ g in 200 μ l of acetone. For short-term studies, TPA was applied twice, three and six days after DMBA treatment and mice sacrificed 24 hours after the last treatment. Mice were housed with littermates and examined weekly for papilloma formation.

2.4 Results

To investigate the mechanism of E2F1's tumor suppressive abilities, we performed immunohistochemistry on K5 E2F1 transgenic skin for ARF, p53, and p21. We found that K5 E2F1 mice have an increased percentage of epidermal cells positive for expression of ARF, p53, and p21 when compared to wild type mice (Figures 2.1 and 2.2). Further, when K5 E2F1 mice were crossed onto an *Arf*-null background, p53 and p21 staining in the epidermis was reduced (Figures 2.1 and 2.2). Additionally, loss of *Arf* leads to an increase in epidermal thickness in K5 E2F1 transgenic mice, but there is no effect on apoptosis following short-term carcinogen (DMBA/TPA) treatment (Figure 2.3) (109).

Next we crossed the K5 E2F1 mice onto a p21-null background and examined the epidermis for differences in proliferation, hyperplasia, and apoptosis. In untreated mice, loss of p21 lead to increased proliferation, as measured by BrdU incorporation, whether or not E2F1 was overexpressed, though this change was not statistically significant. Yet, this increase in proliferation did not lead to a

corresponding increase in hyperplasia. In fact, animals null for p21 have decreased epidermal thickness as compared to corresponding control mice. This is true in the absence or presence of E2F1 overexpression, though only significant in transgenic skin, $p=0.017$ (Figure 2.4).

Conversely, the overexpression of E2F1 in p21-null mice does result in increased epidermal thickness when compared with p21-null mice not overexpressing E2F1, $p=0.043$. Moreover, treatment with a short-term DMBA/TPA protocol does not change the trends identified for p21 loss on proliferation or hyperplasia. However, only the decrease in proliferation approaches significance, $p=0.077$ (Figure 2.5). Finally, we examined the result of p21 loss on its upstream effector p53 and found no effect on p53 expression. Nonetheless, apoptosis is increased in K5 E2F1 mice lacking p21, $p=0.0009$, as assayed by caspase-3 activity (Figure 2.6).

Previously, our lab has shown that K5 E2F1 transgenic mice are resistant to two-stage chemical carcinogenesis and this requires both ARF and p53 (109). However, since these experiments were done in an SSIN background, we needed to assess whether K5 E2F1 transgenic mice are resistant to two-stage chemical carcinogenesis on an FVB background before proceeding with experiments in this strain background. So, we initiated 13 wildtype and 13 K5 E2F1 mice with DMBA and promoted twice weekly with TPA for 22 weeks; and mice were scored weekly for papilloma development. Wild type mice were found to have an average of 3 tumors per mouse compared to less than one tumor per mouse for K5 E2F1 mice,

thus establishing the resistance of the transgenic mice to chemical carcinogenesis in an FVB background (Figure 2.7).

Next we assessed the effect of p21 loss on E2F1-mediated tumor suppression. We found that K5 E2F1 mice lacking p21 were resistant to a chemical carcinogenesis protocol. However, mice lacking p21, without the K5 E2F1 transgene, were also resistant to two-stage skin carcinogenesis (Figure 2.8). This result was unexpected and suggests a surprising requirement for p21 in skin papilloma development. Unfortunately, this requirement precludes us from establishing a role for p21 upregulation in E2F1-mediated suppression of skin carcinogenesis.

Finally, to address the possibility that senescence could be involved in tumor suppression by E2F1, we examined the skin of wildtype and K5 E2F1 transgenic mice for β -galactosidase activity. We found a high level of background staining in the FVB strain background as compared to epidermis from SSIN mice (Figure 2.9). Yet, it is unclear why this high level of β -galactosidase activity exists in FVB epidermis. As well, carcinogen treatment did not seem to affect β -galactosidase activity the same way for each strain. DMBA/TPA treatment increased the senescent cells in K5 E2F1 epidermis on an SSIN background, but senescent cells decreased in the same mice on an FVB background. Lastly, we stained K5 E2F1 epidermis with and without p21 for β -galactosidase (Figure 2.10). We could detect no difference in staining in the basal layer, hair follicle, or super basal layers. In all, because of the high background staining we could make no definitive conclusions

about the role of senescence in E2F1-mediated tumor suppression using β -galactosidase activity as a marker.

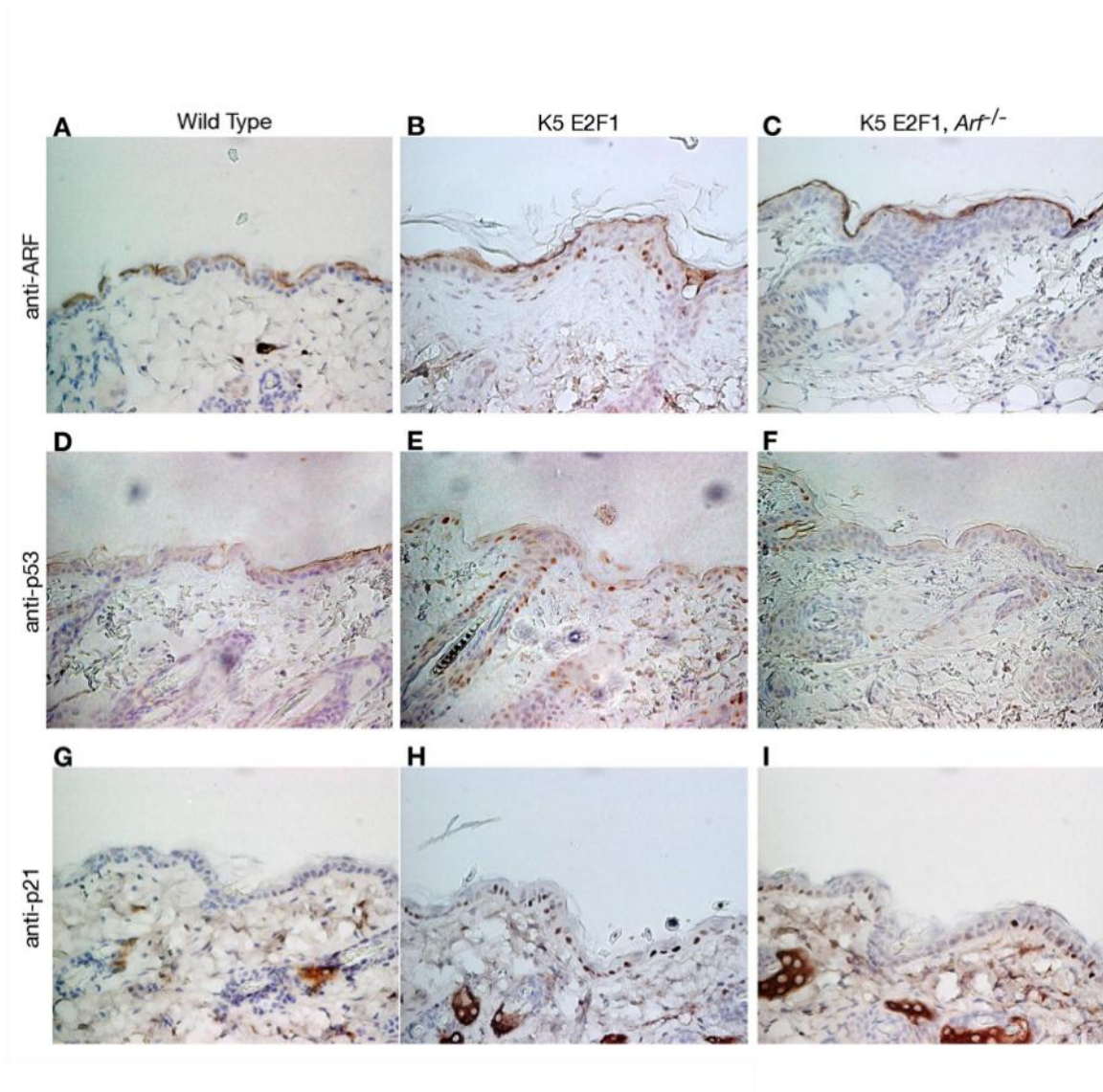


Figure 2.1. K5 E2F1 transgenic epidermis exhibits higher levels of ARF, p53 and p21 positive cells. Skin sections were taken from mice, fixed, and immunohistochemically stained for ARF (a, b, and c), p53 (d, e, and f), or p21 (g, h, and i). The genotypes were wild type (a, d, and g), K5 E2F1 transgenic (b, e, and h), or K5 E2F1 transgenic, $Arf^{-/-}$ (e, f, and i). Photographs were taken at 40x magnification (109).

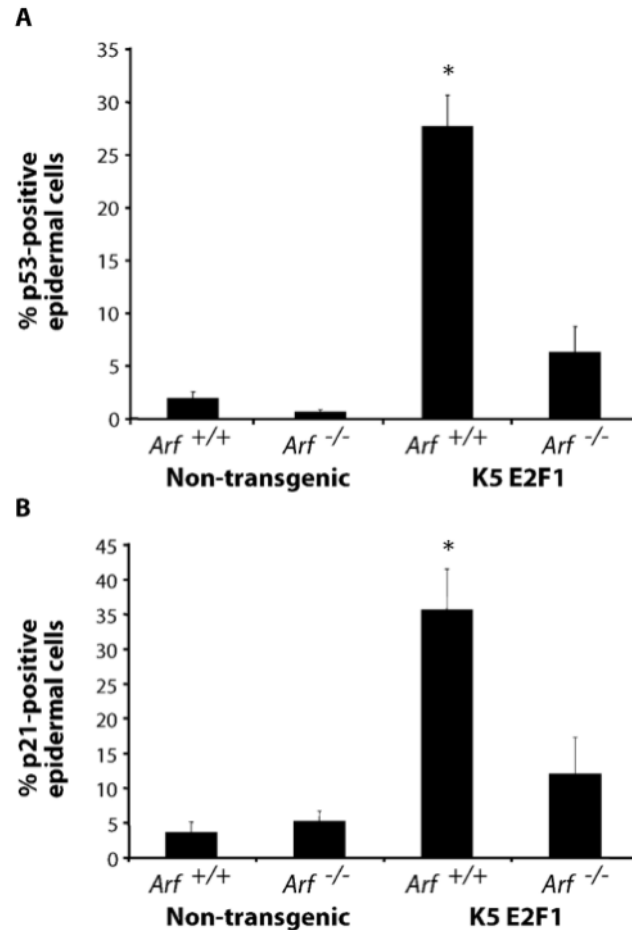


Figure 2.2. Inactivation of *Arf* reduces expression of p53 and p21 in K5 E2F1 transgenic epidermis. (A) Skin sections from nontransgenic and K5 E2F1 transgenic mice, with or without *Arf*, were immunohistochemically stained for p53. The average percentage of epidermal cells that stained positive for p53 was determined microscopically using samples from at least 5 mice for each genotype. (B) Skin samples from the same mice as in (A) were stained for p21 and the percentage of cells staining positive was determined microscopically and graphed. Bars represent standard error. * indicates $p < 0.05$ by t-test (109).

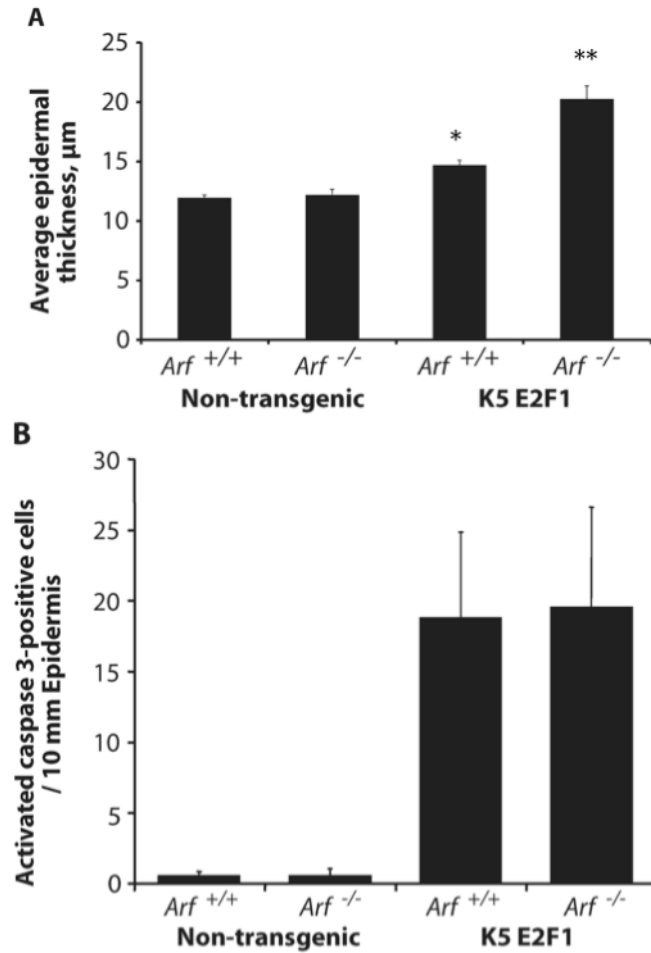


Figure 2.3. Inactivation of ARF induces hyperplasia but does not affect apoptosis. **(A)** The average epidermal thickness was determined by taking 20 random measurements of each dorsal skin sample from 5 mice of each genotype. **(B)** Skin sections were taken from three wild type, two $ARF^{-/-}$, four K5 E2F1, and four K5 E2F1, $ARF^{-/-}$ mice after a short term DMBA/TPA protocol. Skin sections were stained for activated caspase 3 as a marker of apoptosis and the average number of caspase positive cells per 10mm of epidermis is shown. Bars represent standard error. * indicates $p < 0.05$ by t-test (109).

