

**THE ROLES OF THE RB AND P53 TUMOR SUPPRESSOR PATHWAYS IN AN
INTESTINAL TUMORIGENESIS MODEL SYSTEM**

by

Jennifer Ann Markovics

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FACULTY OF ARTS AND SCIENCES

This dissertation was presented

by Jennifer Ann Markovics

It was defended on

November 1st, 2005

and approved by

Paula A. Grabowski, Professor, Biological Sciences

Jeffrey L. Brodsky, Associate Professor, Biological Sciences

Jeffrey Hildebrand, Assistant Professor, Biological Sciences

Javier Lopez, Associate Professor, Biological Sciences, Carnegie Mellon University

Dissertation Advisor: James M. Pipas, Professor, Biological Sciences

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Jennifer A. Markovics, PhD

Cancer is characterized by unregulated growth. SV40 large tumor (T) antigen interacts with cellular proteins to cause transformation and tumors in rodents. Interaction with the retinoblastoma (Rb) family of tumor suppressors allows T antigen to inhibit growth arrest, while T antigen interaction with the p53 tumor suppressor blocks apoptosis.

Transgenic mice that express T antigen (TAg^w) in intestinal enterocytes develop hyperplasia that progresses to dysplasia. Expression of a mutant of T antigen (TAg^{32/3}) that is unable to interact with Rb family members in enterocytes does not result in an intestinal phenotype. Thus, T antigen interaction with Rb family members (pRb, p130 and p107) is required for T antigen-induced intestinal neoplasia. pRb performs the majority of its tumor suppressive properties through repression of E2Fs, a family of transcription factors that regulate the expression of many genes required for S phase. T antigen binds Rb family members, disrupts p130/E2F complexes and induces E2F2 and E2F3a in villi. These E2Fs are bound to the promoters of up-regulated E2F-responsive genes, supporting a role for them in T antigen-induced hyperplasia. Expression of an amino-terminal truncation mutant of T antigen (TAg^{dl1/37}) in villi enterocytes is sufficient to induce hyperplasia, but not progression to dysplasia. We hypothesized the T antigen interaction with p53 is required for the progression to dysplasia. However, T antigen does not bind and stabilize p53 in villi enterocytes. Furthermore, TAg^{dl1/37}/p53^{-/-} mice do not

progress to dysplasia. Therefore, we propose that the p53 is not active in intestinal enterocytes and its inactivation is not required for T antigen-induced progression to dysplasia.

These results suggest that distinct tumor suppressor proteins and pathways function in specific cell types to regulate normal or abnormal mechanisms of proliferation.

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PREFACE

My intentions for the design of this thesis were to provide a simple background of the Rb and p53 tumor suppressor pathways and the intestinal transgenic model system employed in these studies in the introduction. This is by no means a comprehensive analysis. Due to the lengthy nature of studies involving mouse genetics, the initial hypotheses made might be different had they been made more recently. However, they remain to be the hypotheses upon which these studies were based. Therefore, the minimal background is included in the introduction to provide the reader with sufficient knowledge of the field to understand the investigations described in the results chapters, chapters 2-5. A much more thorough analysis of the Rb and E2F pathway is described in the concluding chapter. The results from these studies can both support the classic model of Rb/E2F function as well as lend support to a revised model presented in the concluding chapter. The revised model is presented as a novel and alternative perspective for interpreting studies such as these, not as an irrefutable conclusion.

Here I would also like to add my acknowledgements. First, I would like to acknowledge my mentor, Jim Pipas. I am very grateful that he accepted me into his lab and let me follow my desires to pursue this project in the “mouse group”. I have enjoyed this project very much and I am grateful for the atmosphere that Jim fosters in his lab – a fun, happy lab! His philosophies on life transcend into the lab and I am glad to have been mentored by him. I hope to make you proud, Jim!

When I first came to Pitt, there were two professors on the list that I wanted to work with – Dr. Sáenz-Robles and Dr. Pipas. Soon, I discovered I could not work with Dr. Sáenz-Robles because she was not a graduate professor. However, I lucked out because she works in Dr. Pipas’ lab, and I did get the chance to work with her, after all! Mayte especially helped me as a rookie grad student, teaching me almost every aspect of

the lab, about mouse genetics, transgenics and intestines. I am very grateful for her help and expertise. I also enjoyed our many discussions about the Rb/E2F pathway as well as many other aspects of our work. I enjoy her enthusiasm – it is contagious. Thank you, Mayte, for all that you have done for me!

Next I would like to acknowledge my committee members, Jeff Brodsky, Paula Grabowski, Jeff Hildebrand and Javier Lopez. I appreciate their patience and their interest in my project. Their questions and advice helped me to think critically of my research, which helped me to develop as a researcher. Therefore I am very thankful for their support.

Paul Cantalupo also deserves a special acknowledgement. He is a guru of sorts in the lab, an informatics king. I appreciate the way he is so well organized that he can find a vile of antisera from 10 years ago within moments of asking for his help. He has also been particularly helpful with computer troubleshooting. Thank you, Paul!

I had an overall wonderful experience in the Pipas lab essentially from September 2000 until my departure now in the fall of 2005. I attribute this grand experience to all of my lab mates, with whom I became good friends: Pat Carroll, Liz Laposata, Leigh Kridle and Chevaunne Edwards. Thank you all very much! All the other lab mates that I have not mentioned are also in my thoughts and appreciation as well, thank you. Particularly, I have a lot of faith in Abhi Rathi in her future dissertation – it is sure to be exciting!

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always bailed me out without fear for losing their love! Thank you Mom and Dad, (Carol and Jim). They have taught me a good work ethic and I have acquired my values on education, family, quest for knowledge and the improvement of humanity from them.

Finally, I'd like to acknowledge all of those close to my heart that are not here anymore. To my sister, Jodi, and my nephew, Vinnie, I miss you so very much. Each monumental moment of my life is marked with your absence. To my friend Abbie Rinehart who died as a result of cancer treatment, I miss you, my friend! Her death has inspired me in my art and research endeavors.

This research is dedicated to all those who have died, have suffered or currently are suffering from cancer. I will continue to work hard to make treatment and life better for everyone.

1. INTRODUCTION

1.1. BIOLOGY; THE STUDY OF LIFE, AND THUS THE STUDY OF GROWTH AND GROWTH ARREST

A defining feature of life is the ability to reproduce and pass on genetic material to propagate more life. All living organisms reproduce, from viruses that use the cell's growth potential in order to propagate their genetic material to multicellular organisms such as humans that use specialized cells to combine their genetic material into one fused cell that proliferates and develops to form another multicellular being. The simplest reproductive mechanism is to make an exact copy of oneself. To do this, a cell needs to grow twice its own size, copying everything inside itself and distribute the material to both offspring. These activities correspond to the observed phases of the cell cycle: the first growth phase (G_1), the synthesis phase (S) where critical components are copied like DNA and centrioles, the second growth phase (G_2), and the mitotic phase (M) where the cell splits itself in half. A cell that is not dividing is considered to be in growth arrest, G_0 , which often occurs as cells exit mitosis and before the first growth period. While it is important for a cell to replicate, it is also just as important for a cell to be able to stop its growth. This ability to growth arrest is particularly important for multicellular organisms that must form specific cellular structures and tissues, since they must be of specific size proportions to maintain their shape and their function. The phenomenon of cancer indicates that the

regulation of cellular growth and growth arrest in each cell is vital to metazoans, particularly mammals.

1.2. CANCER; THE IMPORTANCE OF CELLULAR GROWTH AND GROWTH ARREST

Cancer is a leading cause of death in the developed world. It has been considered a "microevolutionary" process where a single cell which has acquired particular genetic mutations is selected for overgrowth within a multicellular organism (Cairns, 1975; Powell, 1976; Gatenby and Vincent, 2003). Malignant cancers that can lead to death are also characterized by the ability of these cells to acquire additional changes that allow for their migration to and colonization of secondary tissues in the organism where they continue to overgrow and alter normal physiological processes carried out by that tissue.

Cancer perturbs the normal regulation of growth and growth arrest in a cell. The cell has evolved defense mechanisms against abnormal growth that must be blocked in cancer cells. This block leads to selection for overgrowth and allows for additional changes that result in metastasis throughout the organism, resulting in the destruction of normal tissues. These multiple steps that a cancer cell must undergo are within the hypotheses of multi-step or multi-stage models of cancer (Armitage and Doll, 1954; Peto, 1977). In theory, distinct steps in both morphological change and change in patient health are the result of particular genetic mutations in each cancer, like colon cancer (Vogelstein and Kinzler, 1993).

In order to understand how cancer arises, it is imperative to understand how a cell changes so that it proceeds through these stages of tumorigenesis. The first step towards cancer

occurs when a cell is altered, thus allowing it to bypass the normal regulation of growth arrest. As most cancers begin with a genetic alteration that goes unrepaired, one possibility is that this first step disengages the normal DNA repair pathways. However, since there are conditions that do not lead to cancer in which the DNA repair pathways are altered, such as xeroderma pigmentosum, Cockayne syndrome and the photosensitive form of trichothiodystrophy, disruptions in these pathways do not always lead to cancer (de Boer and Hoeijmaker, 2000). While disruption of DNA repair pathways may not be the critical first step of cancer, changes in these pathways often occur at later stages of cancer, and these may drive further genetic changes. In this study, the focus is on the pathways that regulate cellular growth and growth arrest.

1.3. STUDYING MECHANISMS OF GROWTH AND GROWTH ARREST IN CELLS

The simplest model of growth is that every cell divides in the same controlled way. Under this assumption, cells that grow in an organism and cells that grow outside the organism, on plastic tissue culture plates, for instance, perform the same tasks and undergo the same or similar changes in order to grow. Even if the assumption is wrong, there must be some overlap in these functions as the result is similar – two cells are produced from the division of a single cell. Since more variables can be controlled in an artificial environment such as tissue culture versus the complex environment of a tissue in an organism, it is of no surprise that most of the current knowledge in the field of growth and the cell cycle is derived from studies in tissue culture. It is likely that findings from these studies will not tell the whole story of growth control of all the cells in complex multicellular organisms. The very nature of selecting for cells that can grow on plastic dishes results in a skewing of the analyzed population. Only particular cell types are able

to live and grow on dishes, and even these may become altered when selected for growth *ex vivo*. There is an uncertainty as to whether or not these cells exist in the same form in the organism, coined 'The Uncertainty Principle of Cell Culture' by James M. Pipas (Ahuja et al., *in press*). Despite these caveats, tissue culture studies provide knowledge of at least one possible way that cells are able to control growth. Therefore, a brief explanation of growth control in tissue culture is in order.

A cell that is not growing, but is still metabolizing and surviving is growth arrested. Most eukaryotic cells are growth arrested in a $2n$ (n = number of sister chromosomes) state with a single nucleus. The DNA is loosely packed, some regions undergoing active transcription in a euchromatic state, while other areas have low to no transcriptional activity. In cell culture, this is considered the G_0 stage of the cell cycle. Most "normal" cells in culture reach G_0 as the result of growth arresting signals such as contact inhibition, serum starvation, loss of contact with the extracellular matrix, or other signals. Cells that growth-arrest in G_0 have the potential to release from growth arrest and enter the cell cycle upon reversal of the signal. A cell that is incapable of re-entering the cell cycle, but continues to metabolize and survive, is considered to have entered senescence, a term, until recently, exclusive to tissue culture.

Cells are initially cultured by dissection of the animal, mechanical disruption of the tissue and enzymatic degradation of the extracellular matrix, followed by selection for growth on culture dishes in the presence of serum containing an undefined milieu of growth factors and nutrients. The cells that grow under these conditions vary according to the tissue type they were derived from and the method by which they were obtained. These primary cell cultures can contain many different cell types and are thus aclonal.

Primary cell cultures undergo a limited number of doublings and then enter senescence. Continued culture of these cells will result in a cell culture event called crisis, where >99.9% of the cells die. However, a few cells acquire genetic alterations that allow for their continued culture. These cells grow in clonal clusters and can undergo indefinite doublings in culture. These established cell lines are considered immortal. One benefit to studying established cell lines is that they are clonal, thus each cell is genetically identical. However, one caveat of studying these cells is that they inevitably contain genetic alterations in pathways that regulate growth and growth arrest.

Established cell lines retain the ability to growth arrest like primary cells, but so far none have been stimulated into senescence (Chondrogianni et al., 2003; Boucher et al., 2004). However, some cell lines derived from tumors can be stimulated into senescence (Wells et al., 2000b; Oruetxebarria et al., 2004). Cells that lose the ability to growth arrest upon any of the previously described growth arresting stimuli, such as serum deprivation and contact inhibition, are “transformed.” Since most transformed cells are tumorigenic when placed in animals, a common hypothesis is that these same pathways that are altered in these cells are also altered in cancer cells.

Another benefit of studying cells in culture is that they can be manipulated to grow relatively synchronously. Many cell types have the ability to be synchronized by either the addition of drugs that arrest the cells or by serum starvation, which arrests cells in G_0 . Then, with the release from the drug or addition of serum, the cells enter the cell cycle at the same time and are thus “synchronized.” The cells are only synchronized, however, for one or two doublings. Non-transformed cells arrest upon contact with neighboring cells. Thus, as the cells double and

reach higher confluence, the synchronicity will be lost as each cell becomes contact inhibited and arrested, and the slight differences in synchronicity are amplified.

1.4. THE CELL CYCLE AND CHECKPOINTS

It was first discovered in 1983 that there are proteins termed cyclins whose levels cycle during cell growth (Evans et al., 1983). Cyclins are master regulators of the cell cycle, performing their role by binding to and activating the cyclin-dependent kinases (cdks). The cdks phosphorylate key targets to activate or de-activate their substrates, thus turning the wheels of the cell cycle.

When a growth-arrested cell receives growth signals, signaling pathways are induced that lead to the activation of transcription factors that activate the expression of cyclin D. The translated cyclin D protein binds and interacts with cdk4, causing the cell to progress into the first growth period of the cell cycle (G_1). Historically, it was believed that the rest of the cell cycle then played out like the toppling of dominoes. However, it is more accurate to view the cell cycle like the sequential churning of gears in a mechanical clock with multiple levels of control that can either lock the clock at a specific position or even cause it to self-destruct. At particular points in the cell cycle, feedback mechanisms survey the progress of the cell and assess whether or not it should proceed to the next stage of the cell cycle. If the cell does not meet specific criteria for passing through these "checkpoints", it either arrests or dies by apoptosis.

There are three major cell cycle checkpoints: 1) The G_1/S phase checkpoint ensures the cell has the appropriate signals and size. 2) The G_2/M phase checkpoint ensures the fidelity of the copied DNA before entry into mitosis. 3) The meta/anaphase checkpoint ensures the appropriate alignment and adhesion of chromosomes to the spindle so that each daughter cell

inherits the appropriate number of chromosomes, allowing for the end of mitosis and subsequent cytokinesis, or cell death by apoptosis. Different molecules and pathways regulate these checkpoints. These molecules are controlled, at least in part, by multiple cyclin/cdk complexes. They also regulate cyclin/cdk complexes in a negative feedback mechanism. These negative feedback mechanisms are inactivated in cancer cells, thus allowing them to bypass these checkpoints and proceed through the cell cycle. Hence, the study of cancer cells has elucidated key molecules that regulate these checkpoints. Therefore, studying the molecular mechanisms of checkpoint regulation in both normal and abnormal cells continues to provide critical insight into how cancer proceeds. The more that is known about how cancer cells behave, and how that is different from normal cells, the more treatments can be created that specifically target cancer cells and not all dividing cells non-specifically.