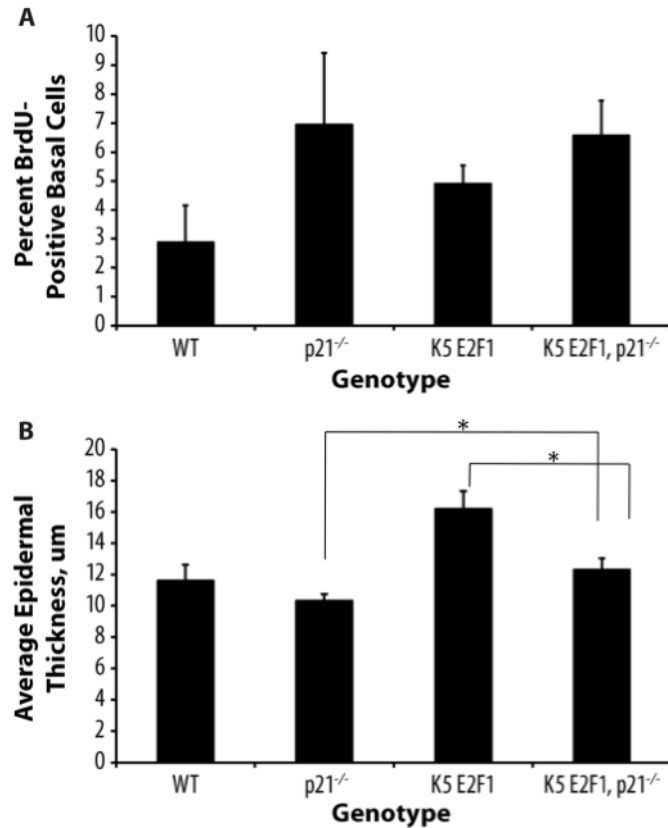


Figure 2.4. Inactivation of p21 increases BrdU-positive cells but not hyperplasia in K5 E2F1 transgenic epidermis. (A) Skin sections from non-transgenic and K5 E2F1 transgenic mice, either with or without p21 as indicated, were immunohistochemically stained for BrdU. The average percentage of epidermal cells that stained positive for BrdU was determined microscopically using samples from six mice for each genotype. **(B)** The average epidermal thickness was determined by taking 20 random measurements of each dorsal skin sample from the same mice as above. Bars represent standard error. * indicates $p < 0.05$ by t-test.

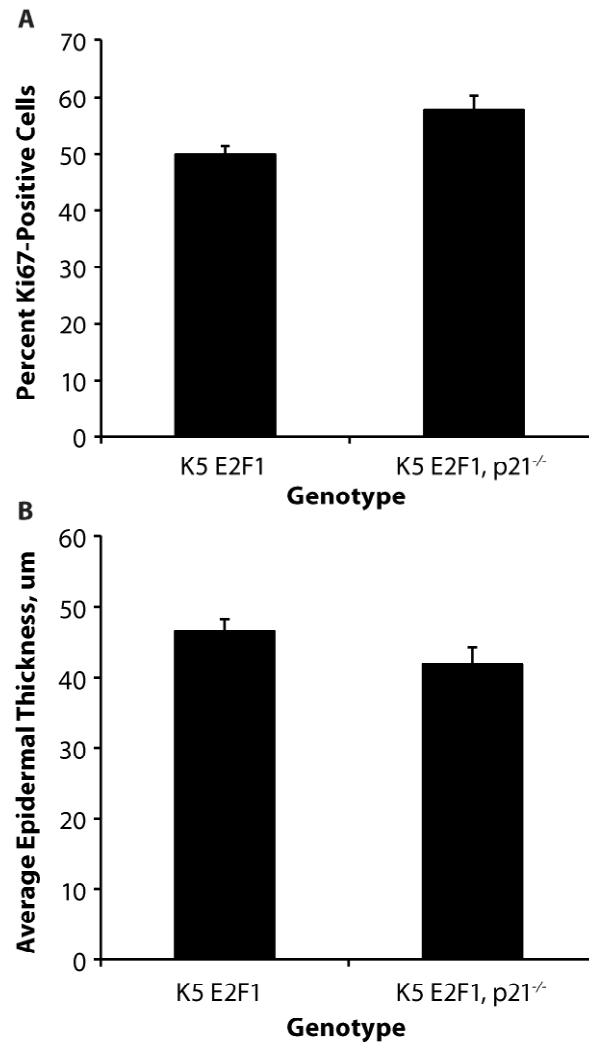


Figure 2.5. Short term DMBA/TPA treatment increases proliferation but not epidermal skin thickness in K5 E2F1 transgenic mice lacking p21. (A) Dorsal skin sections were collected and immunohistochemically stained for Ki67. The number of Ki67-positive cells per 1000 basal cells was calculated. Percentages are averages from at least 3 mice per genotype. **(B)** The average epidermal thickness was determined by taking 20 random measurements of each dorsal skin sample from the same mice as above. Bars represent standard error. No significance differences were detected by t-test, $p < 0.05$.

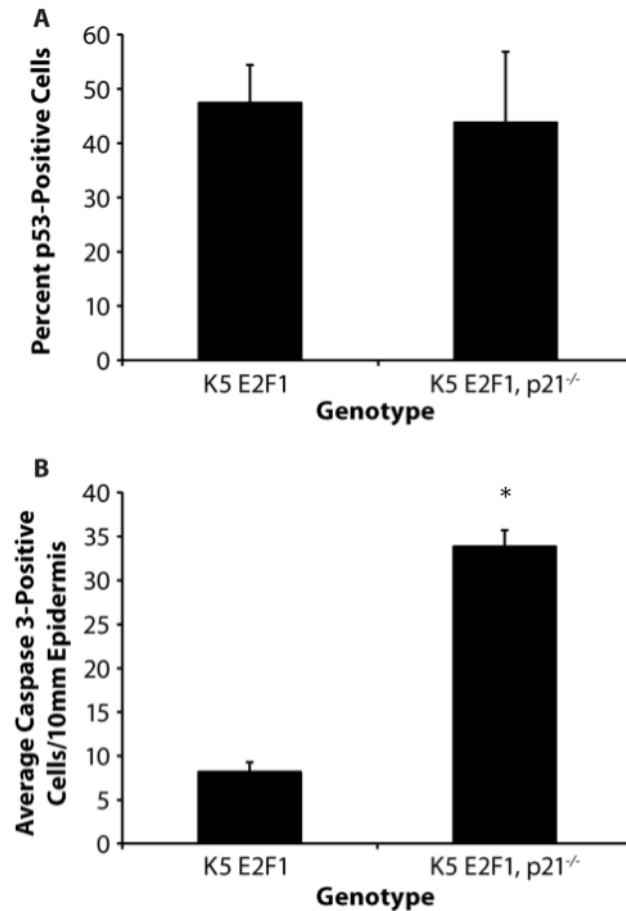


Figure 2.6. Apoptosis increases in K5 E2F1 transgenic epidermis when p21 is lost, but p53 levels are unchanged. (A) Dorsal skin was collected after short-term treatment with DMBA/TPA. Skin was stained for p53 and the number of p53-positive cells per 1000 basal cells was determined microscopically. At least three mice per genotype were analyzed. **(B)** Skin sections from the same mice as above were immunohistochemically stained for activated caspase 3. The average number of positive cells per 10 mm of epidermis was calculated. Bars represent standard error. * indicates $p < 0.05$ by t-test.

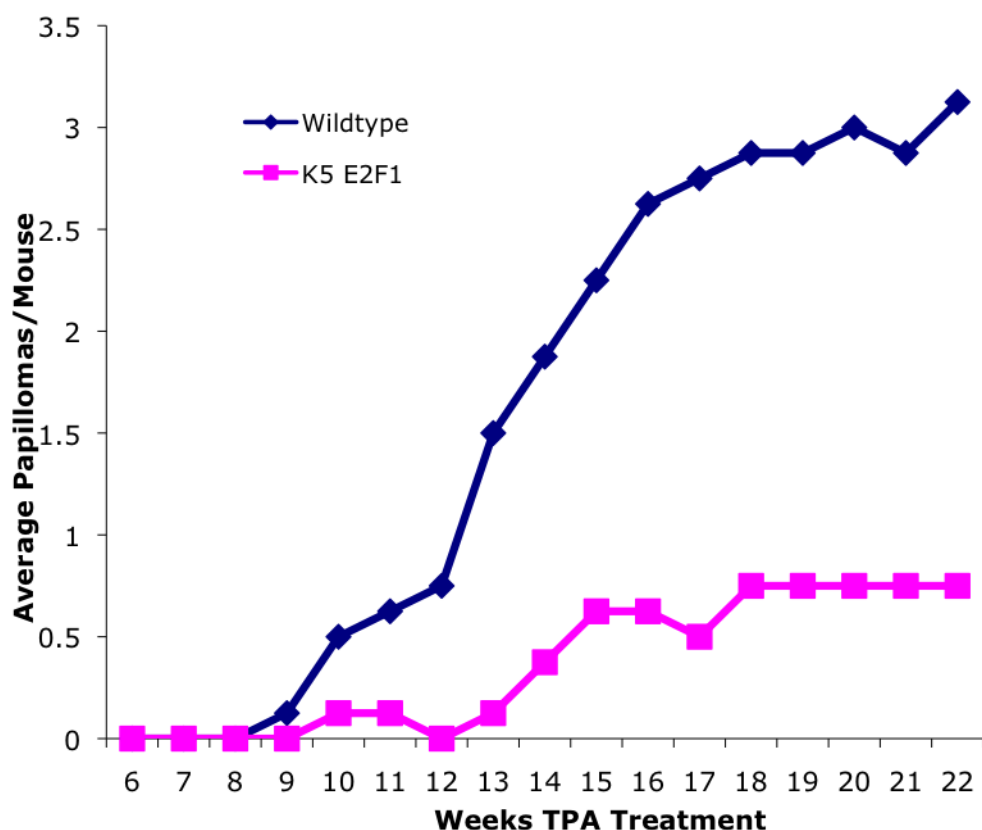


Figure 2.7. K5 E2F1 transgenic mice on an FVB background are resistant to two-stage chemical carcinogenesis. Mice were shaved 1-2 days prior to treatment. Wildtype (n=13) and K5 E2F1 (n=13) transgenic mice were initiated with 50 nmol DMBA at 6-8.5 weeks of age. Beginning two weeks after initiation, mice were treated twice weekly with 2 μ g of TPA for 22 weeks. Tumor formation was assessed weekly beginning 6 weeks after initiation and the average number of papillomas per mouse is plotted.

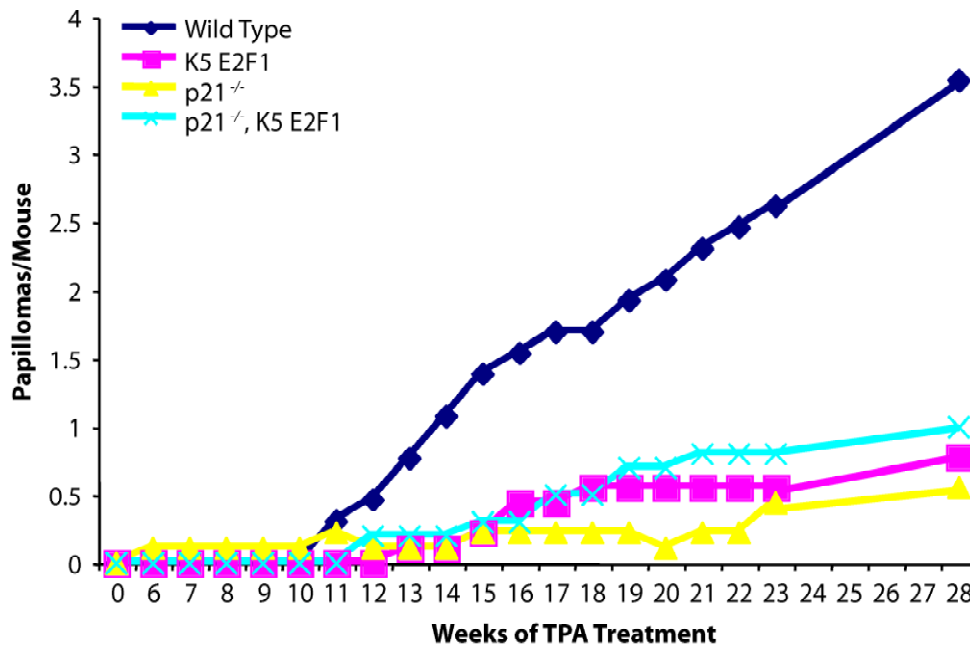
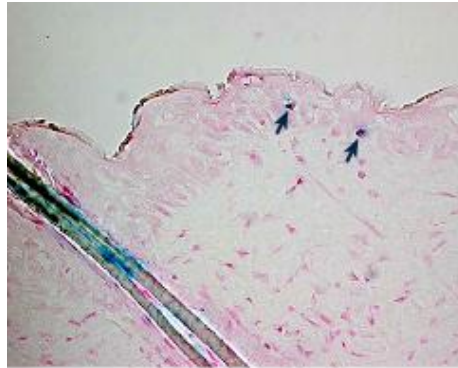


Figure 2.8. Suppression of two-stage carcinogenesis by E2F1 does not require p21. A total of 13 wildtype, nine p21^{-/-}, nine K5 E2F1 transgenic, and 10 K5 E2F1 transgenic p21^{-/-} mice in the FVB background were initiated with 100 nmol of DMBA at 6-8 weeks of age. Two weeks later, mice were treated with twice weekly applications of 2.0 µg of TPA for 16 weeks and 4.0 µg of TPA for another seven weeks. Mice were examined weekly for papilloma formation; and, the average number of papillomas per mouse is plotted.