1.5. G₁/S CHECKPOINT IS THE MAJOR REGULATED ENTRANCE INTO THE CELL CYCLE

The most critical checkpoint that must be passed for entry into the cell cycle, and thus for a cancer cell to override, is the G₁/S checkpoint. In fact, a key breakthrough in the field of the molecular biology of cancer was the discovery of the tumor suppressor gene, the retinoblastoma susceptibility gene (*RB*), which is a key regulator of the G₁/S checkpoint. Hereditary retinoblastoma is a rare childhood cancer that is acquired by the heterozygous inheritance of a mutant allele of the *RB* gene; the functional allele is lost at some early point in development of the retina and results in cancer. The recessive nature of the mutation indicates that the gene product acts as a tumor suppressor. The characterization of *RB* as a tumor suppressor was

confirmed by the discovery that both alleles are often found mutated in sporadic cases of the disease. pRb, the protein encoded by the *RB* gene, is a nuclear phosphoprotein that associates with DNA (Lee et al., 1987b; Lee et al., 1987a). The retroviral introduction of the cloned *RB* gene into retinoblastoma and osteosarcoma cells, which contain two mutated alleles of *RB*, demonstrated that pRb is capable of suppressing the neoplastic phenotype of these tumor cells (Huang et al., 1988). An indication that pRb is a general regulator of the cell cycle and “tumor suppressor” in most cells, not just the retina, was the discovery that it interacts with many viral oncogenes. Viral oncogenes are genes encoded by tumor viruses that behave in a dominant manner to cause cellular transformation.

1.6. DNA TUMOR VIRUSES; TOOLS FOR STUDYING CANCER

Viruses cannot reproduce themselves on their own, simply because they are too small to make everything that is required for their reproduction. Viruses come in many different sizes with various genome sizes. Larger viruses encode their own polymerases, but even these viruses need to commandeer the host cell for its metabolism and certain biosynthesis pathways. They have evolved to economically pack their genetic material that encodes what they need for survival – the proteins that encapsulate the viral genome as well as whatever other proteins they need for entry into the host cell and manipulating the host cell to copy their genetic material and allow for viral propagation. In their natural hosts, viruses can either live dormant, either integrated into the host chromosome or as a separate episome, or they can manipulate the host cellular machinery to make more viral particles and release the viruses for infection into other cells (Fields, 1985).

Since most adult cells are growth arrested, and thus not in S phase, they do not contain the enzymes and nucleotides necessary to replicate its DNA. Viruses have evolved to bypass the host cell's normal block to making these materials, in effect disabling the G₁/S checkpoint. Studying viruses has led to the discovery that some viruses have the ability to cause transformation. This discovery opened a door for studying these viruses, which encode very few genes as compared to the cell, as a tool for understanding how cancer can occur (O'Shea, 2005). In particular, the small DNA tumor viruses like adenovirus and human papillomavirus (HPV) have been studied both because of their small size and also because of their associations with human diseases. HPV16 was discovered to be the causative agent of human cervical cancer (Tsunokawa et al., 1986; zur Hausen, 2000). An even smaller DNA tumor virus, Simian virus 40 (SV40), a polyomavirus, contains a genome that is only 5243 bp in length. SV40 causes a productive infection in its natural host, the growth-arrested kidney cells of the monkey, *Rhesus macaque* (Shah, 1985). Although its association with human disease remains controversial, there are human viruses, such as the BK and JC viruses, that are related to SV40 and are more clearly associated with human disease (Imperiale, 2001). SV40 was discovered as a contaminant of the polio vaccine in 1960 that was distributed to countless millions of people from 1955 to 1963 and has been studied extensively since (Bookchin and Schumacher, 2004).

Cancer has historically been considered a disease that arises from genetic mutations in somatic cells. Therefore, studying a genetically unaltered cell that is infected with a virus or that expresses a viral oncogene may not appear to be an appropriate model for studying cancer. Two pieces of evidence should dispel this concern: 1) More frequently, new viruses are being discovered that cause human cancers. For example, Epstein-Barr virus causes Burkitt's lymphoma as well as a subset of Hodgkin's disease and other lymphomas, while Human

Herpesvirus type 8 causes Kaposi's sarcoma in immunodeficient individuals (zur Hausen, 2001). 2) Studying the cellular molecules with which the viral oncogenes interact enabled the discovery of cellular gene products that play key roles in regulatory pathways that are perturbed in cancer. Many of these same genes or other genes in these pathways have also been found mutated in most cancer cells, confirming that they are bona fide targets for genetic mutations that cause cancer.

1.7. RB IS A TUMOR SUPPRESSOR THAT REGULATES E2F TO CONTROL THE G₁/S CHECKPOINT

At about the same time that the *RB* gene was discovered, pRb was shown to interact with the E1A oncoprotein encoded by Adenovirus (Whyte et al., 1988). Subsequently, viral oncogenes E7 from HPV and large tumor antigen (T antigen; TAg) from SV40, were also discovered to interact with pRb (DeCaprio 1988; Dyson 1989; Munger 1989). The transformation properties of these three oncogenes depend, at least in part, on this interaction with pRb (DeCaprio 1988; Whyte 1988; Dyson 1989; Munger 1989). All three of these oncoproteins bind pRb through a consensus sequence, the LxCxE motif (x = any amino acid residue) (Chellappan et al., 1992). The region on pRb where this motif binds overlaps with positions of naturally occurring inactivating mutations of the *RB* gene, suggesting that all three oncogenes target a site of pRb that is important for its tumor suppressive function (Hu et al., 1990). This connection between viral oncoproteins and cancer also reveals a common mechanism for disrupting Rb tumor suppression function, thereby suggesting the use of viral oncogenes as tools to study this function of pRb.

pRb is a nuclear phosphoprotein that regulates the G₁/S phase transition by binding and inhibiting E2F activity (Cobrinik, 2005). E2F (**E2** factor) is a transcription factor that was first discovered through its activity of binding to and transactivating the E2 promoter of Adenovirus (Kovesdi et al., 1986; SivaRaman et al., 1986; Kovesdi et al., 1987). E2F heterodimerizes with DP (**d**imerization **p**artner) to bind DNA in a sequence-specific and methylation-specific manner (Yee et al., 1989; Girling et al., 1993; Helin et al., 1993b). There are E2F-responsive binding sites on the promoters of cellular genes, such as dihydrofolate reductase (DHFR), thymidine kinase 1 (TK1) and b-myb - many of which are required for DNA replication and cell cycle control (DeGregori, 2002; Stevaux and Dyson, 2002a). E2F regulates the expression of these genes through multiprotein complexes that change in composition but are present throughout the cell cycle (Yee et al., 1987). E1A disrupts pre-existing, higher-order E2F complexes, releasing “free” E2F to activate transcription, dependent on its pRb binding domain (Reichel et al., 1988; Bagchi et al., 1990; Mudryj et al., 1991). E2F activity can be stimulated either by expression of viral oncogenes, such as E1A, or the addition of serum to cells, placing it downstream of growth factor signaling pathways (Mudryj et al., 1990).

The phosphorylation status of pRb changes throughout different stages of the cell cycle, suggesting phosphorylation as a regulatory mechanism of pRb (Buchkovich et al., 1989; DeCaprio et al., 1989). pRb is unphosphorylated in growth-arrested cells. As cells progress through G₁ and into S phase, multiple phosphorylated forms of pRb are detected which remain throughout the cell cycle. SV40 is tropic to infecting growth-arrested cells in the kidneys of monkeys, which contain hypophosphorylated pRb. Therefore, it is not surprising that SV40 T antigen preferentially binds to the hypophosphorylated form of pRb (Ludlow et al., 1990).

A simple opposition model for the mechanism of pRb/E2F function emerged from these early studies of viral oncogenes. This model states that unphosphorylated pRb binds and represses E2F in growth arrested cells; upon binding by oncogenes or phosphorylation, pRb releases E2F from repression, thus allowing for its transactivation and progression into S phase (Fig. 1) (Weintraub et al., 1992). This model also explains how cancer cells might achieve deregulated entry into the cell cycle: either by mutation of *RB* in corresponding encoded regions of the protein that are necessary for its interaction with E2F or by mutations in other genes allowing for the constitutive phosphorylation of pRb. Cloning and expression of naturally occurring mutants of pRb demonstrated that these mutant proteins are indeed unable to inhibit E2F transactivation (Helin et al., 1993a).

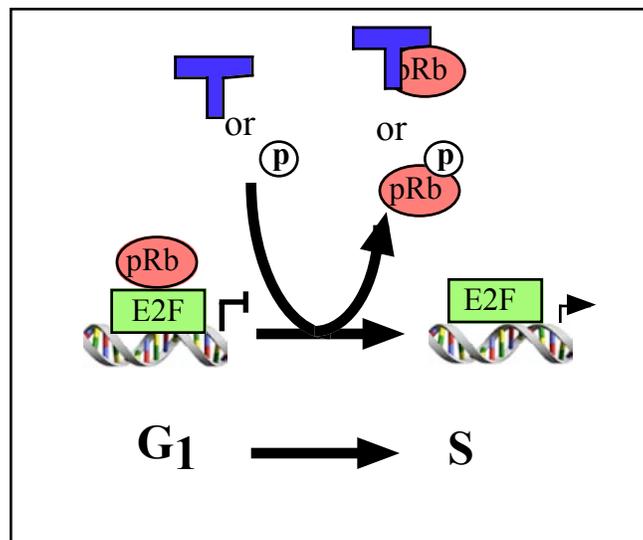


Figure 1. Simple opposition model of Rb/E2F function. In growth arrest, G_0 , Rb is bound to E2F, repressing E2F-responsive gene promoters. Upon interaction with viral oncogenes, such as SV40 T antigen, or phosphorylation, Rb releases from E2F. This “Rb-free” E2F subsequently transactivates S phase gene synthesis, sending the cell into the cell cycle and proliferation.

1.8. RB AND E2F ARE FAMILIES OF PROTEINS WITH SIMILAR STRUCTURES AND FUNCTIONS

In mammals, pRb is one of three family members with similar structures. The other two family members, p107 and p130, were discovered by cross-reactivity with antibodies to pRb and homology in the functional domains of pRb (Hu et al., 1991; Mayol et al., 1993). These domains, A and B, fold to form a globular domain where they interact with E2Fs as well as viral oncogenes (Fig.2a). An alternatively spliced form of p107 also exists which does not contain the spacer region or B pocket (Kim et al., 1995). Without these regions, this alternatively spliced form of p107 is incapable of interacting with E2F, suggesting that this form has an alternative function other than repressing E2F transactivation. All three Rb family members are synonymously called “pocket proteins”. Each pocket protein is capable of repressing E2F-mediated transactivation (Classon and Dyson, 2001). The pocket proteins have different cell cycle kinetics in expression levels and in forming complexes with E2F, all of which can be disrupted by viral oncogenes. Very little pRb is found in a DNA binding complex with E2F throughout the cell cycle, as assessed by electromobility shift assays (EMSAs). p107 forms a complex with E2F in S phase cells and p130 is in a complex with E2F exclusively in G₀ and G₁ cells (Chittenden et al., 1993; Schwarz et al., 1993). These results partially explain the different multi-protein E2F DNA binding complexes observed throughout the cell cycle. Additionally, these data present a conundrum for the simple opposition model of Rb/E2F function; how can a repressive p107/E2F complex be present during late G₁ and S phase when E2F is required to transactivate S phase gene transcription?

This conundrum is partially explained by the discovery that E2F is also a family of structurally related transcription factors that fall into three subcategories: the “activating” E2Fs,

(E2F1-3), the “repressing” E2Fs, (E2F4 and 5), and the less understood “dominant-negative” E2Fs, (E2F6-8) (Dimova and Dyson, 2005; Maiti et al., 2005). The first cloned E2F, E2F1, was isolated by its ability to bind pRb and retain E2F activity (Helin et al., 1992; Kaelin et al., 1992). E2F2 and E2F3 were discovered by screening cDNA libraries for low-stringency hybridization to a cDNA encoding E2F1 as well as by screening for DNA binding to E2F consensus sequences (Ivey-Hoyle et al., 1993; Lees et al., 1993). All three E2Fs are expressed in a cell-cycle dependent manner, with highest expression in G₁ and S phases (Li et al., 1994). Since the timing of their expression coincides with the timing of E2F transactivational activity, a hypothesis emerged that these E2Fs are responsible for that activity. These E2Fs (1-3) were coined the “activating” E2Fs primarily because their transient over-expression can drive cells into S phase, while their constitutive over-expression can mediate transformation of cell lines, demonstrating that they can behave as oncogenes (Singh et al., 1994; Xu et al., 1995; Yang and Sladek, 1995). However, over-expression of E2F1 can also induce apoptosis, a function inconsistently shared by E2F2 and E2F3, demonstrating it to also behave as a tumor suppressor (Shan et al., 1994a; Yang and Sladek, 1995; DeGregori et al., 1997).

The E2F3 locus encodes two isoforms of E2F3 termed E2F3a and E2F3b. E2F3a and E2F3b share the majority of exons, but contain alternative first exons with different promoter regions (Adams et al., 2000; He et al., 2000b) The amino terminus of E2F3a is truncated from E2F3b and therefore E2F3b does not contain the homologous cyclin A/cdk binding domain that E2F1, E2F2 and E2F3a share (Fig.2b). E2F3b expression is also different from the other “activating” E2Fs in that it does not fluctuate during the cell cycle, maintaining relatively low expression throughout. E2F3b is thus considered to play a role in repression of E2F-responsive promoters, although it retains the ability to transactivate transcription and interact with pRb.

More recently, a specific role for E2F3b-mediated repression of specific E2F-responsive gene promoters, independent of pRb, has been described (Aslanian et al., 2004).

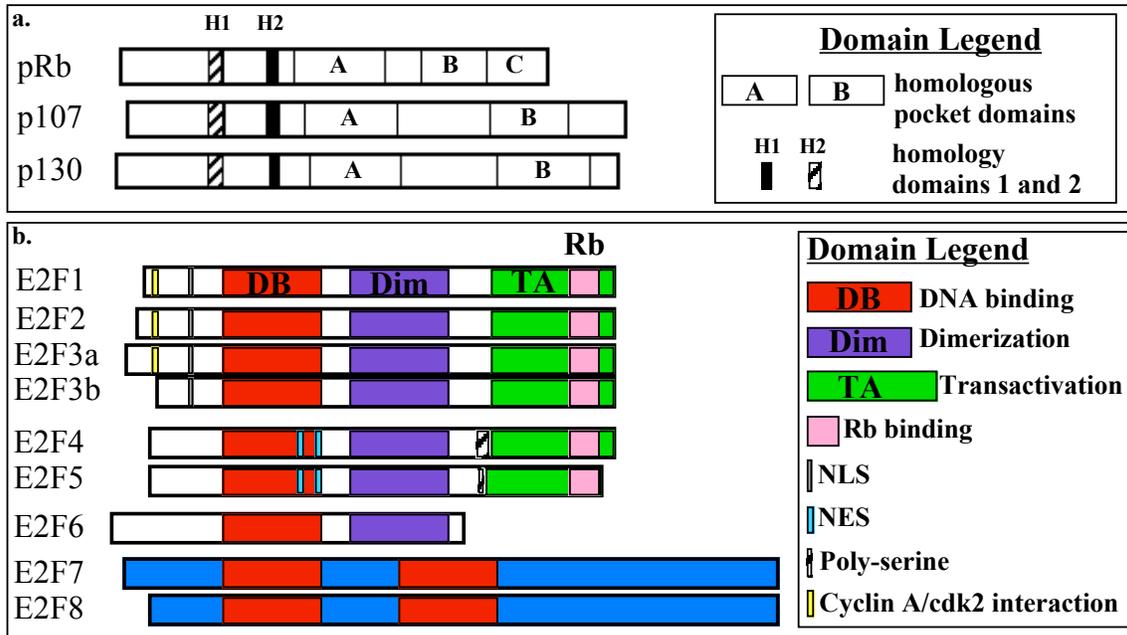


Figure 2. Domain structures of Rb and E2F family members. **a. Rb family domain structures.** The three Rb family members, pRb, p107 and p130, share sequence homology in domains H1, H2, A and B. The A and B domains form a pocket structure where Rb family members interact with the E2F family members. The linker region between the A and B pockets share sequence homology between p107 and p130, but not pRb. pRb also contains domain C in its carboxy terminus which can facilitate a specific interaction with E2F1 (Dick and Dyson, 2003). **b. E2F family domain structures.** All eight E2F family members, E2F1-8, share sequence homology in their DNA binding domains (DB). The E2F family can be subcategorized according to structural similarities of other domains into 4 groups. The ‘activating’ E2Fs, E2F1-3, share homologous domains and motifs such as their nuclear localization sequence (NLS), dimerization domain, transactivation domain and slight differences in their Rb-binding motifs. E2F1 - E2F3a, but not E2F3b, share a homologous amino-terminal cyclin A/cdk2 interaction domain. The ‘repressing’ E2Fs, E2F4 and 5, share homologous domains and motifs such as their nuclear export signal motif (NES), their dimerization domain, a poly-serine region, their transactivation domain and slight differences between their Rb binding motifs. E2F6 shares sequence homology in its DNA binding domain and dimerization domain with the other E2Fs, but is otherwise a divergent outlier. E2F7 and E2F8 share sequence homology in their DNA binding

domains with the other E2Fs, but contain two DB domains instead of one, and otherwise do not share homology throughout the rest of their proteins, except to each other (colored cyan blue).