A



B

| Strain-Genotype | AC/AC | AC/TPA | DMBA/AC | DMBA/TPA |
|---------------------|-------|--------|---------|----------|
| SSIN-WT | 7 | 3 | 11 | 8 |
| SSIN-K5 E2F1 | 7 | 7 | 1 | 20 |
| FVB-WT | 135 | 175 | 121 | 139 |
| FVB-K5 E2F1 | 113 | 160 | 125 | 114 |

Figure 2.9. FVB mice have a high level of β -galactosidase positive background staining when compared to SSIN mice when both are treated with a short-term DMBA/TPA protocol. (A) K5 E2F1 skin on an SSIN background stained for β -galactosidase activity. **(B)** Mice were initiated with 10 (SSIN) or 50 (FVB) nmol of DMBA or vehicle (acetone). Two days later, mice were treated with TPA or vehicle, and again two days after that. Mice were sacrificed 24 hours following the last treatment and dorsal skin sections were preserved in OCT tissue matrix. After sectioning, one to two mice per genotype (wildtype or K5 E2F1) and background strain (SSIN or FVB) were assayed for β -galactosidase activity using the Senescence Detection Kit from Biovision. The number of β -galactosidase-positive cells per 10mm of epidermis was determined microscopically.

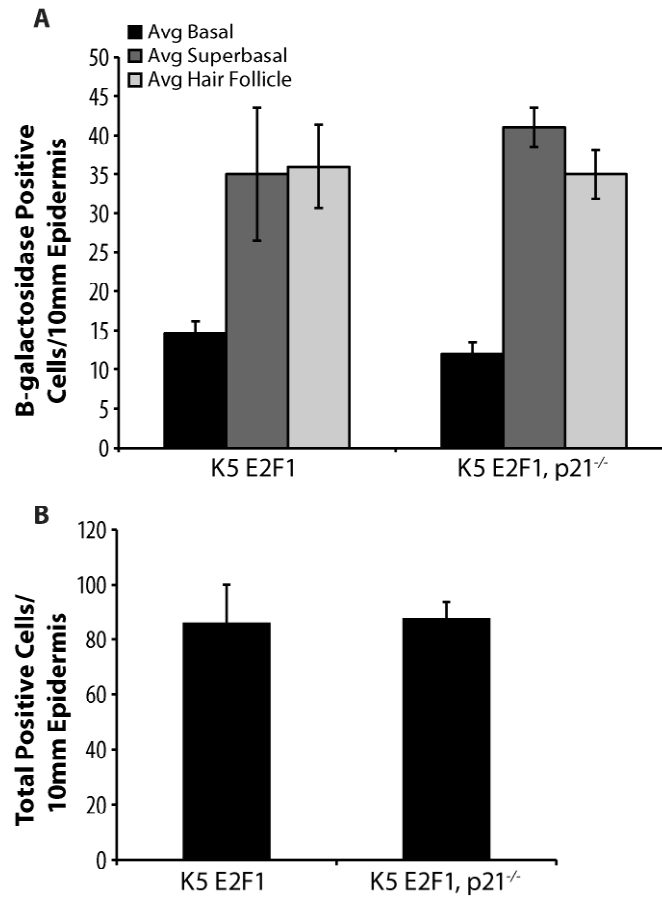


Figure 2.10. β -galactosidase activity is not affected by p21 status in either wildtype or K5 E2F1 transgenic mice. Mice were subjected to a short-term DMBA protocol. Dorsal skin was collected and embedded in OCT tissue matrix. Skin sections were stained using the Senescence Detection Kit from Biovision. **(A)** The total number of β -galactosidase-positive cells in the basal, superbasal and hair follicle compartments were counted and averaged separately per 10 mm of skin. Three mice per genotype were analyzed. **(B)** The total number of β -galactosidase-positive cells in the basal and superbasal epidermis and hair follicle were counted per 10 mm of skin and averaged together for three mice per genotype.

2.5 Discussion

In this study, we investigated the mechanism of E2F1-mediated tumor suppression using the K5 E2F1 transgenic mouse model. Our lab previously showed that this mouse is resistant to two-stage chemical carcinogenesis and that both ARF and p53 are required for tumor suppression in this model using an SSIN genetic background (82, 109). As a primary downstream target of p53 (125), we explored p21 as a likely candidate for a role in this process as well. Using immunohistochemistry (IHC), we found that all three proteins, ARF, p53, and p21 were upregulated in K5 E2F1 epidermis and this was dependent on *Arf* (Figure 2.1 and 2.2). Since loss of *Arf* did not affect apoptosis, we concluded that an alternate mechanism must be responsible for tumor suppression by E2F1 (Figure 2.3). A reasonable choice for that mechanism was senescence. In fact, unpublished data from our lab shows increased β -galactosidase activity in K5 E2F1 transgenic skin, an indicator of senescence. Furthermore, this increase in senescence cells was lost in the absence of either *Arf* or p53, consistent with tumor experiments. Since p21 is a common mediator of senescence, we asked the question whether p21-induced senescence could be responsible for tumor suppression by E2F1. We found that loss of p21 itself conferred resistance to DMBA/TPA induced tumor formation, thus preventing us from making any conclusions about p21's involvement in E2F1-mediated tumor suppression. Furthermore, we also could not determine the role of senescence in E2F1-mediated tumor suppression because no difference

was seen by SA- β -gal staining between p21-null skin and p21-null skin overexpressing E2F1.

Three labs have previously reported on the role of p21 in skin carcinogenesis; yet, each used a different strain of mice and each has drawn a different conclusion. Philipp et al. reported that loss of p21 did not change tumor incidence versus wildtype mice, but it did result in a higher incidence of less differentiated tumors (Black Swiss x 129 mixed background) (126). Weinberg and colleagues described an enhanced propensity to form papillomas in p21 knockout mice, but no change in rate of malignant conversion (129/SvEv background) (127). And finally, Topley et al. showed loss of p21 led to sensitivity to chemical carcinogenesis, but at a lower rate than wildtype mice, and an increased rate of carcinoma formation (SENCAR x NIH Swiss mixed background) (128). Our lab has now generated another unique finding in yet another genetic background of mice. Our data shows that in an FVB background, loss of p21 results in loss of sensitivity to a classical skin carcinogenesis protocol. The mechanism for this result is unclear, but may be due in part to an increase in apoptosis seen in K5 E2F1, p21^{-/-} mice, that seems to occur independent of p53 protein levels. It is worth noting that had we done this experiment in a different background, such as SSIN as we used previously, it is likely that our results would have been different. Thus, these experiments highlight the significance of genetic background in experimental design and the caution with which to interpret such data in the context of signaling pathways.

Chapter 3: E2F1-mediated regulation of miRNAs

3.1 Abstract

MicroRNAs (miRNAs) are a class of noncoding RNAs that regulate protein expression by targeting messenger RNAs for degradation or by inhibiting protein translation. MiRNAs play a key role in development and their importance in cancer formation is becoming increasingly apparent. Research has shown that E2F1 can be regulated by miRNA expression, but whether E2F1 could in turn regulate miRNAs in the skin was unclear. Thus, we chose to investigate the effect of E2F1 on miRNA expression using the K5 E2F1 transgenic model. By northern blot, we discovered that let-7 was expressed at higher levels in epidermis from K5 E2F1 transgenic mice as compared to wildtype mice. Further, when mouse skin was treated with DMBA and TPA, let-7 expression was found to increase. Despite this, microarray data failed to confirm these observations, and in fact, showed let-7 to be higher in wildtype skin. Several other miRNAs, 16 in total, were expressed at higher levels in wildtype skin than in K5 E2F1 transgenic epidermis, while 6 miRNAs were downregulated in E2F1 overexpressing skin. Consistent with the microarray data, western analysis did not show a downstream effect on Ras protein levels, a target of let-7, in wildtype and E2F1 transgenic epidermis with and without carcinogen treatment. Moreover normal human fibroblasts (NHF) and squamous carcinoma cells do not show any effect on Ras when both were infected with an E2F1 adenovirus. We were, however, able to show that E2F1 binds to the miR-301 promoter by chromatin immunoprecipitation (ChIP). In summary these data provide intriguing evidence that E2F1 is involved in miRNA regulation.

3.2 Introduction

MicroRNAs (miRNAs) are a class of small (18-25 nt) non-coding RNAs which regulate protein expression (129). These small RNAs were first discovered in the early 1990s in the nematode *C. elegans*, but have quickly become important as key regulators of an ever expanding list of biological pathways (130, 131). The processes identified to date that are regulated by miRNAs include: differentiation, cell proliferation, apoptosis, development, hematopoiesis, immunity, metabolism, stem cell maintenance, and ultimately cancer development (132-140).

miRNAs are initially transcribed as long precursor molecules (pri-miRNAs) by RNA polymerases II and III (141, 142). These pri-miRNAs are then processed by Drosha to generate pre-miRNAs, which can be transported to the cytoplasm by exportin 5. Once in the cytoplasm, pre-miRNA duplexes are cleaved by Dicer to generate mature miRNAs, which can then be incorporated into the RISC (RNA-Induced Silencing Complex) complex. As part of the RISC complex, miRNAs can regulate protein expression in one of two ways. Degradation of target messenger RNAs occurs by deadenylation; or, translation of target mRNAs is blocked at either the initiation or elongation steps. Preference for degradation or translational inhibition is based on complementarity between the miRNA and its target mRNA, with perfect complementation favoring degradation and imperfect pairing supporting translational inhibition (143, 144). Generally speaking, plant miRNAs tend to have complete sequence pairing, while most animal miRNAs interact with their targets through 5' seed sequences consisting of only 6-8 nucleotides (145).

Mounting evidence has shown a clear role for miRNAs in cancer. The first direct connection between miRNAs and cancer was found in B-cell chronic lymphocytic leukemia (B-CLL) when it was demonstrated that miR-15a and 16-1 are deleted in half, and downregulated in a third, of all CLL cases (146). Subsequently several miRNAs have been identified as tumor suppressors (let-7) or oncogenes (miR-17-92, miR-155, miR-21, miR-372/373) (147-151). Moreover, nearly all types of tumors show some degree of deregulated expression of miRNAs (152).

Several crucial cellular pathways are regulated by miRNAs, including the Rb pathway, notoriously known for being mutated in cancers (153). As key components of this pathway, the E2F transcription factors are direct targets of miRNAs. miR-210 targets E2F3 in ovarian cancer, while E2F6 is regulated by miR-193a in oral squamous cell carcinoma (154, 155). Additionally, E2F1 and E2F3 are targets of miR-34a in colon cancer cells (156). More recent studies have shown that E2F1 can in turn regulate miRNAs. E2F1 overexpression appears to downregulate miR-93 and miR-106b in gastric cancer (157). Furthermore, E2F1-3 are involved in autoregulatory feedback loops with at least two oncogenic miRNA clusters, miR-17-92 and miR-106b-25 (157-160). All of this points to an important link between miRNAs, E2Fs, and cancer.

Our lab formerly generated a transgenic mouse model, which overexpresses the human E2F1 gene under the control of the keratin 5 promoter. This directs expression of E2F1 to the skin and other epithelial tissues (52). As mentioned earlier, this K5 E2F1 mouse is resistant to a two-stage chemical carcinogenesis assay, which relies on an activating mutation in Ras for initiation (82). The miRNA

let-7 is known to negatively regulate Ras, and consequently is down-regulated in tumors that have an activating Ras mutation (151). Thus, we decided to determine if let-7 or other miRNAs are regulated by E2F1 in mouse epidermis and, if so, possibly involved in E2F1-mediated tumor suppression.

3.3 Materials and Methods

3.3.1 Mice: Previously described K5 E2F1 transgenic mice generated by our lab (52) were maintained in the FVB line, $\geq 95\%$ purity. Male and female mice were housed with littermates. Genotyping was performed using sequence specific PCR primers.

3.3.2 RNA Isolation and Northern Blot: Epidermis was either untreated or treated with DMBA and TPA. Treatment consisted of one 50 μm dose of DMBA followed 3 and 6 days later by two TPA treatments of 2 μg each. Mice were sacrificed 24 hours after the second TPA treatment. Total RNA was isolated from wildtype and transgenic epidermis using 10 ml of TRI Reagent per mouse (Applied Biosystems/Ambion, Austin, TX). Epidermis was chipped from frozen whole skin into liquid nitrogen and ground into a powder using mortar and pestle and then combined with TRI Reagent. After purification, RNA was then run on a 15% acrylamide/urea gel, transferred to a positively charged nylon membrane and probed for either the let-7 miRNA (5'-aaccatacaacactactaccta-3') or the U6 snRNA (5'-atttggttcgcttcacgaatt-3'). Probes were labeled with dCTP using the Rediprime II

random prime labeling kit (GE Healthcare Biosciences, Pittsburgh, PA).

Membranes were washed and exposed to Kodak BioMax MS film for imaging.

Densitometry was performed using the StormTM Imager (GE Healthcare Biosciences, Pittsburgh, PA).

3.3.3 Protein Isolation and Western Blot: Epidermis was treated with acetone, acetone/TPA or DMBA/TPA. Treatment consisted of a single 100 μ m dose of DMBA in 200 μ l of acetone, followed 3 and 6 days later by two TPA treatments of 2 μ g each in acetone. Mice were sacrificed 48 hours after the second TPA treatment. Protein was isolated from either wildtype and transgenic epidermis or cells using modified RIPA buffer. Protein was run on an 8% SDS/PAGE gel, transferred to a PVDF membrane using standard methods and assayed for either Ras (05-516, Millipore, Billerica, MA), β -tubulin (sc-9104, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or actin (sc-8432, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The Ras antibody recognizes H-, K-, and N-Ras. Membranes were washed and exposed to Kodak BioMax MS film for imaging. Densitometry was performed using the StormTM Imager (GE Healthcare Biosciences, Pittsburgh, PA).

3.3.4 Chromatin Immunoprecipitation: Chromatin was isolated from NIH 3T3 fibroblasts (CRL-1658, American Type Culture Collection, Manassas, VA) using standard methods and sheared by sonication. ChIP was performed using the Upstate Biotechnology ChIP Assay kit (Millipore, Billerica, MA). Antibodies were for E2F1 (sc-193, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit IgG

(ab46540, Abcam Inc., Cambridge, MA). PCR was performed on 50 ng of each sample using a miR-301 specific primer pair (Forward: 5'-tcattggctgctgctgcttc-3', Reverse: 5'-ttgacattcctcagaccgctg-3').