E2Fs 1-3 preferentially form complexes with pRb, but E2F4 and E2F5 were identified by their abilities to interact with p130 (Hijmans et al., 1995; Sardet et al., 1995). E2F4 was also isolated by another group through its ability to bind p107 (Ginsberg et al., 1994). These E2Fs are more distantly related to E2Fs1-3 in structure, have weaker transactivational activity and S phase-induction capabilities (Fig.2b) (DeGregori et al., 1997). Also unlike E2Fs1-3, the levels of E2F4 do not significantly change throughout the cell cycle. E2F5 levels are highest in G₁ before the onset of “activating” E2F expression and then decline by S phase (Sardet et al., 1995). Together, these results implicate E2F4 and E2F5 in a repressive complex with p130 as the E2F DNA binding activity associated with G₀ and G₁, leading to their classification as the “repressive” E2Fs (Vairo et al., 1995). In support of this growth repressive activity, the over-expression of E2F4 is sufficient to cause growth arrest and assist in the induction of differentiation of neuronal precursors (Persengiev et al., 1999).

The hetero-dimerization partner of E2F, DP, is also a family of structurally and functionally similar proteins. DP1 preferentially binds E2Fs 1-3 while DP2 was isolated by its preferential binding to E2F4 (Zhang and Chellappan, 1995). Also unlike DP1, DP2 has two alternatively spliced transcripts. A third DP, DP3, also exists (Zhang et al., 1997). The genomic locations of all three DPs, DP1, DP2 and DP3 were mapped. These same chromosomal regions for DP1 and DP2 are associated with abnormalities in two diseases: Burkitt lymphoma-leukemia for the DP1-containing chromosomal region and the blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES) for the DP2-containing region (Zhang et al., 1997). These data suggest the possibility of a role for these factors in human disease.

Using protein motif searches through Genbank, E2F6 was identified by similarity to E2F family members in its DNA binding domain (Trimarchi et al., 1998). Like E2F1-5, E2F6 also requires hetero-dimerization with DP to bind DNA. Unlike the E2F1-5, however, it is a truncated form of E2F, lacking an Rb binding motif and a transactivation domain (Fig.2b). It was hypothesized that E2F6 may act as a dominant-negative E2F. This hypothesis is supported by experiments that demonstrate E2F6 can repress E2F1-transactivation in a reporter assay (Trimarchi et al., 1998). Since E2F6 does not contain an Rb-binding motif, it is hypothesized that this repression must therefore occur in an Rb-independent manner. Two lines of evidence suggest that E2F6 recruits polycomb group (PcG) complexes to modify chromatin structure at promoters. One is the observation that E2F6 null mice, which are embryonic lethal, have skeletal defects which are similar to the homeotic transformations common in PcG protein null mice (Table 1) (Storre et al., 2002). Secondly, E2F6 binds directly to RYBP (**R**ing1 and **Y**Y1 **B**inding **P**rotein) and can be found endogenously in a complex containing additional PcG proteins such as Ring1, Mel18, mph1, and the oncoprotein, Bmi1 (Trimarchi et al., 2001). Recently, E2F6 co-purified in separate complex of PcG proteins EPC1, SinB3 and EZH2, a histone methyltransferase (Attwooll et al., 2005). Although they do not directly bind DNA, PcG complexes are known to play roles in chromatin silencing by methylating histone tails and recruiting methyl-histone binding proteins to form higher-order macromolecular structures such as heterochromatin (Lund and van Lohuizen, 2004). They play critical roles in development, senescence and cancer. A role for E2F6 in cancer remains to be determined.

With the ongoing completion of the genome sequences of several mammalian species, two more divergent E2Fs, E2F7 and E2F8, have been identified by sequence similarities in DNA binding domains (de Bruin et al., 2003a; Maiti et al., 2005). Both E2F7 and E2F8 have two DNA

binding domains and do not dimerize with DP. Instead, they homo-dimerize to bind DNA. The only region of homology between these E2Fs and the rest of the E2F family is in their DNA binding domain. However, E2F7 and E2F8 are very similar to each other (Fig.2b). Over-expression of either E2F7 or E2F8 blocks proliferation. E2F7 has two transcripts with alternative promoters, 'a' and 'b'. E2F7b is the most abundant form of these two transcripts and increases as cells progress into S phase. This expression pattern might be easily explained by the fact that E2F1 and E2F4 associate with the E2F7 promoter *in vivo*, as determined by chromatin immunoprecipitation (ChIP) (Di Stefano et al., 2003). E2F7a, on the other hand, is constitutively expressed. E2F8 also has multiple transcripts – a, b, and c – containing alternative first exons and promoters. There are E2F sites in the c promoter but mutation of any of them does not affect their expression induced by E2F1 (Maiti et al., 2005). Further studies must be performed to clarify the functions of these E2Fs.

In summary, there are three members of the Rb family and 11 different loci of E2F and DP family members; some loci produce multiple transcripts containing alternative first exons and promoter regulation. Altogether, these data suggest a mechanistic switch between different Rb and E2F complexes that are regulated throughout the cell cycle. E2F4 is the best candidate as the pivotal switch since it can associate with all three pocket proteins and is the most ubiquitous E2F, accounting for the majority of endogenous E2F activity (Moberg et al., 1996). E2F4 is in a complex with p130 during G₀, which dissociates during G₁. During G₁, E2F4 is predominantly free of Rb, but begins to increase in association with pRb and p107. The p107/E2F4 complex is most abundant during S phase. These data indicate an intricate regulation of Rb/E2F complexes during the cell cycle, centering about E2F4.

Table 1. Phenotypes resulting from mutational analysis of the Rb and E2F family genes in mice. The genotypes of the mice are abbreviated in the left column. The resulting viabilities and phenotypes of these mice and

embryos are described with their accompanying references to the right. Where there are two different phenotypes, the references are separated, corresponding vertically to their respective phenotypes described to the left.

Genotype	Viability	Phenotype(s)	References
Rb ^{+/-}	viable, premature death ~8.5mos.	- increased tumor incidence such as pituitary and thyroid tumors	(Jacks et al., 1992; Lee et al., 2002)
Rb ^{-/-}	lethal: E13.5	- defects in lens development and erythropoiesis, increased apoptosis in CNS, anemia	(Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992)
Rb ^{loxP/loxP} , Cre ^{Mox2}	lethal: P0.5	- defects in lens development, sk. muscle dysplasia	(de Bruin et al., 2003b; MacPherson et al., 2003)
Rb ^{+/-} ; E2F1 ^{-/-}	viable	- extended lifespan and reduced tumorigenicity as compared to Rb ^{+/-} alone	(Yamasaki et al., 1998)
Rb ^{+/-} ; E2F3 ^{+/-}	viable, premature death ~ 9.5mos.	- suppresses pituitary tumors as compared to Rb ^{+/-} alone, increased incidence of metastatic thyroid carcinomas - slight increase in lifespan as compared to Rb ^{+/-} alone	(Ziebold et al., 2003)
Rb ^{-/-} ; E2F4 ^{-/-}	viable	- extended lifespan and reduced tumorigenicity as compared to Rb ^{+/-} alone	(Lee et al., 2002)
p130 ^{-/-}	- viable - strain-dep. lethal: E13.5	- no obvious defects - strain-dep. defects in growth and differentiation	(Cobrinik et al., 1996) (LeCouter et al., 1998b)
p107 ^{-/-}	viable	- no obvious defects, thickening of bones - strain-dep. defects in growth and myeloid hyperplasia	(Lee et al., 1996) (LeCouter et al., 1998a)
p130 ^{-/-} ; p107 ^{-/-}	lethal: P0.5	- defects in growth, limb and bone development - defects in epidermal differentiation and epithelial/mesenchymal interactions	(Cobrinik et al., 1996) (Ruiz et al., 2004)
p107 ^{-/-} ; Rb ^{-/-}	lethal: E11.5	- accelerated apoptosis in liver and CNS compared to Rb ^{-/-} alone	(Lee et al., 1996)
E2F1 ^{-/-}	viable	- testicular atrophy, reproductive tract sarcomas, lung and exocrine tissue tumors, lymphomas - decreased thymocyte apoptosis	(Yamasaki et al., 1996) (Field et al., 1996)
E2F2 ^{-/-}	viable	- increased lymphocyte proliferation, autoimmunity	(Murga et al., 2001)
E2F1 ^{-/-} ; E2F2 ^{-/-}	viable	- defects in proliferation and maturation in hematopoietic cells, diabetes and tumorigenesis, absence of spermatogenesis	(Zhu et al., 2001; Li et al., 2003b)
E2F3 ^{-/-}	lethal: E13.5	- embryogenesis and growth defect	(Humbert et al., 2000b; Saavedra et al., 2002)
E2F4 ^{-/-}	lethal: P0.5, or viable, premature death ~ 1.5mos.	- craniofacial defects, defects in hematopoiesis, anemia - defect in crypt formation in intestinal epithelium	(Humbert et al., 2000a) (Rempel et al., 2000)
E2F5 ^{-/-}	viable, premature death ~1.5mos.	- hydrocephaly due to increased secretion of cerebrospinal fluid (CSF)	(Lindeman et al., 1998)
E2F4 ^{-/-} ; E2F5 ^{-/-}	lethal: E14.5-P0.5	- growth defect	(Gaubatz et al., 2000)
E2F6 ^{-/-}	viable	- homeotic patterning defect - defect in restriction of male germ cell gene expression	(Storre et al., 2002) (Pohlers et al., 2005)
DP1 ^{-/-}	lethal: E12.5	- trophoblast proliferative defect; endoreplication failure	(Kohn et al., 2003)

1.9. REGULATION OF RB/E2F ACTIVITY

Each Rb family member is regulated by expression level and post-translational modifications such as phosphorylation throughout the cell cycle (Dyson, 1998; Mittnacht, 1998; Nevins, 1998; Ledl et al., 2005). As mentioned previously, the pocket proteins have different patterns of expression throughout the cell cycle. p130 is expressed during cell cycle exit and growth arrest, p107 is expressed during S phase while pRb is expressed throughout the cell cycle with a slight increase during G₁/S phase. p130 expression is regulated post-transcriptionally, as its transcript remains relatively constant regardless of growth status, while p107 expression is regulated transcriptionally by E2F (Smith et al., 1998). Each Rb can interact with and be phosphorylated by multiple cyclin/cdk complexes. pRb specifically binds cyclin D2 and D3 and is phosphorylated by the cyclin D-dependent kinase, cdk4 as well as the cyclin E-dependent kinase, cdk2 (Akiyama et al., 1992; Kato et al., 1993). p107 and p130 share a conserved spacer region between their A and B domains where they interact with cyclin A/cdk2 and cyclin E/cdk2 (Lees et al., 1992; Li et al., 1993). p107 and p130 also share a homologous amino-terminal region that is not conserved in pRb that can inhibit cdk activity (Woo et al., 1997). In general, phosphorylation of Rb family members regulates their growth suppressive activities by causing their disassociation with E2Fs, resulting in progression from G₁ into S phase. The different Rb family members, however, are phosphorylated on distinct residues, resulting in specific changes in protein activity (Graña et al., 1998). For instance, the hyperphosphorylation of pRb causes it to release from E2Fs and associate with the mitotic spindle, whereas the hyperphosphorylation of p130 targets it for proteasome-mediated degradation (Thomas et al., 1996; Bhattacharya et al., 2003). Protein phosphatase 1 specifically binds pRb, suggesting a mechanism by which pRb is activated later in the cell cycle and growth arrest (Kovesdi et al., 1987). Alternatively, tubulin-

associated pRb is de-phosphorylated upon anaphase of mitosis, resulting in the cytoplasmic distribution of hypo-phosphorylated pRb in the daughter cells (Thomas et al., 1996). Thus, there are two mechanisms that can activate pRb by de-phosphorylation later in the cell cycle.

E2F activity is regulated in several different ways. One mechanism for the regulation of E2F activity is through nuclear-cytoplasmic shuttling. Although E2F1-3 all have nuclear localization sequences, E2F4 and E2F5 do not. Immunofluorescence and subcellular fractionation studies revealed that E2F4 and E2F5 shuttle between the cytoplasm and the nucleus in a cell-cycle dependent manner (Magae et al., 1996; Lindeman et al., 1997). This shuttling is achieved by association of E2F4 and E2F5 with pocket proteins or DPs. Both proteins contain nuclear export signals, although experiments demonstrate the nuclear export of E2F5 does not depend on this sequence (Apostolova et al., 2002). The export of both E2F4 and E2F5 is achieved through a CRM1-dependent mechanism. As of this report, cytoplasmic functions of E2F4 or E2F5 remain to be determined.

The observation that the steady-state protein levels of the “activating” E2Fs fluctuate throughout the cell cycle suggests that there is a mechanism that degrades these proteins as cells progress out of S phase. Several studies of E2F1 suggest that there are regions in both the amino and carboxy termini of the protein that target it for ubiquitination and proteasome-mediated degradation. First it was demonstrated that a carboxy region of E2F1 is sufficient to target a heterologous protein for ubiquitination and proteasome-mediated degradation (Campanero and Flemington, 1997). pRb binding blocks E2F1 ubiquitination and this stabilizes the protein. The ubiquitin ligase, SCF^{SKP2} and Cul1 bind E2F1 within the first 41 amino acids of E2F1. This F-box containing complex mediates the degradation of E2F1 at the S/G₂ phase transition. Deletion of the first 41 amino acids of E2F1 results in its stabilization (Harper and Elledge, 1999; Marti et

al., 1999). This is not the only SCF complex that mediates E2F1 degradation. *In vitro* experiments demonstrate that E2F1 can be ubiquitinated by multiple cullin ligases that do not interact with SKP1 and that the interaction with SKP1 is not required for E2F1 ubiquitination (Ohta and Xiong, 2001). It is possible that these same complexes can also mediate the degradation of E2F2 and E2F3, although this remains to be determined.

As previously mentioned, E2Fs can also be regulated by phosphorylation. The “activating” E2Fs (1-3a) contain homologous cyclin/cdk binding domains in their amino termini and have been shown to form a DNA binding complex with cyclin A. Cyclin A is a late S/G₂ phase cyclin which complexes E2F with cdk2 and causes the phosphorylation of E2F. This phosphorylation event causes the decrease in E2F1 DNA binding activity, thereby inhibiting E2F-mediated transactivation (Krek et al., 1994; Xu et al., 1994).

E2Fs 1-3 can also be acetylated by p300/CBP (Marzio et al., 2000). In contrast to phosphorylation, acetylation results in increased DNA binding and transactivational activity. This acetylation occurs on conserved residues in the amino termini of these “activating” E2Fs. E2F4, E2F5 and E2F6 do not have these conserved amino acids and are not acetylated by p300/CBP. E2F1 can also be acetylated by P/CAF, which results in increased E2F1 protein half-life (Martinez-Balbás et al., 2000). pRb-associated HDAC1 can de-acetylate E2F1, demonstrating another mechanism by which pRb can reduce E2F activity. Interestingly, p300 can also acetylate pRb, but this acetylation does not affect the ability of pRb to mediate growth arrest or repress E2F function (Nguyen et al., 2004). The acetylation of pRb is required for its induction of myogenic differentiation, suggesting that this modification causes a switch between cell cycle regulation and differentiation functions of pRb.

Another method of regulating E2F activity is through modulation by interacting factors. Many E2F-responsive promoters like those of DHFR and TK1 contain both E2F and SP1 binding sites. These two transcription factors can interact with each other and act together to synergistically transactivate promoters (Karlseder et al., 1996; Rotheneder et al., 1999). The interaction between E2F and SP1 requires the DNA binding domains of both transcription factors plus the amino-terminus of the “activating” E2Fs and the carboxy-terminus of SP1. Interestingly, the synergistic transactivation does not require that both SP1 and E2F binding sites be on the promoters (Lin et al., 1996). These data suggest that one transcription factor can bind to the promoter and tether the other transcription factor for synergistic transactivation.

1.10. THE DOGMA OF RB/E2F REGULATION THROUGHOUT THE CELL CYCLE

A classic model of Rb and E2F regulation throughout the cell cycle has been proposed from the collection of results presented here and elsewhere (Fig.3). Many years of research and reviews repeat the same model of Rb/E2F regulation over and over, so much so that it has almost become dogma (Dyson, 1998; Trimarchi and Lees, 2002; Attwooll et al., 2004). This dogma states that repressive p130/E2F4 and p130/E2F5 complexes occupy E2F binding sites on promoters in G₀. Upon progression from G₀ to G₁, these complexes switch to pRb/E2F4, some p107/E2F4 as well as Rb-free E2F4 complexes. At the same time, the dogma states that “activating” E2Fs are expressed and become bound to pRb on E2F-responsive promoters during G₁. Two phosphorylation events take place during G₁ and at the G₁/S checkpoint – one by cyclin D/cdk4 and another by cyclin E/cdk2. The second phosphorylation event results in the release of pRb from E2F. Upon release and transactivation of genes necessary for S phase by the

“activating” E2Fs, S phase is initiated. During S phase, p107 is associated with E2F4 and mediates the shuttling of E2F4 into the cytoplasm for degradation. As cells progress through G₂ and M phase the “activating” E2Fs are phosphorylated by cyclin A/cdk2 complexes, causing their dissociation with DNA. These E2Fs are then degraded. p130 expression is upregulated upon growth arresting signals, bringing E2F4 back into the nucleus and binding E2F-responsive promoters, actively repressing their transcription thus sending the cell into G₀.

This model is supported by experiments demonstrating that the ablation of all three “activating” E2Fs, E2F1-3, results in cell cycle arrest (Wu et al., 2001). Interestingly, these cells arrest in all phases of the cell cycle, indicating a requirement for these E2Fs throughout all phases of the cell cycle. Also supporting the requirement for E2F4 and E2F5 in pocket-protein mediated growth arrest, E2F4 and E2F5 double knock-out MEFs were unable to arrest when signaled by over-expression of the cyclin D-dependent kinase inhibitor, p16INK4a (Gaubatz et al., 2000). However, these MEFs could arrest via over-expression of the universal cyclin-dependent kinase inhibitor, p21^{WAF1/CIP1}, which can use a pocket-protein-independent mechanism to arrest the cell cycle. These results indicate the specificity of E2F4 and E2F5 in pocket protein-mediated growth arrest.

Two independent groups have shown that Rb, p107 and p130 triple knock-out (TKO) cells have shorter lengths of G₁, reduced cell size and are unable to arrest in the presence of growth-arresting signals such as growth factor depletion or contact inhibition (Dannenberg et al., 2000; Sage et al., 2000). However, the two groups did not agree on the oncogenicity of the TKO cells. One group found that the cells did not grow in soft agar because they succumb to apoptosis (Dannenberg et al., 2000). The other group, however, found that the TKO MEFs did grow in soft agar and showed a partially transformed phenotype as evident by focus assay (Sage et al., 2000).

The main difference between the two studies lies in how the Rb null alleles were created. The group that did not observe a transformed phenotype obtained MEFs from Rb knock-out mouse embryos. The other group that observed the partially transformed phenotype used the conditional loxP flanked Rb alleles combined with Cre recombinase to create the null. Since it is known that Cre is not 100% efficient at recombining all loxP sites, it is likely that there is some leakage of Rb expression in these cells. However, if leaky expression of pRb contributes to the partially transformed phenotype, then these results suggest that pRb has some oncogenic functions - in clear contrast to the documented roles for pRb in tumor suppression.

There are several questions left unanswered by this model of the Rb/E2F pathway. First, it remains unclear why there are multiple Rb and E2F family members that are capable of similar functions, even within the sub-groups. Second, it is puzzling that repressive p107/E2F4 complexes rise in S phase, when E2F activity is greatest. Finally, if the “activating” E2Fs are required to drive S phase entry, then how come the loss of E2F1, E2F2 or the combined loss of E2F1 and E2F2 in mice does not result in lethality, but the loss of either “repressive” E2F4 or E2F5 does (Table 1)? To address these questions, it is important to characterize the functions and regulation of different Rb and E2F family members in the complex environment of a multicellular tissue within the organism.

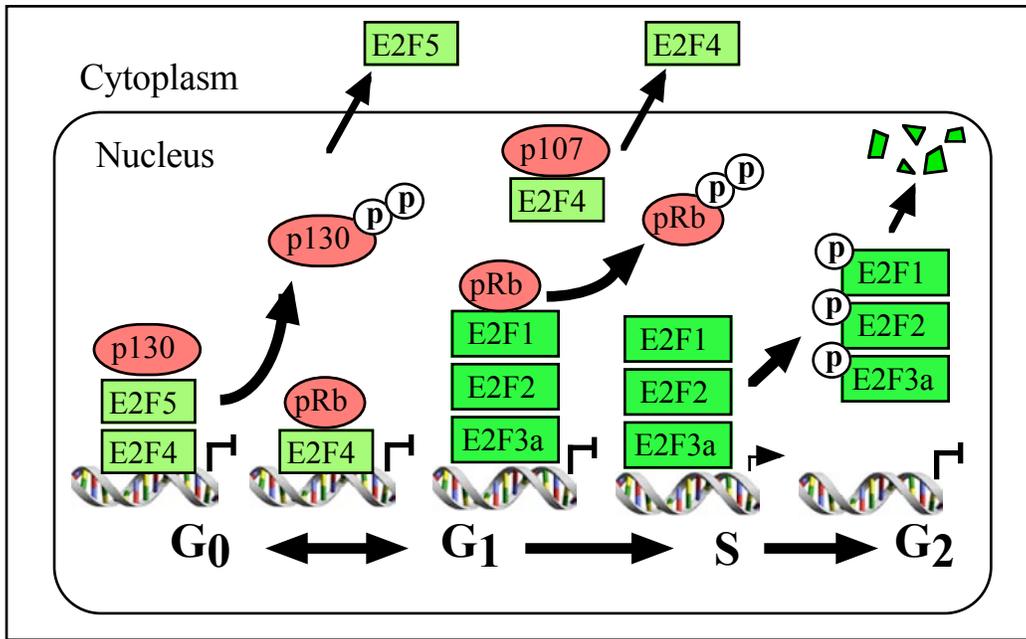


Figure 3. Classic model of Rb/E2F pathway throughout the cell cycle. In growth arrest, G₀, p130/E2F4 and p130/E2F5 complexes are bound to and repressing E2F-responsive gene promoters. Phosphorylation of p130 causes disruption of these complexes. E2F5 is exported from the nucleus for degradation in the cytoplasm, while pRb/E2F4 complexes bind E2F-responsive promoters. As the cell progresses through G₁, these complexes are replaced by pRb/E2F1, pRb/E2F2, and pRb/pE2F3a complexes. Hyper-phosphorylation of pRb causes pRb to dissociate from these complexes, allowing for “Rb-free” E2F1, E2F2 and E2F3a to transactivate E2F-responsive gene promoters. The up-regulation of S phase gene synthesis causes the cell to enter S phase. During S phase, p107 binds E2F4, facilitating its export from the nucleus to the cytoplasm for degradation. E2F1, E2F2 and E2F3a are phosphorylated at the end of S phase, targeting them for ubiquitination and subsequent degradation, thereby causing the de-activation of S phase gene synthesis and the end of S phase. After division, p130 binds to E2F4 and E2F5, importing them into the nucleus to bind E2F-responsive promoters for repression upon cell cycle exit.

1.11. MOLECULAR MECHANISMS OF RB AND E2F TRANSCRIPTIONAL CONTROL

Two mechanisms have been described for Rb/E2F-mediated transcriptional regulation. One is the mechanism of active repression and de-repression of Rb/E2F complexes, whereas the other mechanism depends on E2F activation (Lukas et al., 1996; Zhang et al., 1999). One study attempted to elucidate the relative contribution of each mechanism to Rb/E2F transcriptional activity by use of microarray experiments comparing E2F1-over-expressing and a transactivation-negative E2F1 mutant over-expressing cells (Young et al., 2003). These authors suggest that different genes are regulated by these two mechanisms, and that they segregate by biological pathway. For instance, the authors concluded that the majority of replication factors are controlled by activation and not de-repression, whereas the majority of DNA repair genes and histones are regulated by de-repression. These data suggest the regulation of some genes, such as those that are part of a defense mechanism or quality-control, such as DNA repair, may be more quickly activated by a mechanism of de-repression, whereas other genes, such as for the replication machinery, may require much more concerted effort through E2F transactivation.

Rb-mediated repression has two phases, an acute phase and a chronic phase (Angus et al., 2004). The acute phase involves the repression of replication elongation factors, while the chronic phase involves repression of the replication machinery, itself. These results suggest that there are two types of Rb-mediated repression. These two mechanisms involve the passive, simple blocking of the transactivation activity of E2F through direct binding in their transactivation domains, or active repression by the recruitment of chromatin remodelers. These remodelers are either ATP-dependent, such as the BRG1/BRM complex which alters nucleosomal structure, or ATP-independent, such as HDACs, which de-acetylate histone tails,

normally associated with tight binding of DNA to histones and transcriptional repression (Zhang et al., 2001). These chromatin modifiers such as histone deacetylases (HDAC class I) and histone methyltransferases bind Rb via their LxCxE motifs, in the same region as where the viral oncogenes bind (Flemington et al., 1993; Hagemeyer et al., 1993; Helin et al., 1993a; Harbour and Dean, 2000; Frolov and Dyson, 2004). Interestingly, although the germ-line deletion of pRb is embryonic lethal, knock-in mutants of Rb that cannot interact with LxCxE-motif containing partners but retain all other pRb functions are viable and do not appear to have any significant defects (Balsitis et al., 2005). These data indicate that the viability of the *RB* knock-out mouse does not depend on Rb's interaction with these chromatin modifiers through their LxCxE motifs. In fact, these data may indicate that the repressing function of pRb, not inherent to simple binding to E2F transactivation domains, is not a significant function required for viability. Alternatively, different genes may require different types of Rb-mediated repression. In melanocytes, phosphorylated pRb is present on apoptotic gene promoters, but not on cell cycle genes, suggesting differences in pRb function at different gene promoters (Halaban et al., 2005). Therefore, it is possible that “active repression” by Rb is required for the repression of some genes, possibly genes not required for viability, while “passive repression” by Rb is required for the repression of other genes, such as those required for viability.

pRb can also mediate the permanent silencing of E2F-responsive gene promoters through chromatin remodeling and heterochromatin formation (Narita et al., 2003). This function is associated with the ability of pRb to cause senescence. In addition to mediating changes in histone and nucleosome structure, pRb also recruits the DNA methyltransferase, DNMT1 (Robertson et al., 2000). Through DNMT1, pRb can cause the methylation of DNA at E2F promoters, causing them to be silenced. E2F binds DNA in a methylation-specific manner, such

that the methylation of DNA decreases E2F binding in a promoter- and E2F-specific manner (Campanero et al., 2000). Because of the controversial existence or non-existence of DNA demethyltransferases, these modifications are believed to be very stable, only disappearing upon dilution through multiple rounds of DNA replication without maintenance of the methylated marks (Lande-Diner and Cedar, 2005).

E2Fs activate the transcription of genes first by binding the promoter and causing a bend in the DNA (Cress and Nevins, 1996). Rb/E2F/DP complexes do not cause a net bend different from the natural bend of the promoter DNA. It is suggested that this bend may facilitate the interaction of upstream regulators, such as SP1, with general transcription factors. E2F also recruits transcriptional co-activators such as histone acetyl transferases (HATs) like P/CAF, GCN5/TRRAP (a component of the SAGA chromatin remodeling complex), and p300/CBP (Frolov and Dyson, 2004). Thus, the activation and repression of E2F-responsive gene promoters center extensively around the interplay of histone acetylation and de-acetylation by HATs and HDACs.

1.12. AUTO-REGULATION OF THE RB/E2F PATHWAY REVEALS BOTH NEGATIVE AND POSITIVE FEEDBACK MECHANISMS

The cell cycle is temporally coordinated; the beginning of each phase requires activation of the factors required for that phase, while the ending of each phase requires repression of these factors so as to move on to the next phase properly. For instance, the replication of the genome must occur once and only once in most cells during S phase so that the cell can divide properly during mitosis. In fact, loss of E2F3 results in duplication of the centrosomes and aneuploidy

after cellular division, indicating that a normal function of E2F3 is to repress extra centrosomal duplication (Saavedra et al., 2003). One method by which cells achieve cyclic behavior is through feedback loops. The Rb/E2F pathway has both positive and negative feedback mechanisms that are both auto-regulated and coordinately regulated with other pathways. Here, the auto-regulatory positive and negative feedback mechanisms will be discussed with E2F as the main mediator through which these mechanisms occur.

The “activating” E2Fs, 1-3a, are expressed in a cell cycle regulated fashion, with highest expression at late G₁ and S phases, similar to most, if not all, E2F-regulated genes. Therefore, it was not surprising to find that there were E2F binding sites in the promoters of E2F1, E2F2, and E2F3a (Johnson et al., 1994; Sears et al., 1997; Adams et al., 2000). There are two E2F binding sites in each of these promoters. Analysis of the E2F1 promoter demonstrated that these two sites are both required for proper expression and are functionally different (Johnson et al., 1994; Araki et al., 2003). The site furthest from the transcriptional start site is required for repression and binds E2F4 and p130, while the closer site to the transcriptional start site is required for activation and binds E2F1 and E2F3 (Araki et al., 2003). It seems likely that all three of these E2Fs are regulated by repression of their promoters in growth arrest and early G₁ through p130/E2F4/DP complexes, and upon growth signals, the release of p130 from these complexes de-represses their promoters. After this initial up-regulation, these activating E2Fs can then positively up-regulate their own transcription by binding their own promoters.

The positive auto-feedback of the “activating” E2Fs is balanced by negative auto-feedback through E2F regulation of the *RB* and *P107* promoters, which can then result in the inhibition of E2F activity (Fig.4a). p107 is expressed in a similar pattern as other E2F-regulated genes, as it is highest in S phase. There are also two E2F binding sites in the *P107* promoter.

Similar to the other E2F-responsive promoters containing two E2F binding sites, the site further from transcriptional initiation is required for repression, while the site closer to the start site functions for activation. However, both sites are required for full promoter activity (Zhu et al., 1995). Unlike p107, pRb expression does not fluctuate through the cell cycle. Therefore, it makes less sense that E2F may regulate its expression. However, E2F1 was shown to stimulate *RB* promoter activity that required the DNA binding and transactivation domains of E2F1 (Shan et al., 1994b). Reconstitution of Rb in Rb-null cells caused repression of *RB* promoter activity. This result is consistent with a negative auto-feedback mechanisms in which pRb represses its own promoter (Shan et al., 1994b). Thus, the upregulation of Rb by E2F is countered by the repression of this upregulation by pRb, itself.