3.3.5 Microarray: RNA was isolated from the epidermis of one wildtype and one K5 E2F1 transgenic mouse using 10 ml of TRI Reagent per mouse. Epidermis was chipped from frozen whole skin into liquid nitrogen and ground into a powder using mortar and pestle. After RNA isolation with TRI Reagent, further purification was done using RNeasy columns (QIAGEN, Valencia, CA) and a modified protocol. Briefly, 350 μ L of RLT buffer was added to the RNA sample and vortexed. Then, 3.5 volumes of 100% ethanol were mixed with the sample and run through a column in 700 μ L aliquots, spinning for 15 seconds at $\geq 8000 \times g$. Columns were washed with 500 μ L RPE buffer and eluted in RNase-free water. RNA integrity was verified (RIN=7.8) and then analyzed using the Exiqon miRCURY LNATM microRNA Array for all known human, mouse, and rat miRNAs (Vedbaek, Denmark).

3.4 Results

Our lab previously generated the K5 E2F1 transgenic mouse model, which is resistant to a two-stage chemical carcinogenesis protocol driven by an initiating mutation in H-ras (82). Subsequently, reports have shown that Ras protein levels can be negatively regulated by the miRNA let-7 (151). To study the possible role of miRNAs in E2F1-mediated tumor suppression in mouse epidermis, we examined

let-7 levels by northern blot. We have found that let-7a & c expression was increased in K5 E2F1 epidermis as compared to wildtype skin (Figure 3.1). This difference was 1.5-fold vs. 1.0 for let-7a and 1.7 fold vs. 1.0 for let-7c. Moreover, treatment with chemical carcinogens (DMBA/TPA) increased this difference, 2.7-fold vs. 1.2-fold for let-7a and 3.0-fold vs. 1.8-fold for let-7c. However, when western blots were conducted to look at the downstream effects of let-7 expression on Ras, ERK 1/2, and phospho-ERK protein levels, we found no significant differences in either mouse skin or cultured cells when E2F1 was overexpressed or not (Figures 3.2, 3.3, and data not shown).

To examine the global effect of E2F1 overexpression on miRNAs in mouse skin, we analyzed RNA from both wildtype and E2F1 transgenic epidermis for all known murine miRNA sequences using the Exiqon miRCURY LNA™ microRNA Array. Microarray data showed several differences in miRNA expression between wildtype and K5 E2F1 skin. miRNAs with 1-fold or greater difference in expression level are shown (Figure 3.4). E2F1 seemed to upregulate miR-15a, 451, 331, 434-3p, 195, and 300 as compared to wildtype, while let-7a, let-7c, and miRs 130a, 26b, 467a, 30c, 100, 143, 200a, 454, 129-3p, 142-3p, 122a, 350, 450, and 301 were downregulated in K5 E2F1 epidermis. The reason for the inconsistency between the Northern blot and microarray data for let-7 is unclear at this point. However, it is worth noting that Bueno et al. recently confirmed regulation of the let-7 cluster by E2F1 and E2F3 (161). It is also worth noting that attempts were made to confirm the microarray data for miR-301 and miR-15a, the miRNAs with the greatest fold

change, by northern blot. However, these attempts were unsuccessful, possibly due to the lack of overall abundance of these miRNAs in skin.

Finally, chromatin immunoprecipitation (ChIP) experiments were conducted to ascertain whether E2F1 directly regulates any of the identified miRNAs by binding to their respective promoters. We found no evidence that let-7 was E2F1 regulated, at least at the sites we examined. However, ChIP experiments do show that E2F1 binds to the miR-301 promoter (Figure 3.5). Further experiments are needed to confirm regulation of miR-301 by E2F1 and the physiological consequences of such a relationship.

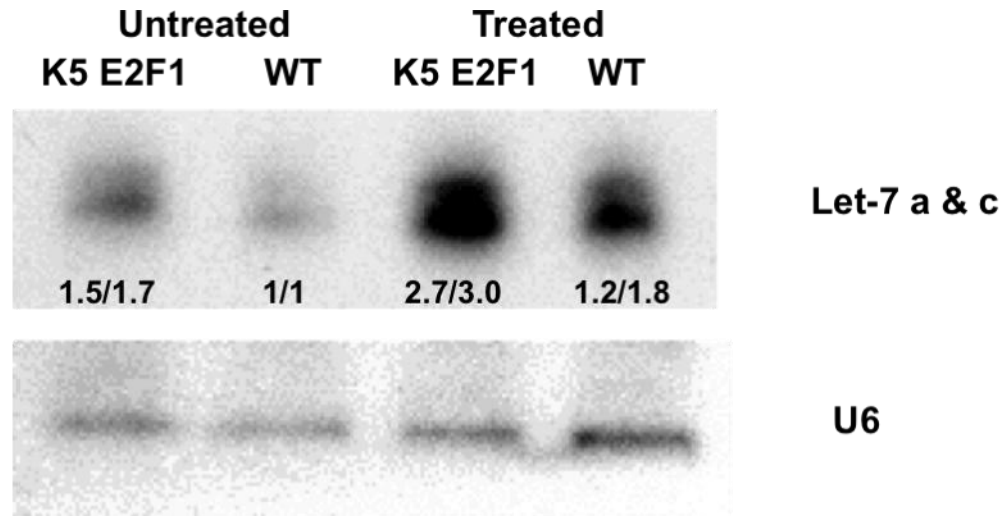


Figure 3.1. Let-7a & c expression is increased in K5 E2F1 epidermis. Skin from wildtype or K5 E2F1 transgenic mice was treated with DMBA/TPA or acetone vehicle (untreated). RNA was isolated from the epidermis, run on a 15% PAGE gel and transferred to a nylon membrane. Northern blotting was done with either a let-7 miRNA or U6 snRNA specific probe. Densitometry is shown for let-7a & c, respectively, compared to U6 as a loading control. Northern blotting was done in duplicate.

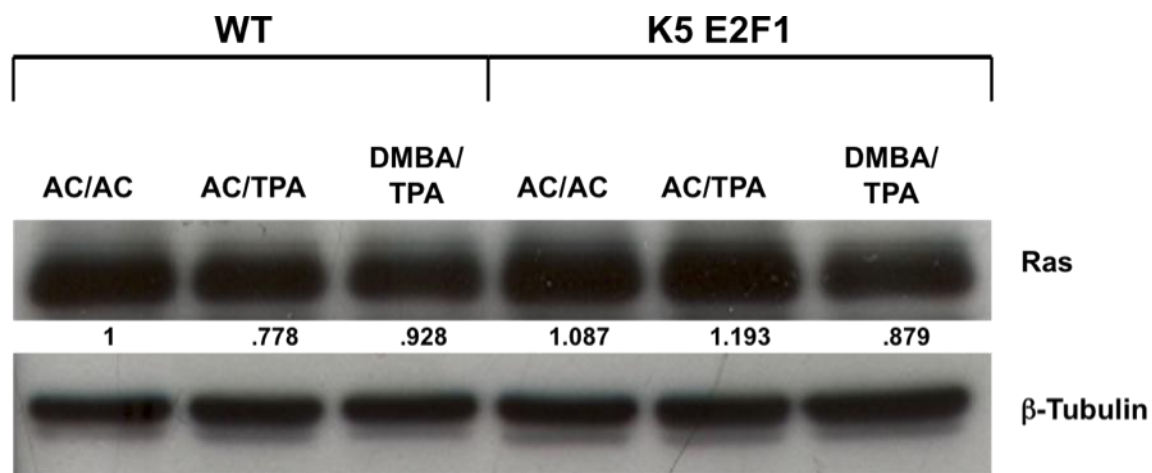


Figure 3.2. Ras levels are not significantly different than wildtype in K5 E2F1 epidermis. Epidermis from either wildtype or K5 E2F1 mice was treated with acetone (AC), acetone/TPA (AC/TPA) or DMBA/TPA. Protein was isolated from whole skin by scraping, run on an 8% SDS/PAGE gel, and transferred to a PVDF membrane. Antibodies were for Ras (recognizes H-, K-, and N-Ras) or β-tubulin. Densitometry is shown.

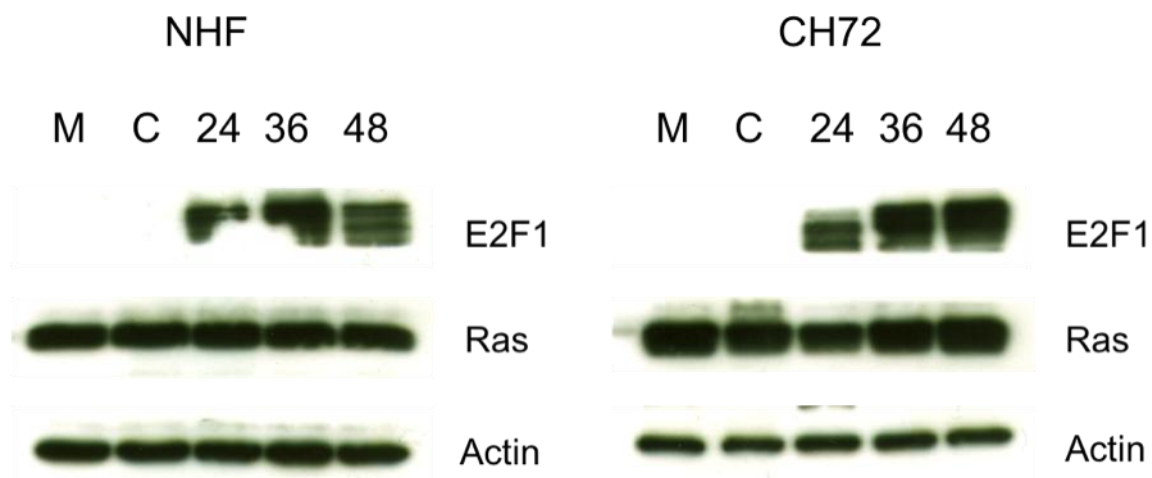


Figure 3.3. Ad-E2F1 expression does not affect Ras levels in cultured cells.

Normal human fibroblasts (NHF) or CH72 keratinocytes were mock-infected (M) or infected with a control (C) or Ad-E2F1 adenovirus. Protein was collected at 24, 36, and 48 hours. Antibodies were for E2F1, Ras (recognizes H-, K-, and N-Ras), or Actin.

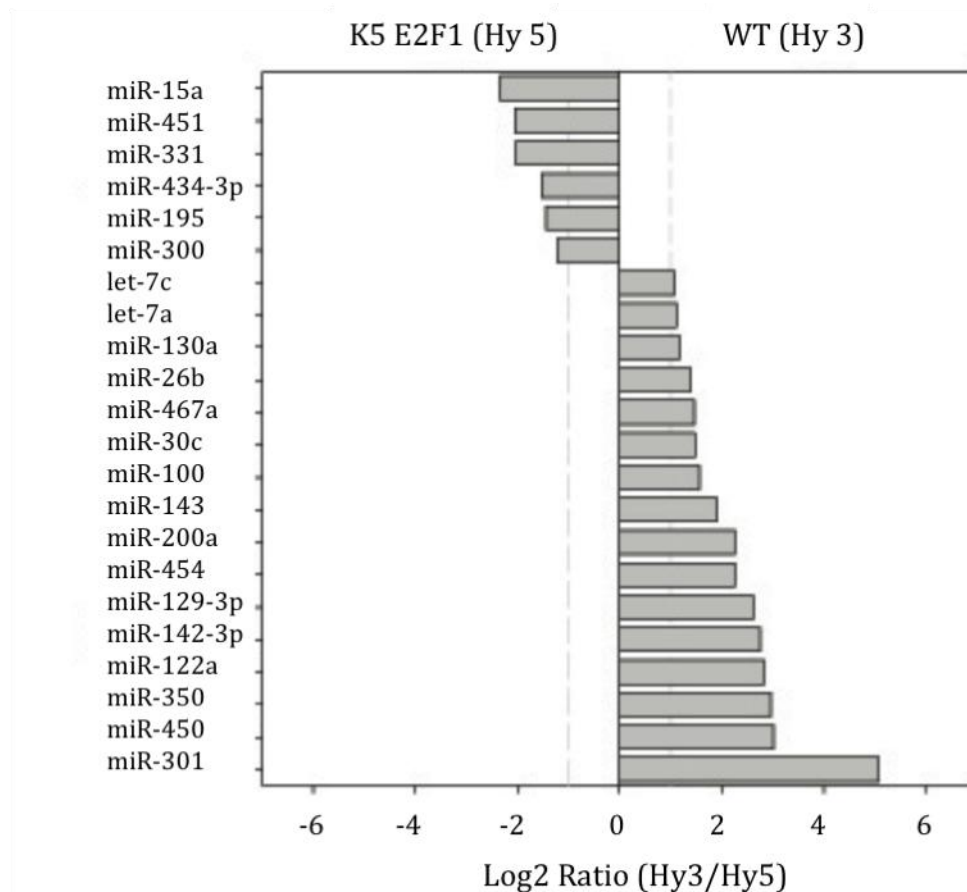


Figure 3.4. K5 E2F1 transgenic epidermis differentially expresses certain miRNAs when compared to wildtype skin. RNA was isolated from either wildtype (WT) or K5-E2F1 epidermis and analyzed using the Exiqon miRCURY LNA™ microRNA Array. Results for differentially regulated murine miRNAs are shown as log2 ratio of Hy3/Hy5, which corresponds to fold change. Hy3 and Hy5 refer to fluorophores used to label each genotype.

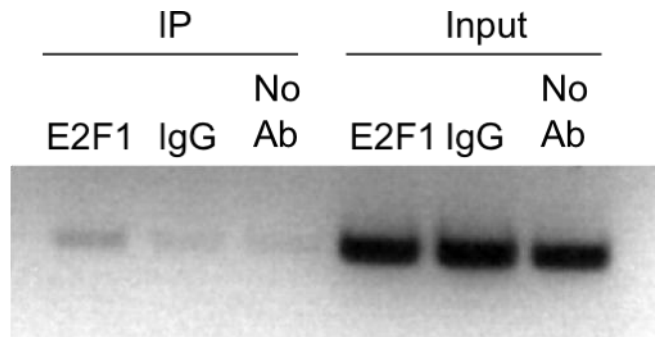


Figure 3.5. E2F1 is found at the miR-301 promoter. Chromatin immunoprecipitation (ChIP) was performed on NIH 3T3 cells using the Upstate Biotechnology kit. Chromatin was immunoprecipitated with an E2F1 antibody, an IgG control, or no antibody (No Ab). PCR was performed on 50 ng of DNA from each immunoprecipitation (IP) or input DNA using miR-301 specific primers. PCR was performed in duplicate.