Recent studies have unveiled another proto-oncogene, gankyrin, as mediator of pRb degradation (Higashitsuji et al., 2000). Gankyrin is over-expressed in many human hepatocellular carcinomas (HCCs). Expression of gankyrin causes growth of cells in soft agar as well as tumorigenesis in nude mice (Higashitsuji et al., 2000). Gankyrin is expressed at the G1/S phase transition in normal, non-cancerous hepatocytes (Iwai et al., 2003). It binds specifically to pRb, not p107 or p130, through an LxCxE motif in its fifth ankyrin repeat and enhances the phosphorylation of pRb by cdk4/6 (Higashitsuji et al., 2000). Crystal structural analysis of gankyrin reveals that it binds to cdk4/6 in the same region as p16INK4a, suggesting a competitive mechanism between these two mediators (Padmanabhan et al., 2004). The authors propose a model for this competition as part of normal regulation through the cell cycle. These data also support a model suggesting dynamic equilibrium of pRb levels throughout the cell cycle. Thus, pRb levels remain constant until the balance gets perturbed somehow.

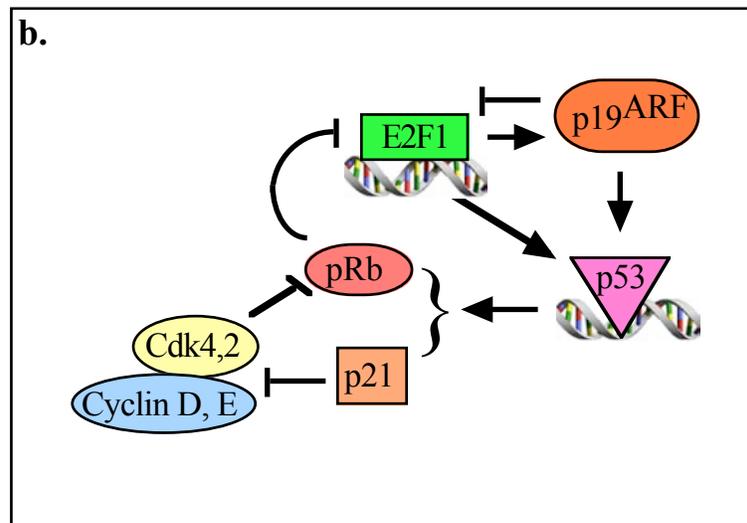
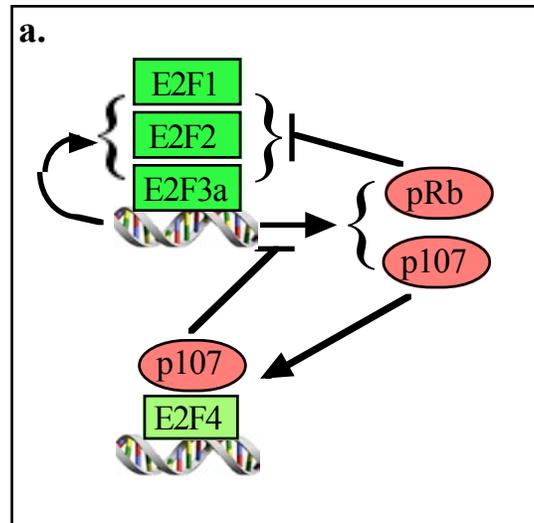


Figure 4. Models of Rb/E2F/p53 pathway regulatory feedback loops. a. Auto-feedback within the Rb/E2F pathway. E2Fs bind to the promoters of ‘activating’ E2Fs 1-3a, up-regulating their own expression in a positive-auto-feedback loop. E2Fs also bind to the promoters of the genes for pRb and p107, activating their transcription. In turn, pRb and p107 repress E2F-mediated transactivation in a negative-auto-feedback loop. **b. Feedback between the Rb/E2F and p53 pathways.** ‘Activating’ E2Fs 1-3a bind to the p19ARF promoter and E2F1 binds to the p53 promoter, and transactivate their transcription. p19ARF activates p53 by blocking its degradation by mdm2. p19ARF also directly binds E2F1, disrupts E2F1/DP1 DNA binding complexes and targets E2F1 for degradation. p53 can bind to the pRb and p21 promoters, transactivating their transcription. In turn, p21 blocks cyclin/cdk complexes that phosphorylate and inactivate pRb, and pRb binds E2F, repressing its transactivational activity.

1.13. CELLULAR DEFENSE MECHANISMS

Cells have evolved mechanisms to combat cellular stresses such as inappropriate entry into the cell cycle, DNA damage, hypoxia or low glucose/energy levels (Moley and Mueckler, 2000; Pietenpol and Stewart, 2002; Ben-Porath and Weinberg, 2004). There appears to be a hierarchy determining which mechanism the cell will employ. First, if a cell is abnormally induced to proliferate, it will push the breaks and signal to the cell to growth arrest. If the cell does not respond, or if the damage is too great, the cell will senesce, an irreversible form of growth arrest, or signal to kill itself by apoptosis as a last resort (Sharpless and DePinho, 2005). Of course, if the cell is unable to perform any of these backup plans, then the organism has a real problem on its hands; in other words, this is when a disease such as cancer occurs.

As mentioned previously, over-expression of E2F1 both induces cells to enter S phase and leads to apoptosis. Viral oncogenes such as adenovirus E1A, HPV E7 and SV40 T antigen activate E2F1 by binding to pRb and disrupting pRb/E2F1 complexes as previously described. Expression of E1A or E7 leads to apoptosis but expression of T antigen does not (Lowe and Ruley, 1993; Howes et al., 1994). A key difference between these oncogenes is that T antigen binds and stabilizes a key regulator of apoptosis, p53. p53 was originally discovered by its robust stabilization in SV40-infected cells and was found to interact with T antigen (Linzer and Levine, 1979). p53 is mutated in many cancers and its over-expression can cause apoptosis, suggesting it is a tumor suppressor (Oren, 1994; Prives and Hall, 1999).

p53 forms a homotetramer that binds DNA in a sequence-specific manner on promoters and activates the transcription of genes necessary for apoptosis, including pro-apoptotic membrane proteins (bax) and caspases (Prives and Hall, 1999). p53 levels are regulated by a negative-feedback loop with mdm2, an E3 ubiquitin ligase. At basal levels, p53 activates the

transcription of mdm2. Mdm2 binds to p53 and ubiquitinates it, thus targeting it for proteasome-mediated degradation. p53 is predominantly activated through inhibition of mdm2 (Levine, 1997). A very interesting locus that is conserved in humans and rodents is the *INK4/ARF* locus. This locus encodes two overlapping genes that share some of the same sequences, but use different open reading frames to translate their respective proteins (Quelle et al., 1995). Although the two proteins, p16INK4a and p19ARF (alternative reading frame), do not share any amino acid sequence homology, they are both capable of inducing growth arrest when expressed in cells (Serrano et al., 1993; Quelle et al., 1995). p16INK4a arrests the cell cycle in an Rb-dependent manner by specifically binding to and inhibiting cyclin D/cdk4 complexes (Lukas 1995).

p19^{ARF} (or p14^{ARF} in humans) (ARF) stabilizes and activates p53 by binding Mdm2 and blocking p53 association with mdm2 (Sherr, 2001). ARF is part of a negative regulatory feedback loop with p53, as p53 down-regulates ARF expression (Stott et al., 1998). Overexpression of E2F1 induces ARF expression. This effect depends on the DNA binding and transactivation domains of E2F1 (Bates et al., 1998). These results suggested that E2F1 directly regulates ARF expression by binding to and transactivating its promoter. Experiments analyzing the ARF promoter demonstrated that E2F1, E2F2 and E2F3 can up-regulate ARF promoter constructs (Parisi et al., 2002). Surprisingly, this up-regulation does not depend on the E2F sites. Recently, it was demonstrated that the ARF promoter is occupied and repressed by E2F3b in normal cycling primary fibroblasts *in vivo* (Aslanian et al., 2004). Cells lacking E2F3 have increased ARF expression, indicating that other repressive E2F complexes cannot functionally replace E2F3b on the ARF promoter. Upon expression of adenovirus E1A protein or overexpression of E2F1, E2F3b binding is decreased and E2F1 and E2F3a are detected on the ARF

promoter. These data give the first functionally specific role to E2F3b, demonstrating it is an important link between the E2F and p53 pathways.

ARF has both pro-apoptotic and growth suppressive functions that are independent of p53 (Weber et al., 2000; Qi et al., 2004). ARF-induced growth suppression cannot be bypassed by inhibition of Rb activity through HPV E7 expression, suggesting that ARF blocks cell cycle progression downstream of Rb function (Weber et al., 2000). Accumulating evidence suggests a role for ARF in the direct inhibition of E2F activity, a mechanism that would bypass the requirement for Rb function. ARF can directly bind E2F1 and relocalize it from the nucleus into the cytoplasm (Eymin et al., 2001; Martelli et al., 2001). ARF can also bind E2F1, E2F2 and E2F3 and cause their proteasome-mediated degradation. In contrast, ARF does not bind E2F6 (Martelli et al., 2001). ARF also binds to DP1, causing its dissociation from E2F1. This removes E2F1 from the DHFR promoter, resulting in the down-regulation of promoter expression (Datta et al., 2005). ARF also induces the proteasome-mediated degradation of DHFR. In contrast, ARF does not influence TK1 stability. Altogether these data suggest that ARF regulates the stability of specific proteins, such as E2F, dhfr and p53, to induce growth arrest or apoptosis (Magro et al., 2004).

The activation of p53 can cause cell cycle arrest and/or apoptosis. To induce cell cycle arrest, p53 directly transactivates the p21 promoter, up-regulating the expression of this universal cyclin-dependent kinase inhibitor (Yamasaki, 2003). p53 also regulates the expression of Rb through p53 binding sites on the *RB* promoter (Osifchin et al., 1994). Interestingly, however, this regulation is concentration-dependent. At low levels of p53, *RB* promoter activity is induced, but at higher levels of p53, it is repressed back to basal levels. Altogether, these activities reveal a complex interplay of feedback mechanisms between the Rb and p53 pathways (Fig.4b). E2F

activates p53 directly or through activation of ARF. ARF directly inhibits E2F activity. p53 indirectly inhibits E2F by up-regulating pRb or p21 expression. p21 blocks pRb phosphorylation by cyclin-dependent kinases, thus activating pRb to repress E2F.

Perhaps this is a general property of p53 – to behave as a tumor suppressor at low concentrations, but as an oncogene at high concentrations. For instance, it has long been appreciated that p53 can remain at undetectable levels when in its auto-regulatory feedback loop with mdm2. Upon activation, the levels of p53 increase only modestly and remain at low, barely detectable levels (Aslanian et al., 2004). Naturally occurring mutations of p53 or expression of viral oncogenes such as SV40 large T antigen stabilize p53 and result in robust levels of the protein that are easily detectable (Pipas and Levine, 2001). The classic hypothesis is that T antigen interactions with p53 block its activity, effectively rendering p53 as a null protein. An alternative hypothesis is that T antigen stabilizes p53, manipulating it for an alternative purpose that somehow benefits the virus and consequently causes transformation. Evidence to support this comes from knock-in experiments where naturally occurring mutations of p53 were knocked into the p53 locus of genetically engineered mice (Olive et al., 2004; Lang et al., 2004). These mice develop different spectra of tumors than p53^{+/-} mice including metastasis. MEFs derived from these mice also show differences from null or heterozygous loss of p53. These results indicate that these mutant alleles, which were previously believed to encode non-functional p53 proteins, do indeed retain some gain-of-function activity. Therefore, it is possible that T antigen, which stabilizes p53, may also be manipulating p53 to perform new, oncogenic functions rather than merely inactivating it.

Until recently, cellular senescence was only observed in cell culture, possibly an artifact of prolonged culture and a result of telomere shortening (Ben-Porath and Weinberg, 2004).

Recent studies suggest that senescence is not only a cell culture artifact, but that it is a cellular defense mechanism against deregulated cellular proliferation and can be induced by oncogenes *in vivo* (Braig et al., 2005; Chen et al., 2005b). In fact, benign tumors such as HPV-infected skin warts or pre-malignant lymphomas that express markers of senescence are examples of cells that have successfully enacted this defense mechanism (Collado et al., 2005; Michaloglou et al., 2005). A lack of senescent markers in malignant tumors and reversal of transformed phenotypes in cancer cell lines by induction of senescence support a role for senescence as an initial cellular defense against tumorigenesis (Wells et al., 2000b; Braig et al., 2005; Collado et al., 2005). Many systems examined so far require an intact pRb/p16INK4a pathway to induce senescence, while other systems depend on other genes such as *P53* and *ARF*, or *SUVARHI* (Wells et al., 2000b; Narita et al., 2003; Braig et al., 2005; Chen et al., 2005b). Together, these genes may all constitute one or two parallel senescence pathway(s). Future experiments will have to determine the mechanism(s) of how these pathways converge or do not, how they cause senescence and what molecular events disrupt this defense mechanism to cause tumorigenesis.

1.14. CELL GROWTH AND GROWTH ARREST IN THE MULTICELLULAR ORGANISM

Much of the data that support the models of how the Rb/E2F and p53 pathways function and become perturbed to cause cancer have been accumulated from studies in cell culture. Considering that growth *ex vivo* is under selection when obtaining these cells, it is uncertain how growth regulatory pathways are altered as a response. Therefore, it is important to investigate these pathways in the complex system in which they normally exist, in their natural environment

of cells in the organism. Biological specificities of the different Rb and E2F family members may also be revealed by studies characterizing their regulation and activities *in vivo*.

One of the obstacles that studies of cellular growth control in the organism must deal with is the fact that cells are in various stages of growth. Some cells are capable of proliferation while others are not. It remains unresolved if growth in each cell of the organism is regulated by the same mechanisms. Therefore, it is important that studies in the organism consider the cell type, proliferation status, and developmental time point in which to analyze.

Proliferating cells are most abundant during development. As the organism grows with more cells, these cells also change and become different cell types, a process called differentiation, which is often associated with exit of the cell cycle and growth arrest. However, it is also clear that cells begin the process of differentiation before completely exiting the cell cycle and arresting. Cells that are both capable of differentiation as well as self-replication are called stem cells. Stem cells often give rise to progenitor cells that are incapable of regenerating themselves but proliferate to give rise to differentiated cells. Each of these cell types is differentiated to some degree, but only the terminally differentiated cells are growth arrested. A major question of development is during what phase of the cell cycle do cells differentiate? It has long been assumed that cells exit the cell cycle before they differentiate. However, if this were the case, then stem and progenitor cells would be at the same level of differentiation since they have not exited the cell cycle, a clear conundrum in the field.

The developing organism contains essentially three different proliferation cell types: pluripotent stem cells, pluripotent progenitor cells, and growth-arrested cells. The growth-arrested cells can also be subcategorized into cells with the potential to re-enter the cell cycle and cells that are permanently growth-arrested, often considered “terminally differentiated” cells. For

example, hepatocytes are growth arrested cells but retain the ability to re-enter the cell cycle, whereas terminally differentiated neurons or mature muscle cells cannot be stimulated to re-enter the cell cycle.

Development of an organism is marked by overall growth and changes in morphology until the organism reaches its defined size and shape. By this time, most proliferation ceases and most cells of the organism are growth arrested and differentiated. However, the adult organism still contains many cells that continually proliferate and many other cells that retain the potential to proliferate in case of the need for regeneration. The cells that continue to proliferate are in constant balance of growth and death in order to maintain the overall morphology and size of the adult tissue, a process called homeostasis. It remains unknown how the regulation of proliferation of a tissue during its development differs from regulation during its homeostasis.

The mammalian tissues that undergo constant proliferation and homeostasis include hematopoietic cells, the epidermis, the intestinal epithelium, and the gametes with their associated helper cells. The gametes are distinct from other cell types because they are derived from the primordial germ cells that split early in development from the rest of the somatic cells. Since the gametes undergo meiosis, their proliferation is uniquely regulated. The majority of cells in the adult are in a state of growth arrest. Most tissue types can undergo a certain amount of regeneration in response to damage, theoretically by activating hidden niches of tissue stem cells. The most widely studied and characterized tissue that undergoes regeneration is the liver, which can reform itself to its normal size even after resection of 2/3 its tissue. However, it is clear that most tissues can undergo regeneration to some degree. The differences between these cell types are of great interest since some theories suggest that cancer develops exclusively from

cells that retain the ability to proliferate (Sell 2004). If these differences could be discerned, then the mechanisms that prevent proliferation could be used to treat cancer.