3.5 Discussion

In this study, we investigated whether E2F1 could mediate control of miRNA expression in mouse epidermis. We found several differentially regulated miRNAs between wildtype and K5 E2F1 transgenic epidermis each of which needs to be explored further. Our data also confirms a report by others that E2F1 overexpression regulates at least two isoforms of let-7, a and c, in mouse skin, the significance of which remains to be seen (161). We could not however prove direct regulation of let-7 by E2F1 leaving open the possibility that intermediate signaling molecules exist between these two proteins.

We also found that E2F1 binds the miR-301 promoter, though the significance of this finding is also unclear at this time, since little is known about the function of miR-301. We do know that miR-301 is located in intron 1 of Ska2 and participates in a positive feedback loop with MEOX2 and the ERK/CREB pathway on Ska2 expression (162). Ska2 is a member of the spindle and kinetochore complex and seems to be important for maintenance of the metaphase plate and spindle checkpoint (163). So, it is possible that deregulation of miR-301 expression could result in chromosomal abnormalities. Indeed, four reports have shown that miR-301 expression is differentially regulated between tumors and normal tissue of the pancreas, liver, bile duct, and lung. However, whether miR-301 is up or down regulated seems to be tissue specific (164-167). Also of note, miR-301 expression is decreased in p53^{-/-} embryos, though this may be due to a decrease in miRNA processing which seems to be regulated by the p53 family (168).

In conclusion, we have now shown that at least some miRNAs are indeed regulated by E2F1 in mouse epidermis. We have also shown that one of these miRNAs, miR-301, is a direct E2F1 target. Given the diverse nature of these molecules it is likely that some miRNAs identified by us will be proven important in epithelial cell homeostatic processes and possibly tumor development.

Chapter 4: The Role of E2F1 Serine 29 in the DNA Damage Response and Tumor Suppression

4.1 Abstract

The transcription factor E2F1 is induced and stabilized in response to DNA damage through its phosphorylation at serine 31 by ATM and ATR, and subsequent interaction with 14-3-3 τ . E2F1's phosphorylation also leads to its association with TopBP1 and localization to DNA damage-induced foci. To study the role of E2F1 in the response to DNA damage, our lab has generated a unique mouse model in which serine 29 of E2F1 has been replaced by an alanine (S29A), preventing phosphorylation. Initial experiments indicate that the E2F1 S29A protein is not stabilized in response to ionizing radiation (IR) and cannot localize to sites of DNA damage. As well, mutation of S29 results in an accumulation of endogenous DNA damage, mimicking total E2F1 loss. Additionally, there is a repair deficiency in *E2f1*^{S29A/S29A} mutant cells as compared to wild type cells when exposed to either IR or neocarzinostatin (NCS). Further, the viability of *E2f1*^{S29A/S29A} MEFs is reduced, and higher levels of apoptosis are observed in response to UVB. To further study the role of E2F1 serine 29 in the DNA damage response and tumorigenesis, mice are being aged to examine the formation of spontaneous tumors. Previously, our lab has shown that E2F1 acts as a tumor suppressor in the K5 Myc transgenic mouse model. To examine the importance of E2F1's ability to respond to Myc-induced DNA damage in suppressing tumorigenesis, we have crossed the *E2f1*^{S29A/S29A} knockin mouse onto a K5 Myc transgenic background and found no difference in tumor incidence with wildtype E2F1 or E2F1 S29A; yet tumor spectra and multiplicity vary slightly. Skin studies examining proliferation in the skin of

these mutant mice indicate similarities to E2F1 knockout mice, while apoptosis appears to be lower in the *E2f1*^{S29A/S29A} mutant than the knockout in response to Myc. Taken together, our data indicate a role for E2F1 at sites of DNA damage, which requires its phosphorylation at serine 29, and may promote DNA repair.

4.2 Introduction

E2F1 is a member of the E2F family of transcription factors. It is a multifaceted protein that has roles in a number of important cellular processes including cell cycle, apoptosis, proliferation, and the DNA damage response (DDR). E2F1's role in the DDR first emerged when it was shown that E2F1 protein levels increased in response to a variety of DNA damaging agents (169-172). Subsequently, it was shown that stabilization of E2F1 is dependent on ATM activity. In fact, E2F1 can be phosphorylated by ATM/ATR in response to DNA damage at serine 31 (29 in mice). Moreover, phosphorylation at this site is required for ATM-induced stabilization of E2F1 (173). Yet, the mechanism of E2F1 stabilization was not worked out until later.

E2F1 is stabilized in response to DNA damage by the binding of 14-3-3 τ which inhibits E2F1's ubiquitination and degradation. Binding of 14-3-3 τ to E2F1 requires its phosphorylation at serine 31 by ATM. As well, 14-3-3 τ is required for the induction of the apoptosis inducing genes, p73, Apaf-1, and caspases by E2F1 in response to DNA damage (174). Thus, the induction of apoptosis in response to DNA damaging agents is one function of E2F1.

Further studies have recognized a possible role for E2F1 at the actual sites of DNA damage. A yeast two-hybrid screen identified TopBP1 (DNA topoisomerase II-binding protein I) as an E2F1 binding partner. TopBP1 localizes to DNA damage-induced foci after IR treatment along with other proteins implicated in the DDR, including BRCA1 and 53BP1 (175-177). The first and second BRCT domains of

TopBP1 are important for binding DNA breaks (178). The 6th BRCT domain of TopBP1 is important for interaction with E2F1, while the N-terminus of E2F1 is required for this interaction. This interaction is unique to E2F1, induced by DNA damage, requires phosphorylation of E2F1 by ATM, and represses E2F1-induced transcription, S-phase entry, and apoptosis (179).

To study the role of E2F1 in the DNA damage response *in vivo* and its possible effects on tumorigenesis, we have developed a knockin mouse model where serine 29 of E2F1 is replaced by alanine. This model allows us to study the DNA damage specific effects of E2F1 independent of its role in transcription, since E2F1's role in the DDR requires its phosphorylation at S31 and our modified E2F1 is still functional in transcription. We found that E2F1 S29A is not stabilized in response to DNA damage and does not go to DNA damage induced foci. As well, E2F1^{S29A/S29A} cells accumulate endogenous DNA damage and are slower to repair than wildtype cells after NCS treatment. Finally, K5 Myc, E2F1^{S29A/S29A} mice have a different tumor spectrum, more tumors per mouse, and more aggressive tumors than K5 Myc mice with wildtype E2F1. All of this points to a role for E2F1 in tumor suppression through its participation in the DNA damage response.

4.3 Materials and Methods

4.3.1 Mice: K5 Myc mice were previously described (106, 107). As well, *E2f1*^{-/-} mice have been previously described (88, 89). Both lines were maintained at ≥ 95% pure FVB. *E2f1*^{S29A/S29A} mice were generated in our lab as follows. The

mouse *E2f1* locus was cloned into a vector after long-range PCR using isogenic 129 ES cell DNA as a template. A unique Avill site was introduced into exon 1 upon site-directed mutagenesis using a kit from Stratagene (Agilent Technologies, Inc., Santa Clara, CA). This mutagenesis also changed the protein coding sequence from a serine at position 29 to an alanine. After homologous recombination, positive clones were identified by Southern blotting and injected into recipient females to generate two lines (1 & 2). Chimeras from each line were bred with C57 Bl/6 females to confirm germline transmission and crossed to a FLPeR mouse (Stock # 003946, The Jackson Laboratory, Bar Harbor, ME) to remove the PGK selection cassette. Resulting *E2f1*^{S29A/S29A} mice on a mixed background were used for studies. K5 Myc mice were crossed to *E2f1*^{S29A/+} mice to generate wildtype, K5 Myc, *E2f1*^{S29A/S29A} knockin, and K5 Myc, *E2f1*^{S29A/S29A} knockin mice. Mice were checked weekly for spontaneous tumor development.

4.3.2 Immunohistochemistry: IHC was performed according to the methods in Chapter 2 for BrdU and Caspase-3. TUNEL was performed with the Tdt-FragEL DNA Fragmentation Detection Kit (QIAGEN, Valencia, CA). Slides were deparaffinized and rehydrated in dH₂O, incubated with 3% H₂O₂, and rinsed. Subsequently, slides were incubated with proteinase K (20 µg/ml) for 20 minutes, rinsed in dH₂O followed by PBST. Next, slides were equilibrated in Tdt equilibration buffer for 10-30 minutes and incubated with Tdt labeling reaction buffer for 1 hour at 37°C. After rinsing in PBST, stop buffer was added for 5 minutes. Following another PBST wash, tissue was blocked for 10 minutes in blocking buffer.

Conjugate was added for 30 minutes; slides were rinsed with PBST and developed with DAB.

4.3.3 Comet Assay: The comet assay, or single cell gel electrophoresis (SCGE) assay, was performed using the assay kit from Trevigen, Inc. (Gaithersburg, MD). Briefly, after treatment (NCS, 100 ng/ml), cells were harvested and embedded in low melting point agarose on glass slides and incubated overnight at 4°C in lysis buffer. Mouse adult fibroblasts (MAFs) were subjected to the neutral assay, while keratinocytes were processed with the alkaline method. For the alkaline assay, slides were incubated in alkaline buffer (200 mM NaOH, 1 mM EDTA) before electrophoresis; while for the neutral assay, this step was skipped. After washing, samples were electrophoresed at 19 volts for 5-20 minutes in TBE. Following fixation and drying, cells were stained with SYBR Green. Nuclei were visualized and images captured using a fluorescent microscope. Olive moment of at least 95 nuclei per slide was calculated using COMETSCORE software (Tritek Corp., Sumerduck, VA).

4.3.4 Chromatin Immunoprecipitation: ChIP was performed essentially as in Chapter 3 Materials & Methods. MAFs were generated from wildtype or *E2f1*^{S29A/S29A} knockin mice using standard procedures. MAFs were infected with an I-*Ppo* 1 expressing retrovirus, induced with tamoxifen (1 µM), and collected 8 hours later. PCR was performed using primers specific to the chromosome 10 I-*Ppo* 1 cut site.

4.3.5 Immunofluorescence: Cells were fixed after IR treatment or Ad-Myc infection to induce double-strand breaks, DSBs. For immunofluorescent staining, fixed cells were incubated with 3% BSA, washed, and then incubated with appropriate primary antibodies, E2F1 (C20, sc-193 (1:50) or KH95, sc-251 (1:50), Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or γ H2AX (BL179 (1:4000), Bethyl Laboratories, Montgomery, TX; 05-636 (1:4000), Millipore, Billerica, MA). Following incubation with a fluorescently tagged secondary antibody (A-11037 or A-11029 (1:400), Invitrogen Corporation, Carlsbad, CA), slides were stained with DAPI and coverslipped before visualization. Appropriate fluorescent images were captured using a Nikon eclipse 80i microscope equipped with an X-cite 120 fluorescence illumination system and Metamorph image analysis software.

4.4 Results

In order to study the role of E2F1 in the DNA damage response, independent of its role in transcription, we have generated *E2f1*^{S29A/S29A} knockin mice (Figure 4.1). Two independent ES cell clones were used to generate chimeras that were then bred to homozygosity. These mice carry a single amino acid substitution at position 29 (31 in humans) of the E2F1 gene resulting in the exchange of serine for alanine. This results in a protein incapable of being phosphorylated at this position. However, we could not independently verify lack of phosphorylation because no antibody could be raised to specifically identify this mutant. Nevertheless, we are

confident that phosphorylation is inhibited based on the well accepted function of serine to alanine mutants and the biochemical phenotypes we observed.

E2f1^{S29A/S29A} knockin mice are phenotypically similar to wildtype mice and develop normally. We have yet to observe the phenotypic abnormalities observed in *E2f1*^{-/-} mice including testicular atrophy, exocrine gland hyperplasia, and defective thymocyte apoptosis (87, 88). We did observe that *E2f1*^{S29A/S29A} mice have lower body weights than wild type mice on a mixed background, although this difference is not significant, $p=0.065$ at 45 weeks (Figure 4.2).

In contrast to the lack of noticeable physical defects, *E2f1*^{S29A/S29A} mice have significant defects in the biochemical activities of the E2F1 protein. First, E2F1 S29A cannot be stabilized in response to ionizing radiation (IR) unlike wildtype E2F1 (Figure 4.3). As well, the E2F1 S29A mutant cannot form foci in response to IR or go to sites of DNA double strand breaks as assayed by ChIP (Figure 4.4 and 4.5). Additionally, there is increased endogenous DNA damage in *E2f1*^{S29A/S29A} keratinocytes as compared to wildtype, $p=1 \times 10^{-7}$. In fact, mutation of serine 29 to alanine results in the same amount of endogenous DNA damage as complete E2F1 loss (Figure 4.6). Finally, *E2f1*^{S29A/S29A} MAFS exposed to the DNA double strand break inducing agent, neocarzinostatin, repair their DNA less efficiently than wildtype cells exposed to the same conditions (Figure 4.7). All of these data point to a significant role for E2F1 in the DNA damage response and DNA repair that requires phosphorylation at serine 29.

Our lab previously identified E2F1 as a tumor suppressor in response to the c-Myc oncogene (107). As well, we reported that K5 Myc overexpression results in

DNA damage-induced foci in transgenic skin (180). We now show that E2F1 loss also causes DNA damage in primary keratinocytes, $p=2.85 \times 10^{-13}$. This damage is more than K5 Myc expression, $p=0.003$. When both conditions are present there is a slight overall increase in DNA damage as compared to the non-transgenic, though this is not significant (Figure 4.8).

To study the effects of E2F1's ability to function in the DNA damage response on K5 Myc-induced hyperplasia and apoptosis, we crossed *E2f1*^{S29A/S29A} onto a K5 Myc background. We first took skin from *E2f1*^{+/+}, *E2f1*^{S29A/S29A}, K5 Myc, and K5 Myc, *E2f1*^{S29A/S29A} mice and stained for BrdU incorporation, TUNEL, and caspase-3 activity. The skin study showed no difference in proliferation or apoptosis in the presence of K5 Myc expression whether E2F1 was wildtype or mutant (Figure 4.9). This is different than what was observed in *E2f1*^{-/-} mice, which had increased levels of apoptosis in a K5 Myc transgenic background (107). We did, however, identify a difference between wildtype and *E2f1*^{S29A/S29A} skin in caspase-3 activity, $p=0.04$, though both genotypes had very low levels of apoptosis.

K5 Myc, *E2f1*^{S29A/S29A} mice were allowed to age to examine the effects of E2F1 S29A mutation on K5 Myc-induced tumorigenesis. Spontaneous tumor studies show there is no significant difference in tumor incidence in response to K5 Myc when E2F1 serine 29 is mutated to alanine (Figure 4.11). The tumor incidence is 26% in K5 Myc mice versus 17% in K5 Myc, *E2f1*^{S29A/S29A} by one year of age. This rate of tumor incidence is consistent with what we previously reported for K5 Myc mice (107, 180). However, at 70 weeks, the tumor incidence is 100% in K5 Myc mice versus 73% in K5 Myc, *E2f1*^{S29A/S29A}, though this is not significant with the

amount of mice in this study. Despite this, tumor spectrum and the number of tumors per mouse, seem to vary slightly when E2F1 is mutated. One K5 Myc, *E2f1*^{S29A/S29A} mouse developed a squamous cell carcinoma within the skin of unknown origin, possibly arising from the skin adnexae. Also, two K5 Myc, *E2f1*^{S29A/S29A} mice developed multiple tumors. One mouse developed 3 squamous cell carcinomas and the other 2 papillomas. This is in contrast to K5 Myc mice, which do not develop multiple tumors. As well, one K5 Myc, *E2f1*^{S29A/S29A} mouse with an odontogenic tumor showed evidence of metastasis to the lungs, again something not seen in K5 Myc mice, suggesting an increase in aggressiveness of tumors lacking a functional E2F1. Finally, we found that E2F1 localizes to foci when DNA is damaged by Ad-Myc overexpression (Figure 4.10). Taken together, these data suggest a role for E2F1 in responding to Myc-induced damage, which may affect tumor multiplicity and aggressiveness.

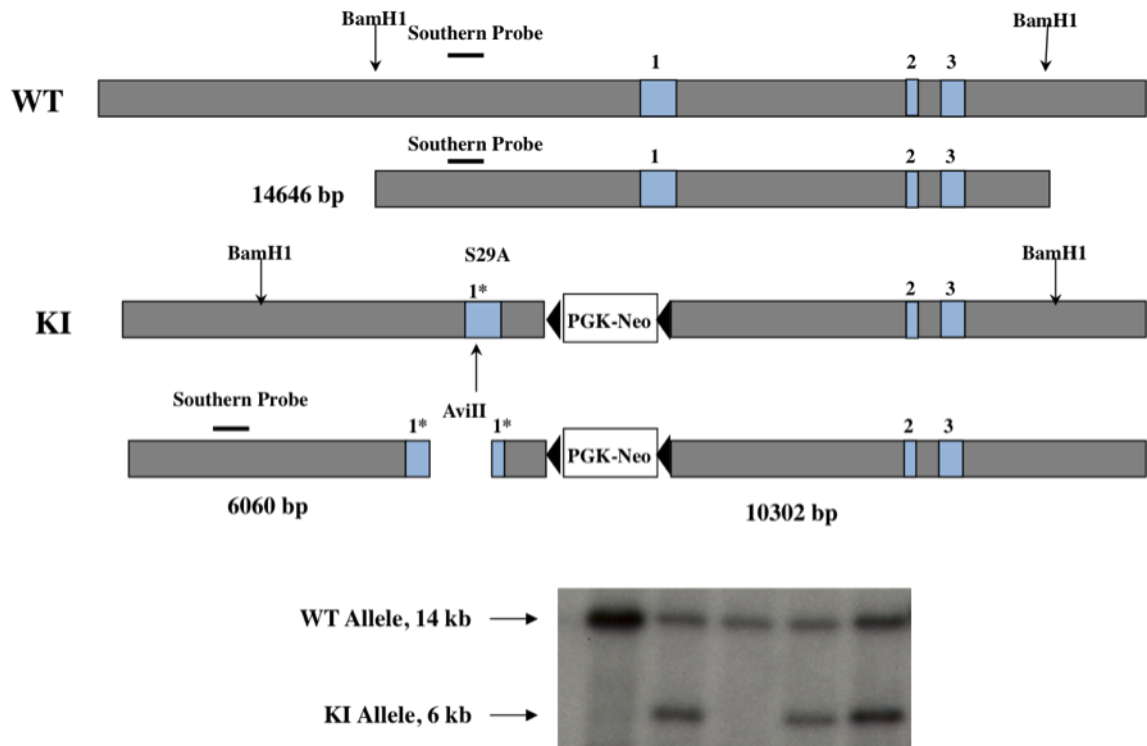


Figure 4.1. Knockin scheme for *E2f1* S29A mice. Wildtype and knockin (KI) alleles are shown. Expected band sizes after digestion with BamHI and AviII are also shown, 14.6 Kb for wildtype and 6 Kb for KI. Targeted ES cell clones are shown after Southern blot. A unique AviII site was introduced by site-directed mutagenesis into the KI allele. A PGK-Neo cassette flanked by FRT sites was used for selection in ES cells and later removed by breeding to a FLPer mouse.

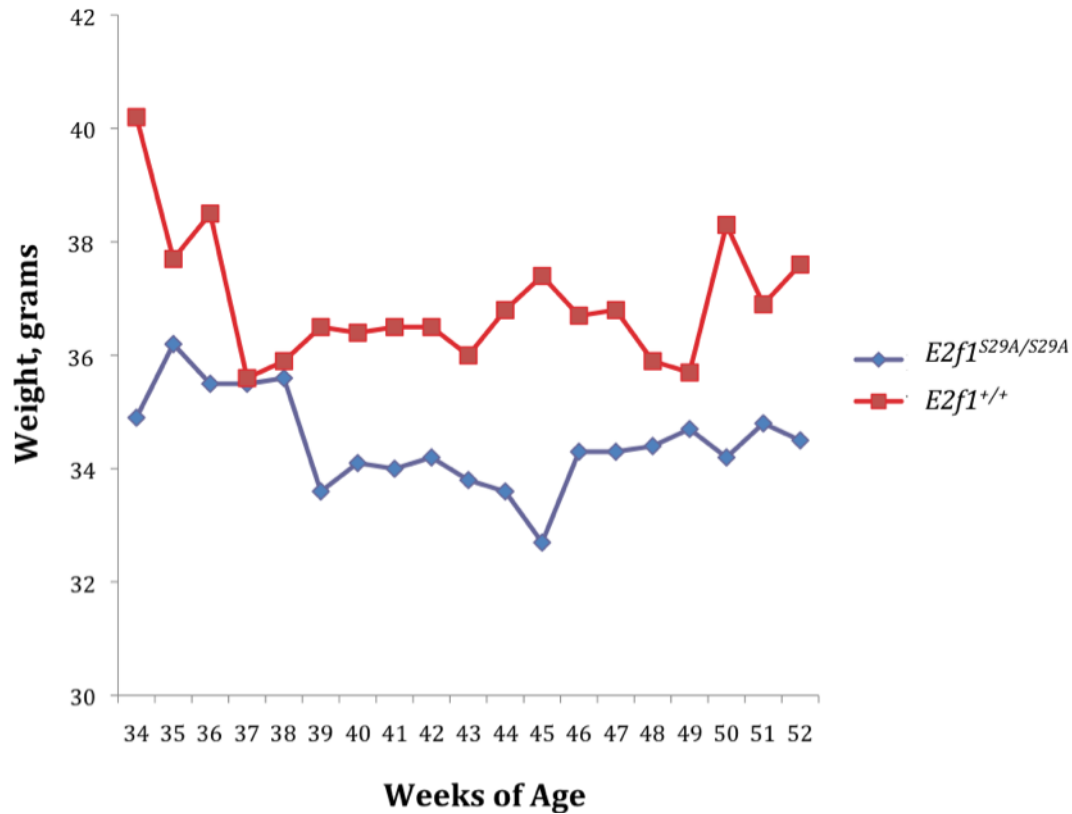


Figure 4.2. *E2f1*^{S29A/S29A} mice have lower body weights than *E2f1*^{+/+} mice on a mixed background. Starting at 34 weeks of age, *E2f1*^{+/+} and *E2f1*^{S29A/S29A} female mice were weighed weekly until one year of age. Weight in grams is shown. The number of mice (n=3 to n=10) varies week to week depending on available mice in age group. p=.065 at 45 weeks.

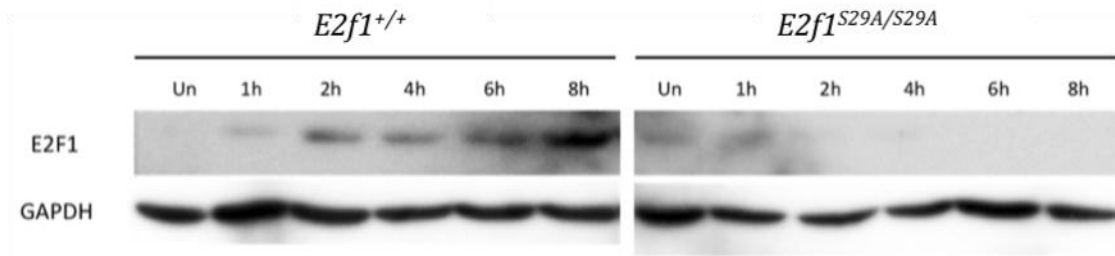
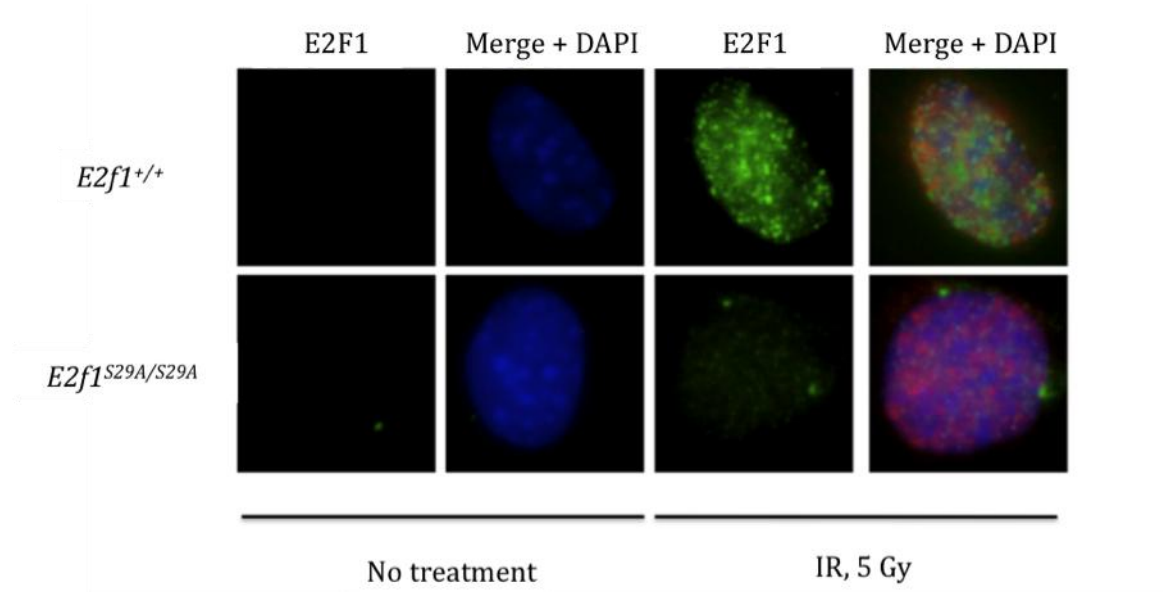


Figure 4.3. E2F1 S29A is not stabilized in response to ionizing radiation.

Mouse adult fibroblasts (MAFs) were isolated from either *E2f1*^{+/+} or *E2f1*^{S29A/S29A} mice. Cells were exposed to 6 Grey of ionizing radiation and protein collected at the indicated time points. Antibodies were for E2F1 or GAPDH as a loading control.

h=hours

A.



B.

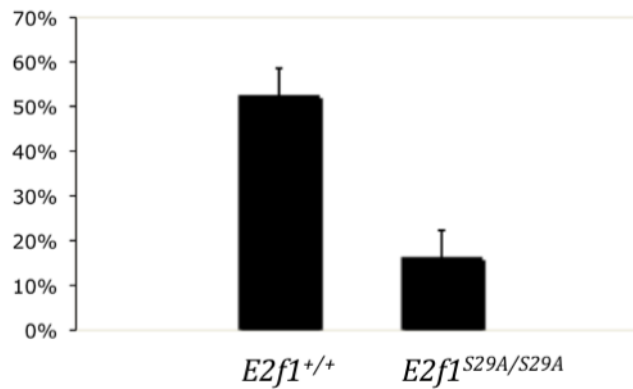


Figure 4.4. E2F1 S29A does not go to DNA double strand breaks induced by ionizing radiation. Mouse embryonic fibroblasts (MEFs) were isolated from d13.5 embryos. After no treatment or treatment with 5 Grey (Gy) of ionizing radiation (IR), cells were fixed and stained for E2F1. Fluorescent images are shown (A). Percentage of cells with E2F1 foci is shown in (B).