1.15. THE MOUSE INTESTINAL EPITHELIUM IS A MODEL SYSTEM TO STUDY BASIC MECHANISMS OF REGULATION OF GROWTH AND GROWTH ARREST IN A MAMMALIAN ORGANISM

The mouse intestinal epithelium is a model system in which to study the regulation of growth in a mammalian organism. The adult intestine undergoes constant renewal as a normal function of homeostasis. In the mouse, most of the cells of the intestinal epithelium are replaced approximately every five days (Sancho et al., 2004). The epithelium is a contiguous, single-layer sheet of cells that convolutes and involutes to form two types of structures; the villus that protrudes towards the lumen, and the crypts of Lieberkün at the base of each villus that invaginate into the underlying basal lamina (Fig.5a). The regulation of homeostasis involves a complex interplay between the mesenchymal cells of the lamina and the epithelial cells, and is controlled by the stem cell towards the base of each crypt (Radtke and Clevers, 2005).

The pluripotent stem cells in the crypts generate the four basic types of differentiated epithelial cells in the small intestine: the absorptive enterocytes, and the secretory goblet, paneth and enteroendocrine cells (Fig. 5b) (Traber and Wu, 1995; Radtke and Clevers, 2005). 95% of the cells in the villi are enterocytes. These are the major absorptive cells of the intestine, responsible for the majority of nutrient intake. There are fewer goblet cells and even fewer enteroendocrine cells, comprising about 1% of total mucosal cells, of which there are at least ten different sub-categories that express different neuroendocrine markers (Schonhoff et al., 2004).

These enteroendocrine cells are important for communication with the nervous and endocrine systems. They secrete hormones as well as neurotransmitters to help maintain homeostasis and other neurological mechanisms to stimulate absorption or distention in the intestine. Goblet cells secrete mucin, the major component of mucous that coats the luminal surface of the epithelium. The goblet cells and enteroendocrine cells are located predominantly in the villi, whereas enterocytes are exclusive to the villi. Paneth cells are exclusively localized to the base of the crypts. Paneth cells are anti-microbial cells that secrete peptides similar to defensins, called cryptdins, which target microbes for phagocytosis. Spatial organization of the cells along the villus/crypt axis is controlled by Ephrin/Eph signaling, downstream of Wnt/ β catenin signaling and modulated by TGF β /BMP signaling (Batlle et al., 2002; Haramis et al., 2004; Hauck et al., 2005).

There are several different signaling pathways that are important for the regulation of proliferation and differentiation of the intestinal epithelial cells, including the Wnt, Notch, TGF β /BMP, and Hedgehog pathways (Sancho et al., 2004; Hauck et al., 2005; Walters, 2005). In brevity, sonic hedgehog (SHH) and indian hedgehog (IHH) are important for intestinal development and radial patterning of the intestine. The Notch pathway is important for differentiation of the specific cell lineages, particularly the secretory lineage. Wnt signaling is important for stem cell renewal and crypt formation, while BMP signaling antagonizes Wnt signaling to inhibit proliferation and formation of the villus compartment (He et al., 2004). Each of these pathways has the ability to impinge on the Rb/E2F pathway to either induce or inhibit proliferation. However, it is unknown how these pathways connect to downstream regulators, such as Rb and E2F. One possible connection is via cyclin D1. β catenin activates the cyclin D1 promoter with LEF1 in cells, downstream of Wnt signaling (Shtutman et al., 1999). A recent

study, however, suggests that this up-regulation of cyclin D1 by β catenin is indirect in the intestine (Sansom et al., 2004). Another target of TCF4/beta-catenin, Cdx1, may directly up-regulate E2F expression, as the ortholog of Cdx, Caudal, does in *Drosophila* (Lickert et al., 2000; Hwang et al., 2002). BMP4 signaling from the mesenchyme to the villus epithelium suppresses proliferation (Haramis et al., 2004; He et al., 2004). Intracellular signaling of TGF β or BMP causes the phosphorylation of Smads in epithelial cells. Smad3 and Smad4 may directly inhibit proliferation by forming a complex with E2F4 or E2F5 and p107. This complex causes the repression of the *c-myc* promoter (Chen et al., 2002). These possible connections require verification in the intestinal epithelium *in vivo*. Future studies need to address the mechanisms by which these pathways influence each other and what roles they play in cancer.

Cancer of the small intestine is rare. Therefore, studies of the small intestine may seem inconsequential for human disease. Contrastingly, colon cancer is one of the most abundant cancers in developed countries. As both organs undergo intense renewal, it is interesting to speculate what differences lead to a greater incidence of cancer in the colon and a low incidence in the small intestine. The small intestine is useful as a model system for the study of cancer because relatively large quantities of dividing or growth-arrested cells can be obtained for molecular analysis. The dividing cells and growth-arrested cells are located in distinct structural compartments that can be separated. Furthermore, the small intestine of mice expressing a mutation in a Wnt pathway gene, *APC*^{min/+} develop phenotypes predominantly in the small intestine, whereas humans carrying a mutation in this gene are predisposed to familial adenomatous polyposis, a form of colon cancer (Moser et al., 1990). Finally, a human colon cancer cell line, CaCo-2, differentiates upon confluence in culture into small intestinal epithelial cell-like structures such as villus-like dome structures, and expresses many enterocyte-specific

markers such as sucrase-isomaltase and alkaline phosphatase (Ramond et al., 1985). These data indicate many similarities and possible default mechanisms in the regulation of proliferation and differentiation between the epithelial cells of the small and large intestines in both mice and humans. Therefore, the small intestine is a useful model system for studying mechanism of growth and growth arrest and could be applicable to human disease.

An interesting connection between the Rb pathway and intestinal epithelial differentiation comes from germ-line deletion studies of *Id2*. *Id2* is a member of the *Id* family of basic helix-loop-helix (bHLH) transcription factors that are known to inhibit differentiation by other bHLH transcription factors and cause proliferation when constitutively expressed (Lasorella et al., 2001; Yokota, 2001). *Id2* specifically interacts with all three members of the Rb family (Lasorella et al., 1996). It can alter Rb-mediated tumor suppressive functions when in stoichiometric excess of Rb. Mice with germ-line homozygous null mutations in *Id2* develop intestinal tumors characterized by severe dysplasia and metaplastic lesions (Russell et al., 2004). During embryonic development, *Id2* loss causes a decrease in differentiation in the villus, particularly in the default enterocyte lineage by E18.5 and P0.5. Instead, the villi contain elevated numbers of proliferating cells and thickened mucosa. Adenomas in adult *Id2*^{-/-} mice contain elevated levels of β -catenin staining with reduced or aberrant staining for other intestinal epithelial lineages. These results implicate *Id2* as a regulator of enterocyte lineage determination and growth arrest during embryonic development of the intestine. The loss of *Id2* affects epithelial homeostasis and causes tumorigenesis later in life. Since *Id2* interacts with the Rb family and plays an important role in enterocyte differentiation and homeostasis, these results suggest that the Rb family may also play important roles intestinal epithelial differentiation and homeostasis.

The targeted germ-line deletions of E2F4 and the universal cyclin-dependent kinase inhibitor, p57^{Kip2}, result in intestinal phenotypes (Yan et al., 1997; Rempel et al., 2000). However, many knock-out mice, such as those for *RB* and *E2F3*, die before the onset of intestinal development (Table 1). Thus, their effects on intestinal development and homeostasis are unclear. To assess the individual contributions of these genes in intestinal development, conditional knock-out mice will have to be engineered. The ablation of other genes, such as *E2F1* and *E2F2*, results in viable mice with no gross intestinal phenotype. These results may reflect compensatory mechanisms in the intestine, or merely indicate that these factors do not play vital roles in the development and homeostasis of the intestine. Clearly, conventional germ-line knock-out mice for the different Rb and E2F family members have not elucidated their normal roles in the development and homeostasis of the intestine. Therefore, to use the intestinal epithelium as a model system for studying the regulation of growth and growth arrest, an alternative approach must be taken. Transgenic mice that specifically express SV40 T antigen in the intestinal epithelium have been generated to probe mechanisms of growth control in this tissue.

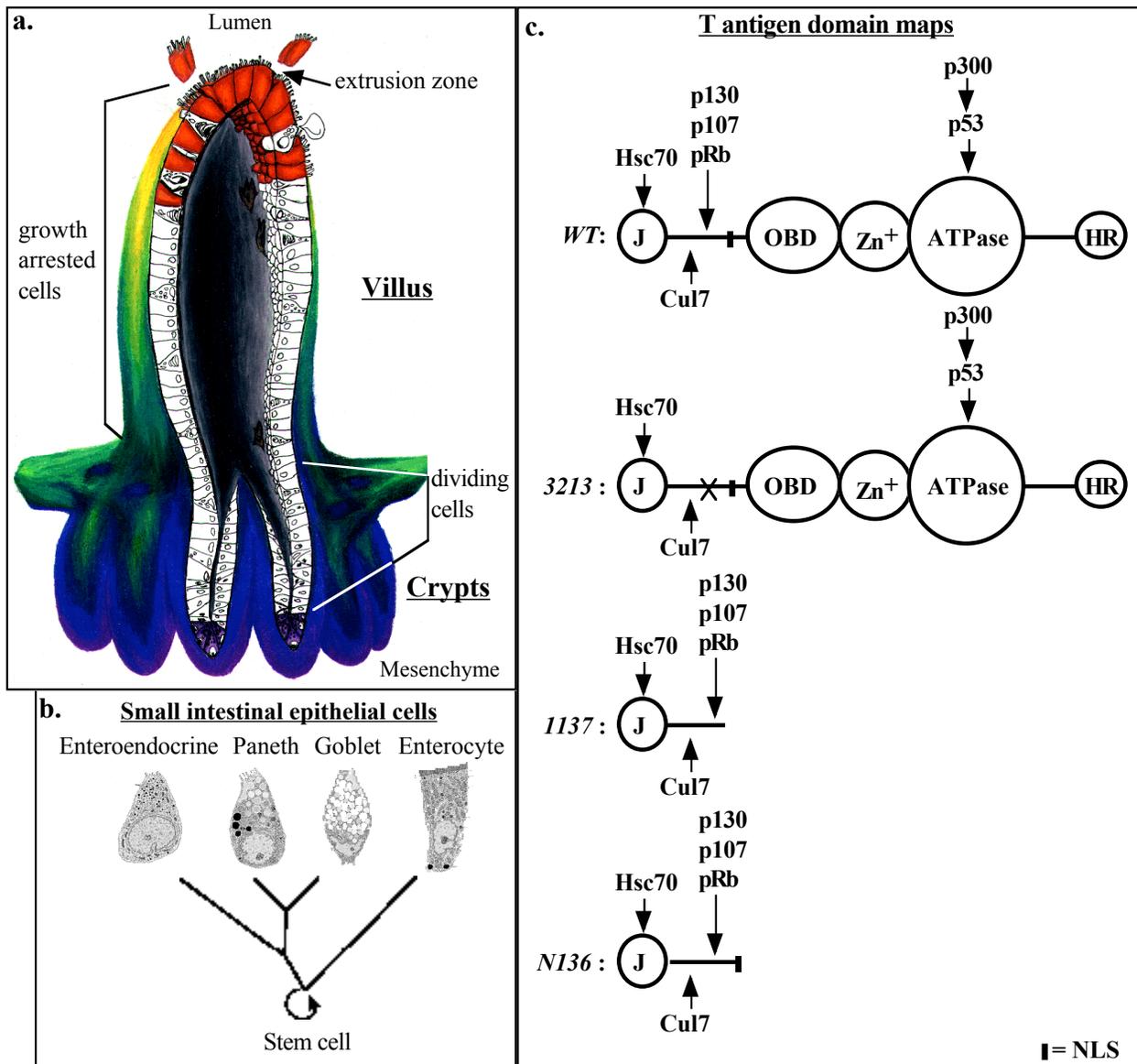


Figure 5. Diagrams of an intestinal tumorigenic model system using SV40 T antigen as a molecular probe. a. Diagram of small intestinal epithelial structures: villus and attached crypts. Dividing cells are located in the crypts, which involute into the mesenchyme. The growth-arrested cells are located in the villus, which protrude into the lumen of the small intestine. **b. Small intestinal epithelial cells and lineage delineation.** All small intestinal epithelial cells are derived from a single stem cell per crypt. Two general lineages are derived directly from the stem cell division, the absorptive cell/enterocyte or the secretory cell lineages. **c. Protein domain maps of SV40 T antigen and pertinent mutants.** Large T antigen contains four globular domains that have been structurally solved; the J domain (J), the origin binding domain (OBD), the zinc interaction domain (Zn⁺), and the

ATPase domain (ATPase). The structure of the host-range domain (HR) has not been solved, but is considered to be globular. T antigen interacts with Hsc70 through the J domain, Cul7 between amino acids 69-83, the Rb family members through the LxCxE motif at amino acids 103-107, and p53 on a surface of the ATPase domain. T antigen mutant *3213* is mutated in the LxCxE motif and cannot bind the Rb family. T antigen mutant *1137* is truncated such that it contains amino acids 1-121. T antigen mutant *N136* is truncated such that it contains amino acids 1-136, which includes the nuclear localization sequence (NLS).

1.16. SV40 T ANTIGEN IS A MOLECULAR TOOL TO INDUCE TUMORIGENESIS IN TRANSGENIC MICE

As previously mentioned, SV40 is a DNA tumor virus capable of causing tumors in rodents and of causing the transformation of various mammalian cell lines. The primary oncogenic proteins of SV40 encoded in the early region of the viral genome are called small and large (t and T) tumor antigens (Ali and DeCaprio, 2001; Sáenz-Robles et al., 2001). Large T antigen is a useful molecular tool to discover cellular factors and pathways that are targeted to cause transformation and/or tumorigenesis in cell lines and animal models. The use of T antigen in these studies has led to the discovery of key regulators and pathways such as pRb and p53.

Large T antigen contains multiple regions that interact with key cellular proteins that are required to induce transformation (Fig.5c) (Ali and DeCaprio, 2001; Sáenz-Robles et al., 2001; Ahuja et al., *in press*). T antigen binds the Rb family of tumor suppressors via its LxCxE motif located at residues 103-107. T antigen causes the disruption of Rb/E2F complexes via an interaction with the host protein, Hsc70, a molecular chaperone that binds T antigen through its J domain (Sullivan et al., 2000). The mechanistic model of T antigen disruption of Rb/E2F complexes states that T antigen binds these complexes through Rb, brings them into proximity of

Hsc70 and stimulates the ATP-hydrolysis-dependent disruption of Rb/E2F complexes, releasing the free E2F from Rb (Sullivan and Pipas, 2002). The model suggests that de-repressed E2F can then cause the transactivation of E2F-responsive promoters and thus send the cell into S phase. The virus subsequently uses the cellular DNA replication machinery expressed during S phase to replicate its genome and propagate more viruses.

The inappropriate induction of S phase activates p53 as previously described. T antigen interacts with p53 on the surface of the ATPase domain, although the binding of p53 and hydrolysis of ATP are distinctly required for transformation and replication, respectively (Peden et al., 1998; Li et al., 2003a). T antigen binds p53, stabilizes it and blocks p53-mediated transcription and consequently, apoptosis (Pipas and Levine, 2001). Thus, in sum, T antigen uses these three regions, the J domain, the LxCxE motif and the p53 binding surface to induce cells to enter the cell cycle and to block apoptosis, essentially causing unregulated proliferation.

Genetic studies suggest that the inactivation of pRb and p53 are insufficient to cause full transformation by T antigen (Sachsenmeier and Pipas, 2001). These results suggest that there are additional cellular targets with which T antigen interacts to cause transformation. Several studies have revealed additional targets that interact with T antigen and some may play important roles in T antigen-mediated transformation. These targets vary in function from transcription regulation, such as the transcription factor TEF-1 and co-activator, histone acetylase p300/CBP, to components of the ubiquitination machinery such as Cul7 and Fbw7, as well as components of the DNA repair and checkpoint pathway, such as Nbs1 and Bub1 kinase (Ahuja et al., *in press*). Importantly, only the interaction between T antigen and Cul7 was demonstrated to be required for transformation (Kasper et al., 2005).

Many of the studies of cells that express T antigen from its genomic template also express small t antigen, as it is a splice variant of the early viral mRNA. Like large T antigen, small t antigen encodes the J domain, but has a unique carboxy terminal region that binds the cellular phosphatase, PP2A (Pallas et al., 1990; Mungre et al., 1994). PP2A is a tertiary complex of a catalytic subunit (C) and two classes of regulatory subunits (A and B). Small t antigen replaces the regulatory B subunits in this complex, leading to reduced phosphatase activity and/or changes in substrate specificity (Yang et al., 1991). Mutants of small t antigen that cannot bind PP2A are defective for transformation in some cell types (Mungre et al., 1994; Hahn et al., 2002). Genetic ablation of specific B subunits of PP2A contribute to transformation similar to small t antigen, again suggesting that they are the key targets of this protein (Chen et al., 2004b; Chen et al., 2005a).

1.17. MODEL SYSTEM: EXPRESSION OF SV40 EARLY REGION IN THE INTESTINAL EPITHELIUM DOES NOT ALTER DIFFERENTIATION

The prevailing goal of studies of transgenic mice expressing SV40 T antigen in specific cell types is to probe both known and unknown growth regulatory pathways within the complex environment of a tissue system in order to ascertain specific, key changes in these pathways that allow for the onset and progression through successive stages of tumorigenesis. It is also important to study the regulation and activities of these pathways in the organism to determine if the known pathways are regulated and function similarly as in cell culture, since the culture environment is vastly different from the environment inside a complex tissue *in vivo*. We have used the early region of SV40, encoding for the large and small tumor antigens, as a tool to

induce proliferation in the enterocytes of the mouse small intestinal epithelium. Transgenic mice were generated to express SV40 T antigen under the regulation of the promoter for the intestinal fatty acid binding protein gene, (*fabpi*) (Hauft et al., 1992). Previously, it had been shown that these promoter elements could drive expression of a reporter construct exclusively in the villus compartment of the small intestinal epithelium with highest expression in the distal jejunum, then decreasing both proximally into the duodenum and distally into the colon, similar to the pattern of expression of the endogenous gene (Cohn et al., 1992). Expression of large T antigen in *fabpi*^{-1178 to +28}-driven transgenic mice extends from the tops of the crypts at the base of the villus to the apical extrusion zone of the villus (Hauft et al., 1992). Co-staining of histological sections demonstrated that these cells express both T antigen and markers for enterocytes. T antigen is also expressed in the follicle-associated epithelial (FAE) cells, which are the epithelial cells directly above the Peyer's patches (Mysorekar et al., 2002). Peyer's patches are organized clusters of gut-associated lymphoid tissue (GALT) that develop in localized regions along the anterior-posterior axis of the small intestine. The FAE is different from the normal intestinal epithelium in that there are fewer goblet cells, enterocytes are reduced in their absorptive activity, and there are specialized membranous/microfold (M) cells (Buda et al., 2005). M cells are specialized transport cells of the intestinal epithelium that undergo "antigen-sampling". The role of T antigen in the FAE cells has not been specifically examined and will not be discussed in this report.

Since T antigen can interact with the Rb family and Id2 can interact with the Rb family, one prediction of T antigen expression in enterocytes is that T antigen disrupts this interaction. Since loss of Id2 results in loss of enterocyte differentiation, another prediction is that expression of T antigen in enterocytes causes their de-differentiation. However, T antigen expression does

not cause de-differentiation. Therefore, if the differentiation function of Id2 requires interaction with the Rb family, T antigen action on Rb in enterocytes occurs downstream of Id2 function.

1.18. TRANSGENIC MICE EXPRESSING SV40 T ANTIGEN IN THE INTESTINAL EPITHELIUM DEVELOP HYPERPLASIA AND DYSPLASIA

Expression of SV40 large T antigen in the enterocytes of the mouse small intestine causes these normally growth-arrested cells to proliferate. Initially, this manifests as a phenotype called hyperplasia, which is characterized by hyperproliferation of the villus enterocytes resulting in longer and thicker villi with more densely packed cells. However, the homeostatic mechanisms that regulate the epithelium remain relatively intact as the overall morphology of the tissue remains similar to normal and the cells retain their differentiated status and functionality (Kim et al., 1993). With age transgenic mice expressing high levels of wildtype, full-length T antigen, (TAg^{WT}), develop intestinal dysplasia (Kim et al., 1994). Dysplasia is a much more severe phenotype characterized by the addition of gross morphological changes in the tissue architecture such as branching of the villi and multi-layering of cells in the epithelium.

Expression of T antigen does not alter the differentiation status of the intestinal epithelium, as evident by the appropriate expression of cell-type specific markers (Hauft et al., 1992). In support, differentiation of post-confluent Caco-2 cells into enterocytes is not blocked by expression of SV40 T antigen (Djelloul et al., 1997). This result is in clear contrast to other systems where the expression of T antigen causes a block in differentiation pathways, such as in the lenses of transgenic mice, myoblasts and pre-adipocytes in culture (Tedesco et al., 1995; Higgins et al., 1996; Chen et al., 2004a). Enterocytes are normally considered “terminally

differentiated” cells. However, if terminal differentiation is dependent on cell cycle withdrawal, as suggested by many researchers in the field, then how can these “terminally differentiated” cells in the TAg^{WT}-transgenic mice retain (or re-obtain) the ability to divide? One possibility is that terminal differentiation, as defined by the end-point of a differentiation pathway in a particular cell type, does not always require cell cycle withdrawal. Another, more complicated, explanation is that T antigen is expressed upon differentiation after cell cycle withdrawal, but T antigen reverses the mechanism of withdrawal, causing the cell to re-enter the cell cycle, but remain terminally differentiated. In order to address this issue, and to understand the mechanism(s) by which T antigen causes hyperplasia and dysplasia in the intestine, it is pertinent to discern how T antigen causes the cells to cycle. Since T antigen is known to interact with Rb family members to cause cells to proliferate in culture, it was hypothesized that a similar interaction with Rb is required for T antigen to cause enterocytes to proliferate in the intestines of transgenic mice.

1.19. DOES SV40 T ANTIGEN CAUSE INTESTINAL HYPERPLASIA BY ACTIVATING THE E2F PATHWAY THROUGH DISRUPTION OF RB FAMILY MEMBERS?

Expression of a mutant T antigen, *3213*, which is incapable of binding the Rb family (Fig.5c) in the intestinal epithelium of transgenic mice does not result in hyperplasia and dysplasia (Kim et al., 1994). Thus, T antigen requires interaction with the Rb family to cause hyperplasia and dysplasia (Fig.6). We hypothesize that this interaction with the Rb family disrupts normal growth arrest of enterocytes by de-repressing E2F-responsive gene promoters, resulting in the activation

of the E2F pathway. Therefore, we hypothesize that T antigen acts through the E2F pathway, at least in part, to cause hyperplasia and dysplasia. To test these hypotheses, the molecular status of the Rb/E2F pathway was analyzed in normal, TAg^{WT}-, and TAg³²¹³-transgenic mouse intestines.

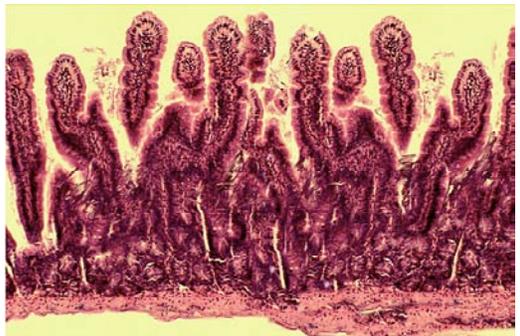
<u>Phenotypes</u>	<u>Transgenics:</u>	<u>Hypotheses:</u>
<p style="text-align: center;">Normal</p> 	<p style="text-align: center;">TAg^{wt}</p> <p style="text-align: center;">TAg¹¹³⁷</p> <p style="text-align: center;">TAg³²¹³</p>	
<p style="text-align: center;">Hyperplasia</p> 	<p style="text-align: center;">↓</p> <p style="text-align: center;">↓</p>	<p style="text-align: center;">disrupt Rb/E2F pathway</p>
<p style="text-align: center;">Dysplasia</p>  <p style="text-align: center;">I</p>	<p style="text-align: center;">↓</p> <p style="text-align: center;">II</p>	<p style="text-align: center;">1. disrupt Rb/E2F pathway</p> <p style="text-align: center;">2. disrupt p53 pathway</p> <p style="text-align: center;">III</p>

Figure 6. Multi-step model of intestinal tumorigenesis induced by T antigen. Column I: Micrographs of histological sections from small intestines of non-transgenic, TAg^{wt} and TAg¹¹³⁷ mice stained with hemotoxylin and eosin depicting normal, hyperplastic and dysplastic phenotypes. Column II: Phenotypes developed in transgenic

mice as indicated by the endpoint of each arrow. Column III: Hypotheses as derived from phenotypes resulting from T antigen mutant transgenics.

1.20. DOES SV40 T ANTIGEN CAUSE INTESTINAL DYSPLASIA BY BINDING AND DISRUPTING P53 FUNCTION?

An amino-terminal truncation mutant of T antigen, dl137, contains the first 121 amino acids of the protein, including the J domain and the LxCxE motif (Fig.5c). dl137 is unable to transform primary mouse embryo fibroblasts (MEFs) or immortal rat embryo fibroblasts (REF52) although it can induce E2F transactivation *in vitro*, suggesting that it disrupts Rb/E2F complexes (Pipas et al., 1983; Srinivasan et al., 1989). Expression of dl137 in the intestinal epithelium of transgenic mice results in the induction of hyperplasia that does not progress to dysplasia (Kim et al., 1994). Thus, hyperplasia and dysplasia require different molecular events, which can be separated genetically. Furthermore, the first 121 amino acids of T antigen are sufficient to induce hyperplasia, suggesting that T antigen interaction on the Rb pathway is necessary *and* sufficient to drive enterocyte proliferation. Finally, the carboxy half of T antigen is required for progression to dysplasia. We hypothesize that T antigen binding to p53 is required for progression to dysplasia (Fig.6). To address this hypothesis, we analyzed the molecular status of p53 and the p53 pathway in the intestinal epithelium and generated transgenic mice expressing dl137 in a p53 null background.

1.21. INTRODUCTION SUMMARY

Cancer is characterized by unregulated cellular growth. Tumors progress through distinct stages caused by specific alterations in cellular pathways. Initial growth of a cancer cell is often caused by the loss of function of tumor suppressor proteins such as pRb and p53. Rb is a repressor of E2F, which is a transcription factor regulating S phase gene synthesis. The loss of Rb function allows for E2F to activate S phase gene synthesis and cause the cell to enter the cell cycle. This unscheduled DNA synthesis can activate p53 to drive transcription of genes required for cell cycle arrest or apoptosis.

Much of the data supporting these models of the Rb/E2F and p53 pathways are from studies of viral oncogene effects in cell culture. SV40 T antigen, a viral oncogene, binds Rb and disrupts Rb/E2F complexes, allowing E2F to drive cells into the cell cycle. T antigen also binds and stabilizes p53, blocking p53-mediated transcription, hence blocking growth-arrest and apoptosis.

Rb and E2F are families of proteins with similar activities. The E2F family is subdivided into two cell-cycle regulatory groups, the “activator” E2Fs (E2F1-3) and the “repressor” E2Fs (E2F4 and E2F5). Molecular studies in cell culture and genetic studies in mice provide conflicting interpretations of the functions for these different Rb and E2F family members. In order to begin deciphering the true functions and regulation of the Rb/E2F pathway, we have undertaken a molecular analysis of these factors in the complex environment of the organism. We have chosen to study the model system of transgenic expression of SV40 T antigen in the enterocytes of the small intestinal epithelium of mice to perturb normal growth control and cause tumorigenesis.

Expression of T antigen in enterocytes causes two tumorigenic phenotypes that are theorized to be the initial stages of the multi-step progression of intestinal cancer, hyperplasia and dysplasia. T antigen interaction with the Rb family of tumor suppressors through its LxCxE motif is required to cause hyperplasia and dysplasia. We hypothesize that T antigen disrupts Rb/E2F complexes, allowing E2F to transcribe S phase genes to drive cells into the cell cycle and cause hyperplasia. Either T antigen interacts with all three Rb family members to achieve this effect or T antigen interaction with a specific Rb family member is sufficient. Similarly, either T antigen disrupts all Rb/E2F complexes, allowing for all five E2Fs to transactivate S phase genes, or T antigen drives cells into the cell cycle by de-repressing and/or activating specific E2Fs. I have undertaken molecular analyses to determine the levels and activities of the different Rb and E2F family members in villus enterocytes from non-transgenic or TAg-transgenic mice, presented in Chapter 4.

Expression in enterocytes of an amino-terminal truncation mutant of T antigen that lacks the p53 binding surface causes hyperplasia that does not progress to dysplasia. We hypothesized that T antigen inactivation of p53 causes the progression to dysplasia. We have undertaken both genetic and molecular analysis of this hypothesis, presented in Chapter 3.

Although both descriptive as well as manipulative, these studies have led me to re-evaluate the current paradigm describing the purpose and activities of the Rb and p53 tumor suppressor pathways. This re-evaluation is presented in the concluding Chapter 6.

2. INTESTINAL EPITHELIAL STRUCTURE FRACTIONATION

2.1. INTRODUCTION

The intestinal epithelium consists of cells in various stages of the cell cycle and of various differentiated lineages. Thus, the analysis of this tissue is complicated by the co-existence of many cell types. It is also important to consider that since T antigen induces proliferation of enterocytes, there are more of these cells in the TAg^w intestine than in the non-transgenic intestine. Thus, an extract from TAg^w intestines will have disproportionately more enterocyte-specific components than the same amount of extract from non-transgenic intestines. To understand the effects of T antigen expression in enterocytes, the analysis of these cells must be performed separately from the other cell types of the intestine. However, intestinal cells do not survive when disrupted into single-cell suspension, probably due to their dependence on cell-cell contact and communication through junctions (Kondo et al., 1984; Louvard et al., 1992). Therefore, I developed an alternative method of isolating these cells, taking advantage of the fact that 95% of villus epithelial cells are enterocytes. This method enriches for villi epithelium separate from crypt epithelium and underlying mesenchyme and was used for the molecular studies presented in this report. Previous methods that have been described for the isolation of villi separate from crypt epithelium are complex and often involve either enzymatic or mechanical disruption, resulting in either a low yield of material and/or degradation of RNA

(unpublished data, J.A. Markovics and M.T. Sáenz-Robles, University of Pittsburgh). The method that I have refined results in a relatively high yield of material (>0.5mg of protein on average) and leaves the RNA intact.

The success of enrichment for the different epithelial structures in collected fractions was initially characterized microscopically. Next, after lysing the cells for protein or RNA isolation, molecular analyses were performed to assess the enrichment of cell type-specific markers in the different fractions.

2.2 MATERIALS AND METHODS

Production and maintenance of transgenic mice. FVB/N transgenic mice containing nucleotides -1178 to +28 of the rat intestinal fatty acid binding protein (Fabpi) promoter linked to SV40 *WT* T antigen, (TAg^{wt}), were obtained from Jeff Gordon (Washington University) and have been described (Kim et al., 1993; Kim et al., 1994). Fabpi-SV40 TAg^{wt} mice were from pedigree number 103 (Kim et al., 1994). Transgenes containing this same promoter region linked to SV40 T antigen mutants, *N136*, (TAg^{N136}), or *3213*, (TAg³²¹³), were constructed by M.T. Sáenz-Robles (University of Pittsburgh) and generated by conventional pronuclear injection at the University of Michigan Transgenic Mouse Facility Service. Fabpi-SV40 TAg^{N136} mice were from pedigree B, while Fabpi-SV40 TAg³²¹³ mice were from pedigree A (M.T. Sáenz-Robles, unpublished data). All pedigrees were maintained by crosses to non-transgenic FVB/N littermates and/or commercially obtained FVB/N mice (Taconic Labs). Standard genetic crosses were performed to obtain mice with various combinations of the Fabpi-SV40 TAg^{dl137} and Fabpi-K-Ras^{Val12} transgenes in p53^{+/+}, p53^{+/-} or p53^{-/-} background, as described in the text. For

simplification purposes, animals are referred in the text as TAg^{wt} (Fabpi-SV40 TAg^{wt}), TAg^{N136} (Fabpi-SV40 TAg^{N136}), or TAg³²¹³ (Fabpi-SV40 TAg³²¹³). The genotype of each mouse was determined using DNA isolated from murine tails followed by polymerase chain reaction (PCR) with protocols detailed elsewhere (Kim 1993; Kim 1994; Chandrasekaran 1996). All routine screens for several murine pathogens, including Hepatitis, Minute, Lymphocytic, Choriomeningitis, Ectromelia, Polyomavirus, Sendai, Pneumonia, and mouse adenoviruses, enteric bacterial pathogens, and parasites were negative.