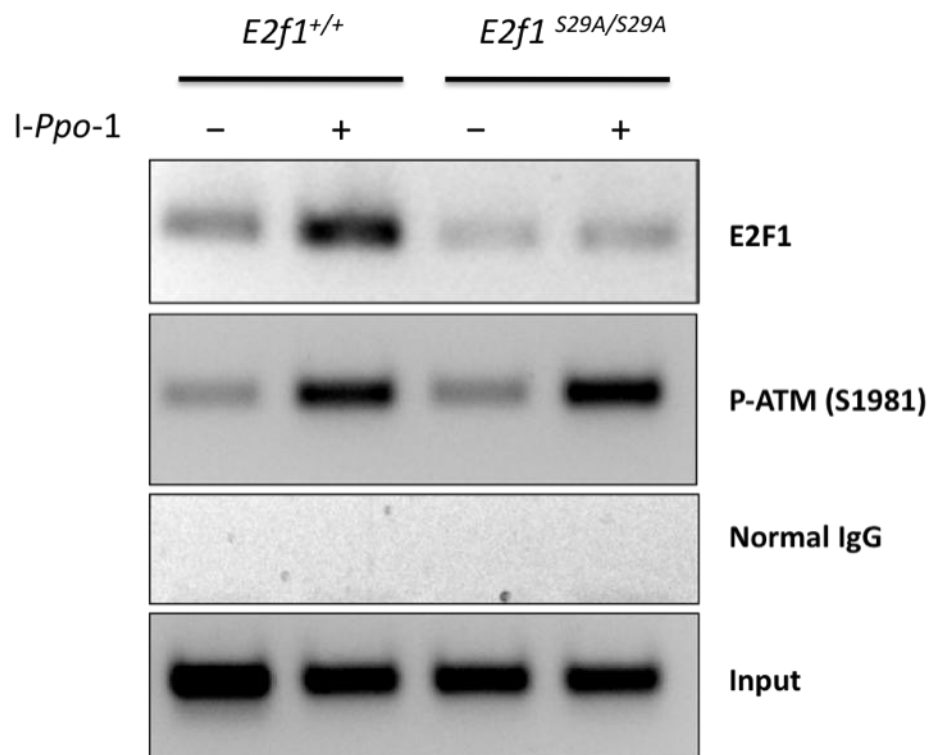


Figure 4.5. E2F1 S29A does not go to sites of DNA damage induced by I-*Ppo*-1. Mouse adult fibroblasts from *E2f1*^{+/+} or *E2f1*^{S29A/S29A} mice were infected with an I-*Ppo*-1 expressing retrovirus. After induction with tamoxifen, double strand breaks were generated at I-*Ppo*-1 recognition sites. ChIP assay is shown with (+) and without (-) I-*Ppo*-1 infection using E2F1, phospho-ATM, and normal IgG antibodies. PCR was performed using primers that specifically amplify the I-*Ppo*-1 site on chromosome 10.

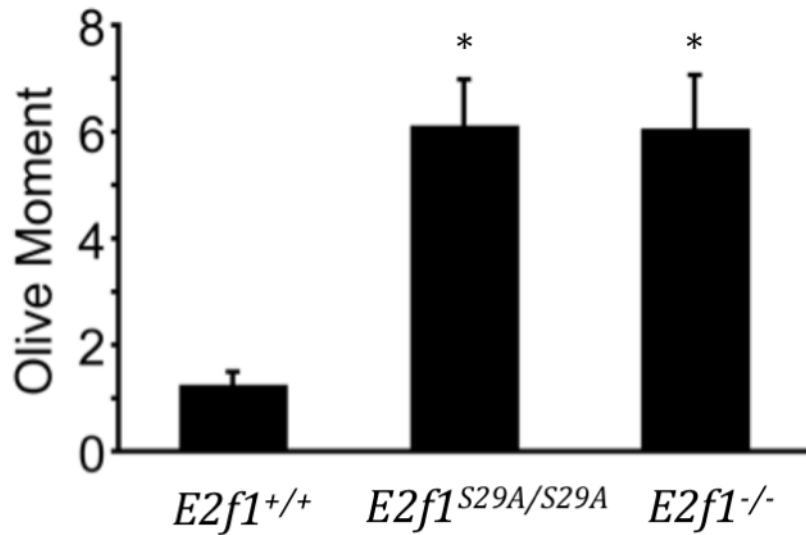


Figure 4.6. Mutation of E2F1 S29A results in endogenous DNA damage equivalent to total E2F1 loss. Primary keratinocytes from *E2f1*^{+/+}, *E2f1*^{S29A/S29A}, and *E2f1*^{-/-} were subjected to the comet assay. After electrophoresis, cells were fixed and stained with SYBR green. At least 150 cells per genotype were analyzed with the COMETSCORE software. Olive moment, the product of tail length and fraction of total DNA in the tail, is shown \pm standard error. * represents $p < 5 \times 10^{-6}$ by t-test.

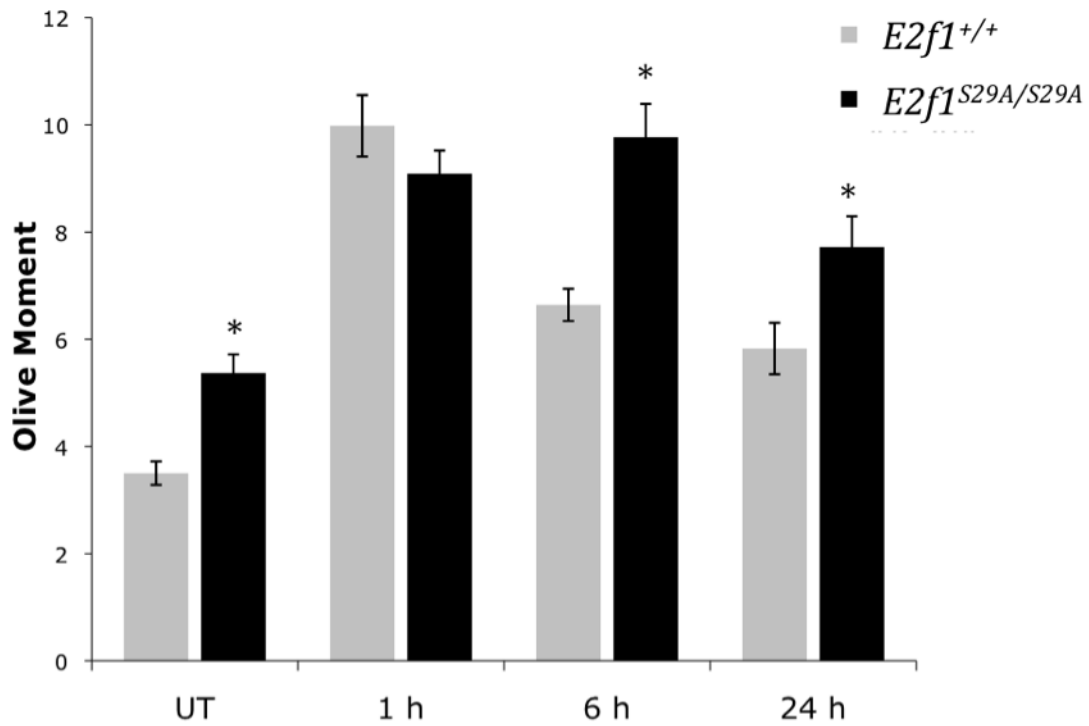


Figure 4.7. *E2f1*^{S29A/S29A} cells repair DNA damage less efficiently than *E2f1*^{+/+} cells. Mouse adult fibroblasts (MAFs) from *E2f1*^{+/+} or *E2f1*^{S29A/S29A} mice were treated with neocarcinostatin (NCS) for 30 minutes and collected at 1 hr, 6 hr, and 24 hr post treatment. MAFs were then subjected to the neutral comet assay. A minimum of 95 cells per genotype and time point, were analyzed with the COMETSCORE software. The average Olive moment, the product of the tail length and the fraction of total DNA in the tail, is shown \pm standard error. * indicates $p < 0.01$ by t-test within each time point.

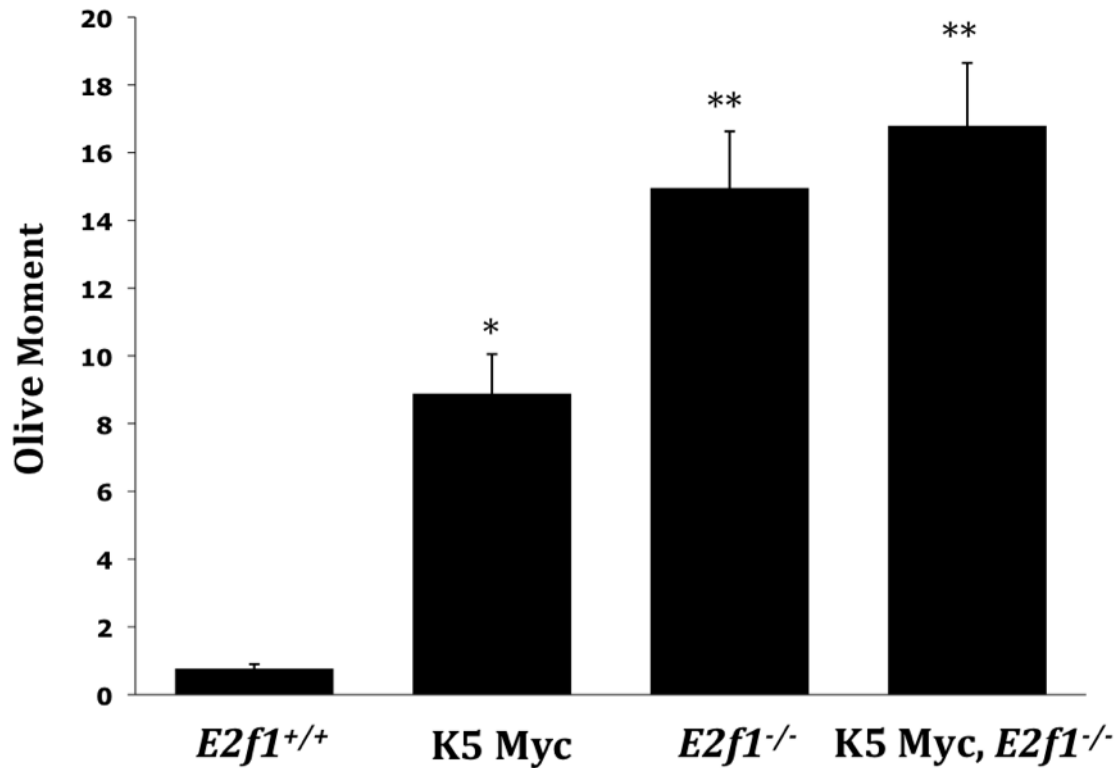


Figure 4.8. K5 Myc and E2F1 loss cause DNA damage in primary cells.

Primary keratinocytes from *E2f1*^{+/+}; K5 Myc; *E2f1*^{-/-}; and K5 Myc, *E2f1*^{-/-} mice were subjected to the alkaline comet assay. After electrophoresis, cells were fixed and stained with SYBR green. At least 95 cells per genotype were analyzed with the COMETSCORE software. Average Olive moment is shown \pm standard error. The asterisk(s) represents $p < 0.004$ by t-test.

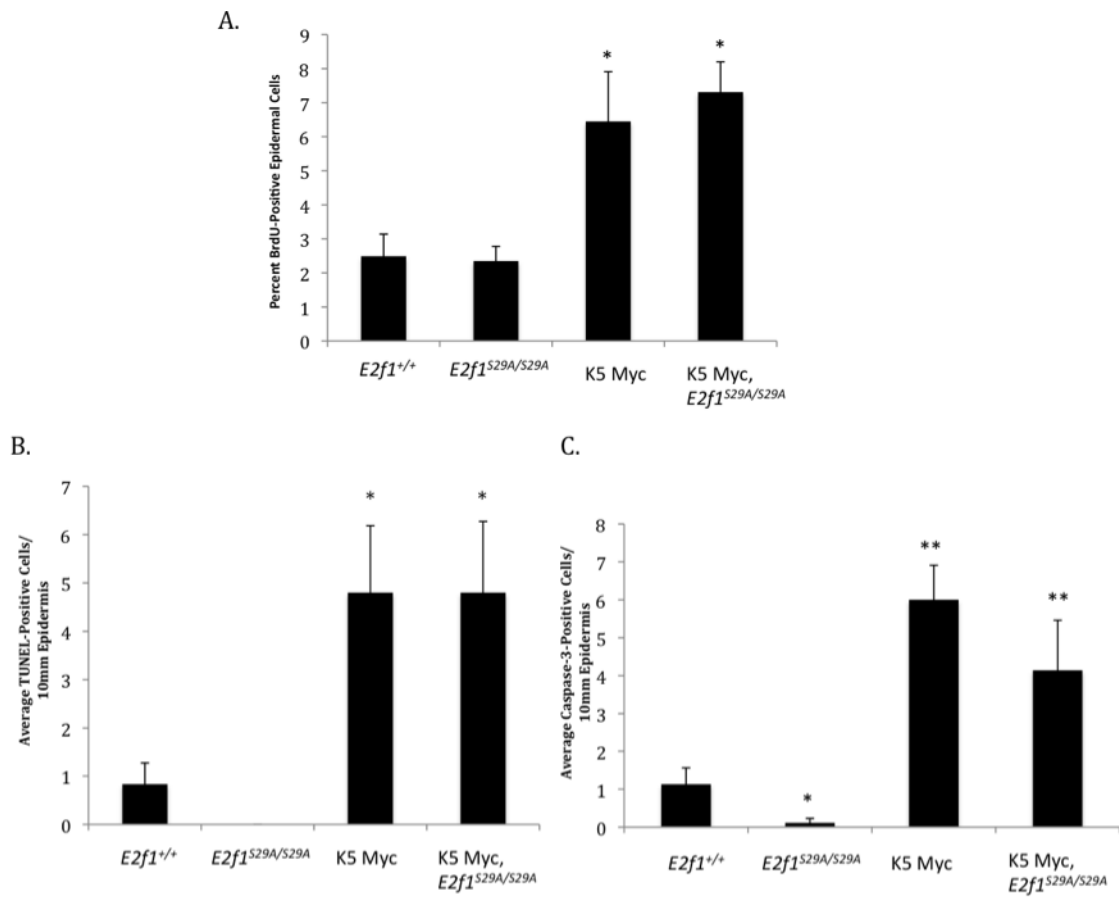


Figure 4.9. E2F1 S29A does not affect proliferation or apoptosis in response to K5 Myc. Epidermis from *E2f1*^{+/+}, *E2f1*^{S29A/S29A}, K5 Myc, and K5 Myc, *E2f1*^{S29A/S29A} mice was collected and analyzed for proliferation and apoptosis.

Averages of at least 5 mice per genotype are shown. (A) shows the percentage BrdU incorporation per 1000 basal cells. (B) shows TUNEL staining. (C) is Caspase-3 immunohistochemistry. Both (B) and (C) are the number of cells per 10 mm of epidermis. Asterisk(s) indicate $p < 0.05$ by t-test.

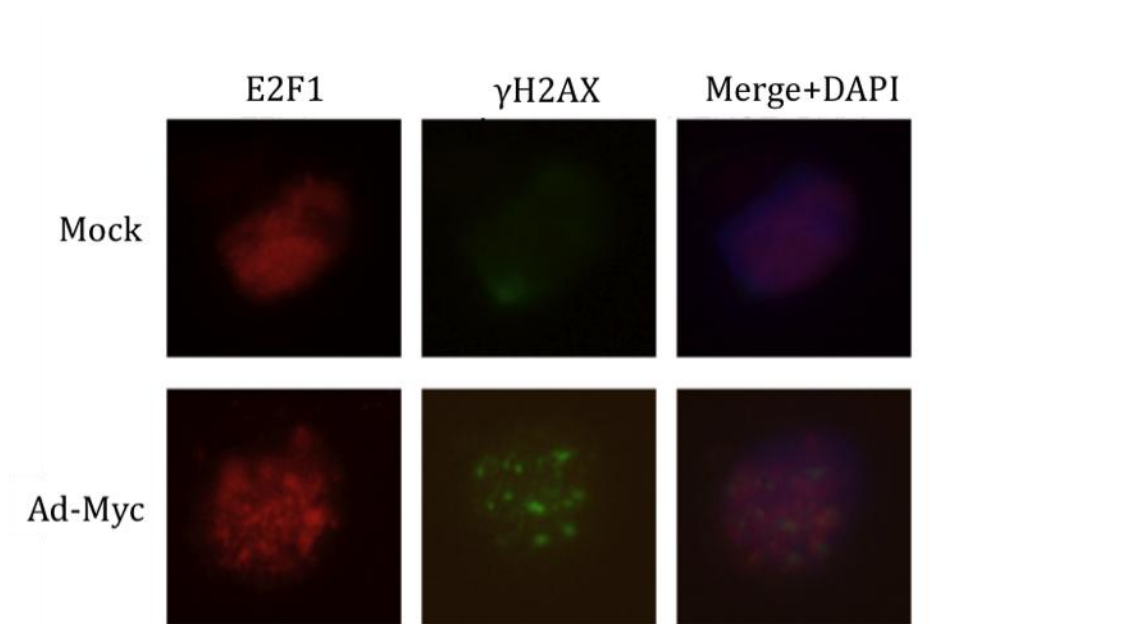


Figure 4.10. E2F1 goes to sites of DNA damage induced by Ad-Myc infection.

Immortalized normal human fibroblasts were serum starved for 24 hours and then infected with an Ad-Myc adenovirus or mock-infected. Forty-eight hours after infection, cells were subjected to immunofluorescence with an E2F1 and γ H2AX antibody. Representative images are shown.

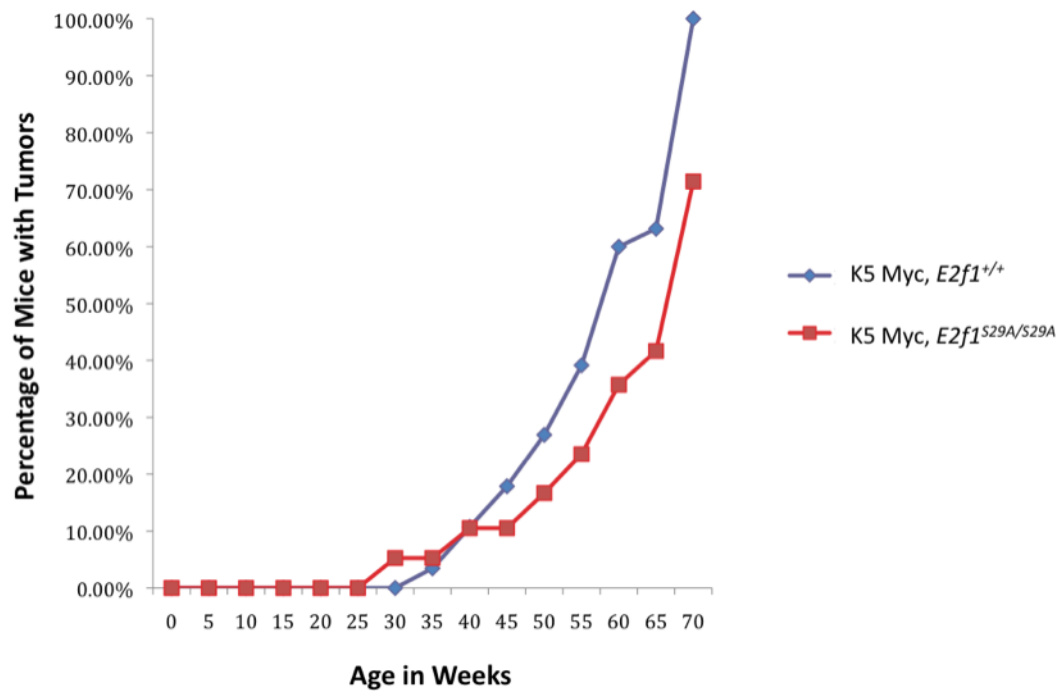


Figure 4.11. E2F1 S29A does not change K5 Myc-induced tumor incidence on a mixed background. K5 Myc and K5 Myc, *E2f1*^{S29A/S29A} mice on a mixed background were aged for spontaneous tumorigenesis. The percentage of mice with K5 tumors versus age in weeks is shown.

4.5 Discussion

In this report we have examined the role of E2F1 in the DNA damage response and tumorigenesis using a unique knockin mouse model in which E2F1 serine 29 is mutated to alanine. These mice develop normally, with only a slightly decreased body mass, and lack the characteristic testicular atrophy, exocrine gland hyperplasia and defects in thymocyte apoptosis seen in *E2f1*^{-/-} mice (87, 88). Since our model only affects E2F1 activities mediated by phosphorylated S29, it is probable that these defects seen in *E2f1*^{-/-} mice are due to non-functional E2F1 transcriptional activity.

When we examined *E2f1*^{S29A/S29A} mice biochemically, we made some interesting observations. E2F1 S29A protein cannot be stabilized in response to DNA damage, nor can it localize to DNA damage-induced foci suggesting it cannot interact with 14-3-3 τ or TopBP1. As well, cells isolated from *E2f1*^{S29A/S29A} mice have high levels of endogenous DNA damage and a defect in DNA repair. These data suggest a role for E2F1 in maintaining genomic stability that requires its function at DNA damage sites.

Finally, we report on the role of E2F1 in response to transgenic Myc expression. We found that mutation of serine 29 to alanine did not affect overall tumor incidence in K5 Myc mice. However, a unique tumor type was identified in K5 Myc, *E2f1*^{S29A/S29A} mice that possibly arose from the skin adnexae. Several K5 Myc, *E2f1*^{S29A/S29A} mice also developed more than one tumor, a finding that was unique to this genotype. In fact, one mouse had 3 squamous cell carcinomas. Consequently,

this mouse also had fight wounds from its littermates. One hypothesis is that wounding promoted the formation of these tumors. Lastly, we found evidence of metastasis in the lungs of a K5 Myc, *E2f1*^{S29A/S29A} mouse with an odontogenic epithelial tumor. Taken together, these data point to a role for E2F1 in suppressing Myc-induced tumorigenesis that requires its phosphorylation at serine 29.

Chapter 5: Future Directions, Summary, and Implications

5.1 Future Directions

5.1.1 The Role of p21 and Senescence in Tumor Suppression by the E2F1 Transcription Factor

In the second chapter, we explored the idea that tumor suppression by E2F1 overexpression could be mediated through the cell cycle inhibitor p21 and its ability to induce senescence. We found that ARF, p53, and p21 levels are all increased in K5 E2F1 epidermis, and loss of ARF inhibits this increase in p53 and p21. Additionally, hyperplasia increases in the absence of ARF in K5 E2F1 transgenic mice, but not apoptosis, both after treatment with chemical carcinogens. Conversely, when p21 is lost, there is a decrease in hyperplasia, and increased proliferation and apoptosis.

We next performed two-stage carcinogenesis assays. Our original studies showing K5 E2F1 mice are resistant to two-stage chemical carcinogenesis in an ARF and p53-dependent manner were done in an SSIN background. So, we first checked the ability of K5 E2F1 to suppress tumorigenesis on an FVB background. We found that K5 E2F1 was indeed still tumor suppressive on an FVB background. However, we could not determine whether p21 status affected tumor suppression by E2F1 since all p21-null mice were resistant to two-stage chemical carcinogenesis. Finally, we examined senescence in K5 E2F1 epidermis by β -galactosidase staining. We found high levels of background staining in FVB mice,

which precluded a conclusion about senescence in E2F1-mediated tumor suppression.

To expand on these findings, it would be interesting to repeat the tumor studies of p21-null mice using the original background strain, SSIN. Given the results of Topley et al who conducted chemical carcinogenesis studies on p21-null mice in a similar background strain, we would expect the K5 E2F1, p21^{-/-} mice in this strain to be sensitive to DMBA/TPA treatment. This strain would also allow us to stain for senescence using β -galactosidase. It is likely that we would find that senescence is mediated by p21 in response to K5 E2F1. On the other hand, if we were to repeat the two-stage studies in an FVB background for the K5 E2F1, ARF^{-/-} and K5 E2F1, p53^{-/-} mice, it is possible that we would get different results.

5.1.2 E2F1-mediated regulation of miRNAs

In the third chapter, we questioned whether E2F1 could regulate the expression of miRNAs, and in turn if miRNAs could mediate tumor suppression by the E2F1 transcription factor. We identified a total of 22 miRNAs that are differentially regulated between wildtype and K5 E2F1 transgenic epidermis. Of these, let-7a & c were shown to be upregulated in K5 E2F1 transgenic epidermis by northern blot. Further, let-7a & c expression increased even more upon treatment with chemical carcinogens in both wildtype and K5 E2F1 skin. However, even though let-7 has been identified as a negative regulator of Ras, we could find no

downstream consequences of let-7 expression on the Ras signaling pathway.

Additionally, we found that E2F1 binds the miR-301 promoter.

The future directions for this project should first be to resolve the conflict that exists between the northern blot and microarray results concerning let-7 expression. Both assays should be repeated, along with realtime PCR using RNA from both wildtype and K5 E2F1 epidermis. Next, the finding that E2F1 binds the miR-301 promoter should be followed up on. Possible experiments include a luciferase reporter assay using the miR-301 promoter to determine if E2F1 affects miR-301 expression. Realtime PCR should be conducted as well. The miR-301 target, Ska2, should be investigated to see if E2F1 affects its expression by western blot or vice versa. As well, the tissue types that have been reported to have differential expression of miR-301 between normal and tumor samples should be analyzed for E2F1 expression. Further, miR-301 expression should be checked in *E2f1*^{-/-} or siRNA knockdown cell lines. Finally, the 22 miRNAs identified by microarray should all be validated individually by northern blot and realtime PCR.

5.1.3 The Role of E2F1 Serine 29 in the DNA Damage Response and Tumor Suppression

In this final group of experiments, we asked whether E2F1's role in the DNA damage response could mediate its tumor suppressive properties. We found *E2f1*^{S29A/S29A} mice were phenotypically normal except for a slightly lower body weight. As well, the E2F1 S29A mutant protein cannot be stabilized in response to

DNA damage, nor can it localize to sites of damage. *E2f1*^{S29A/S29A} keratinocytes and MAFs have increased endogenous DNA damage compared to wildtype cells, and *E2f1*^{S29A/S29A} MAFs do not repair as efficiently as their wildtype counterparts. In response to Myc, tumor incidence is not changed when E2F1 is mutated. However, tumor spectrum, tumor multiplicity and aggressiveness seem to be modulated by E2F1's ability to respond to DNA damage.

Future experiments should focus on the findings concerning tumor spectrum, aggressiveness, and multiplicity. The tumor of questionable origin should be stained for various epithelial markers, such as keratins, to determine where it originated. As well, immunohistochemistry can be used to stain for markers associated with metastasis such as the MMPs (matrix metalloproteases), or Slug and Snail. Another interesting angle to pursue would be a wounding study. Given that the only K5 Myc, *E2f1*^{S29A/S29A} mouse with multiple SCCs also had fight wounds, it is reasonable to assume that the inflammation associated with those wounds might have served as a tumor promoter. Further biochemical studies (co-immunoprecipitations) can be conducted as well to determine which proteins E2F1 might be interacting with at DNA damage sites and how this interaction may affect repair.

5.2 Summary and Implications

In these studies we examined three mechanisms by which E2F1 might mediate its tumor suppressive properties: p21-induced senescence, miRNAs, and

the DNA damage response. When overexpressed, we found that E2F1 acts as a tumor suppressor in response to ras activation through a non-apoptotic mechanism requiring ARF and p53, but not p21. Additionally, we provided intriguing evidence that E2F1 is involved in miRNA regulation. And finally, our data indicates a role for E2F1 at sites of DNA damage, which may promote DNA repair and requires its phosphorylation at serine 29. These studies have identified proteins and signaling pathways that are important in tumor suppression. Ultimately, this information can be used in the future to develop drugs that may target these proteins and pathways and may be used in cancer treatment.

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Vita

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