Intestinal epithelial fractionation. Mice were sacrificed by cervical dislocation, dissected and small intestines removed. After measuring the length, the intestines were cut in thirds, (proximal, middle and distal), and placed in phosphate-buffered saline (PBS). To clean the lumen of the intestines, the intestines were sliced longitudinally and rinsed in PBS. After brief drying on a paper towel, all three intestinal sections were weighed. The intestinal epithelial fractionation was then performed as described, with modifications (Markovics et al., 2005). Each section was placed in a 50mL falcon tube containing 25mL of 1mM DTT in PBS with either 3mM EDTA or 5mM EDTA, depending on the genotype. For non-transgenic and TAg³²¹³ mice, 5mM EDTA was used, while for TAg^{wt} and TAg^{N136} mice, 3mM EDTA was used. These tubes were placed horizontally on the bench so that the intestine could extend completely submerged in buffer, and incubated at room temperature, for 10-25 minutes. Proximal sections were removed from the incubation buffer after 10 minutes, middle sections after 15 minutes, and distal sections after 20-25 minutes. After this incubation, each section was placed in a 15mL falcon tube with 10mL of “release buffer” (1mM DTT in PBS) and gently shaken for several minutes. During this period the epithelium releases, appearing like snow-flakes. This gentle mechanical shaking was

repeated through 3-5 successive tubes of “release buffer”, or fractions. After release, each tube/fraction was placed on ice. When it appeared that either too much epithelium was being released or no more could be released, the intestinal sections were placed in a new 50mL falcon tube with 9mM EDTA and 1mM DTT in PBS and incubated at room temperature for 10-25 minutes. After this incubation, each section was removed and placed in 15mL falcon tubes with 10mL of “release buffer”. The first few fractions entailed medium-gentle shaking until the majority of the epithelium was released. After it appeared that most of the ‘snow-flake’-like epithelium was released, the next one or two fractions entailed extremely vigorous shaking until no more epithelium was released. Release of the majority of epithelium from the underlying mesenchyme and muscle was determined by examination by microscopy. The appearance of shadowy holes in the mesenchyme from where the crypts were removed indicated complete release.

Immunoblot analysis. All samples were lysed on ice using a Tissue Tearor homogenizer (Biospec Products Inc.). The lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 0.5% NP40) contained protease and phosphatase inhibitors (5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, “Complete” EDTA-free protease inhibitors (Boehringer Mannheim), 50 mM NaF, and 1 mM Na-orthovanadate, 10mM β -glycerophosphate). The concentration of solubilized proteins was quantified by a protein assay dye (BIORAD) based on the method published by Bradford (Bradford, 1976). 30 μ g of total protein was analyzed by conventional Western blot techniques. Appropriate dilutions of the following primary antibodies were used: T antigen- mouse monoclonal PAb419 (Harlow et al., 1981); β -tubulin-mouse IgG_{2b} (D-10-HRP) (*sc-5274-HRP*) and vimentin- rabbit polyclonal H-84 (*sc-5565*) (both from Santa Cruz Biotechnology); alkaline

phosphatase- rabbit polyclonal AB904 (Chemicon International), lysozyme- rabbit polyclonal A-0099 (DAKO). Goat anti-mouse A2554, and goat anti-rabbit A0545 (Sigma) were used as secondary antibodies. The peroxidase reactions were developed with ECL-plus reagents according to the associated protocol (Amersham Life Sciences).

RT-PCR Analysis. Whole intestine or intestinal fractions were collected, lysed and homogenized in buffer containing an RNase inhibitor, guanidine isothiocyanate. The total RNA was extracted using the RNeasy kit (Qiagen). 0.75 µg of RNA was reverse transcribed into cDNA using the Superscript First-Strand Synthesis kit (Gibco BRL). The cDNAs were amplified by polymerase chain reaction (PCR) using primers specific for SM22α (5'-ACCAAGCCTTCTCTGCCTCAAC/5'-CACCATTCCTTCAGCCACAC-CTG), alcohol dehydrogenase 5 (Adh) (5'- ATGACAGATGGGGGCGTGGATTAC/5' - TGGAATGGACGAGTGGAGATTTC), SV40 T antigen (5' - ACCTGACTTTGGAGGCTTCTGG/5' - GAGTCAGCAGTAGCCTCATCATCAC), intestinal fatty acid binding protein (ifabp) (5' - TGATTGCTGTCCGAGAGGTTTC/5' - GAGACCAGTGCTGATAGGATGACG), glyceraldehyde -3 -phosphate dehydrogenase (GAPDH) (5'-TGCACCA CCAACTGCTTAG/5'-GATGCAGGGATGATGTTC), intestinal alkaline phosphatase (5'-GAAAGCAGGAAAATCCGTAGGTG/5'-CCCTCCACAAAG AGATAAAAGCC), L-mannan binding protein (L-MBP) (5'-GCCAAGGGAGAAAAGGGAGAAC/5'-CCAAAAAAGAGTCAGAGCAGGGG), or EphB3 (5'-TCCAATGTGAATGAGACCTCGC/5'-AGTTCTTCTGGCTGGTTACAGTGG).

Exponential PCR amplifications of GAPDH and ifabp products were obtained as follows: 5' at 94°C; 22 cycles of 94°C for 30", 55°C for 30", and 72°C for 30", and a final extension step

of 7' at 72°C. The GAPDH and ifabp reactions rendered products of 176bp and 201bp, respectively. Amplification for the Adh5, T antigen, alkaline phosphatase, L-MBP, EphB3 and SM22a reactions used similar conditions, except the annealing temperatures were 58°C, 74°C, 57.3°C, 58°C, 59°C and 57.8°C, respectively. The alkaline phosphatase reaction rendered a 525bp product within exponential range at 30 cycles, while the Adh5, L-MBP, EphB3 and SM22a reactions rendered products of 152bp, 563bp, 487bp and 200bp, respectively, all within exponential range at 25 cycles. The T antigen reaction rendered a product of 147bp, within exponential range at 22 cycles.

Quantification of RTPCR products. PCR products were resolved by agarose gel electrophoresis and visualized by staining with GelStar® nucleic acid stain (Cambrex). They were then digitally photographed and analyzed using the public domain NIH Image program, version 1.63 (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). A measurement of band intensities, using equal area measurements per band, were generated for each sample and surrounding background. For normalization, each sample-background 'b' measurement was subtracted from the foreground 'f' ($S = b - f$) then this difference obtained from the negative control (no RNA) ($C = b - f$, should be 0) was subtracted from each sample ($S_{\text{norm}} = S - C$). Each normalized value was divided by the normalized value for the sample, non-transgenic villi (v) (NTv), ($S' = S_{\text{norm}} / \text{NTv}_{\text{norm}}$). To determine the relative sample intensity value (S_{RV}) for comparison of each PCR reaction, each value obtained after this previous control was divided by the value of that sample obtained for the previous control from the control reaction, Adh5 ($S_{\text{RV}} = S'_{\text{rxnX}} / S'_{\text{Adh5}}$). The average of these measurements from two to four pairs biological replicates from non-transgenic and TAg^{wt} mouse

intestinal fractions (3-4 for 'v', 'c', and 'm' fractions) were calculated and graphed using the Excel software from Microsoft Office X for Macintosh. The average deviations were calculated and graphed as plus and minus error bars. p values were calculated using the students' t-test.

2.3. RESULTS

2.3.1. Intestinal epithelial structures can be isolated by incubation in non-enzymatic chelating buffer and mechanical release.

After incubation in buffer containing EDTA and gentle shaking, the intestinal epithelium is released from the underlying mesenchyme and muscle, like a glove lifting off of a hand. The first fractions release the villi structures, while the next fractions release a mixture of villi and crypts, and the last fractions release crypts (Fig.7a). These fractions were analyzed by microscopy, revealing the enrichment for these structures in their various fractions (Fig.7b). The villi/crypt (v/c) fraction is enriched in an intermediate zone at the base of the villus (arrows), however it contains many contaminating crypts (arrowheads), complicating the analysis of this fraction. Differences between non-transgenic and TAg^{wt}-transgenic intestinal structures are immediately evident. Villi from TAg^{wt}-transgenic intestines are longer and wider at the base. The crypts of TAg^{wt} intestines are longer as well (Fig.7b).

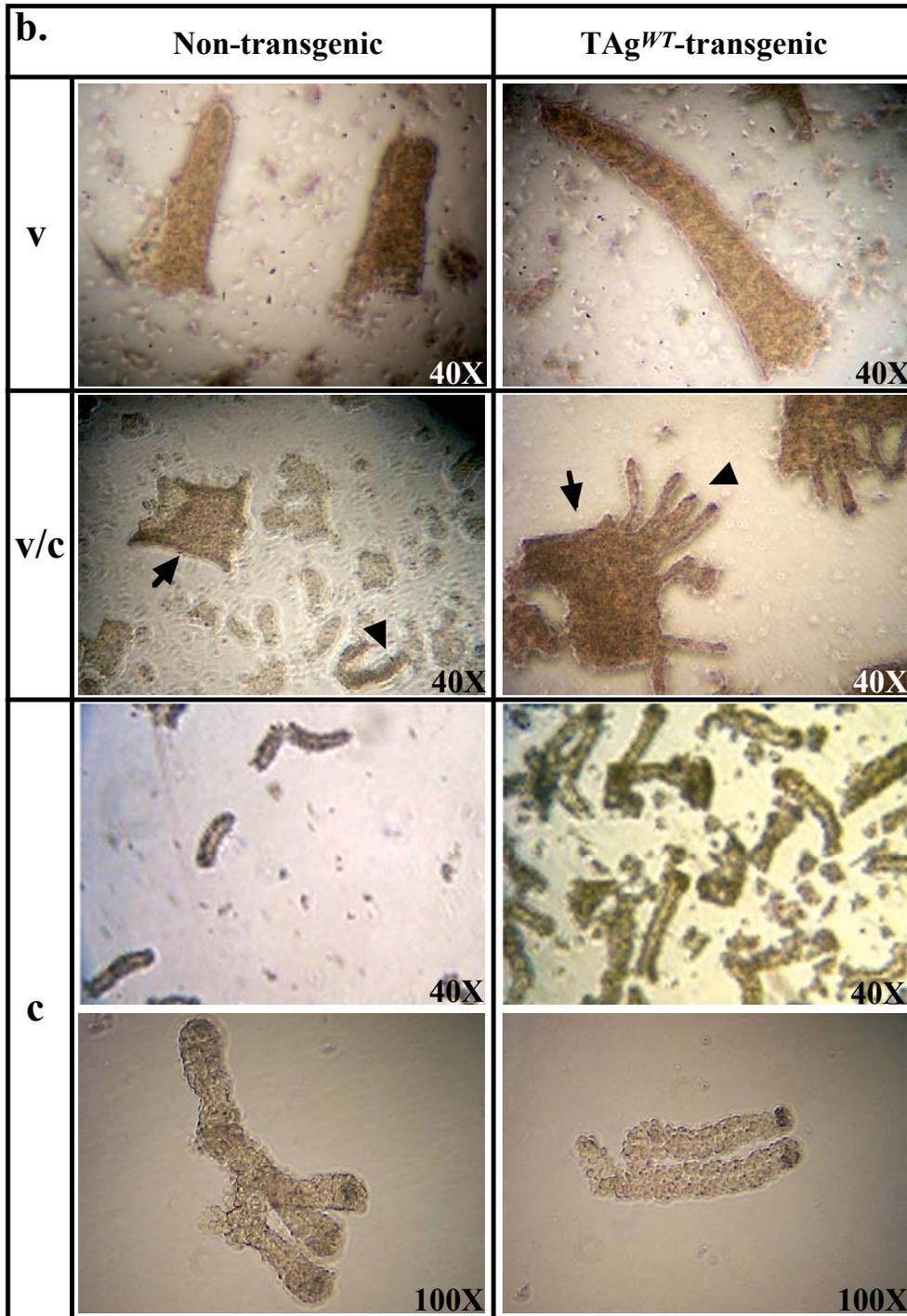
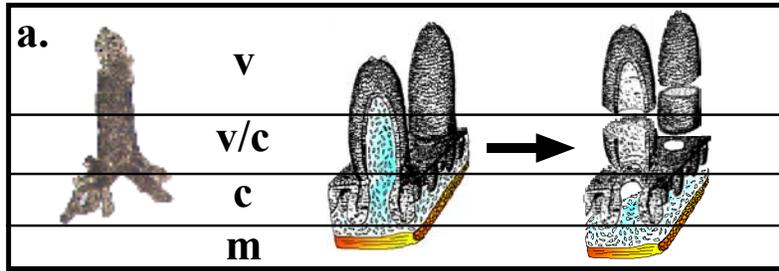


Figure 7. Microscopic characterization of intestinal epithelial fractions. a. Diagram of fraction enrichment. A micrograph of an intact villus with crypts connected is on the left, with a three dimensional diagram of a section of intestine in the center and a diagram of how the intestinal epithelium is fractionated and enriched on the right. The 'v' fraction is enriched for villi, their tips in particular, the 'v/c' fraction is enriched in cells at the base of the villus, the 'c' fraction is enriched for crypts, and the 'm' fraction is the remaining underlying mesenchyme and muscle of the intestine. **b. Micrographs of epithelial fractions.** Villi 'v' fractions from both non-transgenic and TAg^{wt} mice are enriched in villi structures, as depicted at original magnifications of 40x. The 'v/c' fraction contains both villi cells and contaminating crypts (arrow-head), but is enriched for an intermediate zone at the base of the villus (arrows). The crypt 'c' fraction is enriched for crypts in both genotypes. Micrographs at original magnifications of 40x and 100x are presented. The darker cells at the bottoms of the crypts are granule-containing paneth cells.

These fractions were collected and immediately lysed for isolation of either protein or RNA. The enrichment method was further verified for selection of the appropriate epithelial cells by immunoblot and semi-quantitative reverse-transcriptase PCR (RT-PCR) analyses. Different cell type-specific markers are localized to specific epithelial compartments based on protein activity, protein expression and/or transcript expression. For example, the enzymatic activity of sucrase isomaltase (SI) is localized to the villus, but its protein is expressed equally along the crypt-villus axis. In this case, the difference in expression and activity is due to post-translational modifications (Beaulieu et al., 1989). There are only a few markers detectable with commercially available antibodies that react with proteins expressed exclusively in one compartment. The markers used in this study were alkaline phosphatase as a marker for villus enterocytes and lysozyme as a marker for paneth cells localized at the base of the crypts. Although most cells express lysozyme, paneth cells express especially high levels this enzyme so it can be used as a marker for crypts at high dilutions of antibody. Although cycling cells are located exclusively in the crypts in non-transgenic intestines, markers for cell cycle-specific proteins were not used

since cycling cells are located in both the villi and crypts of TAG^{wt}-transgenic intestines. Antibodies against vimentin are used to show the localization of mesenchymal myofibroblasts (Pucilowska et al., 2000). Desmin, was also used to identify the localization of the muscle in the mesenchyme/muscle (m) fraction (data not shown). Figure 8a shows the relative enrichment of these markers in their appropriate fractions from each TAG-transgenic and non-transgenic intestines. In all TAG-transgenics and non-transgenic, the levels of alkaline phosphatase are highest in the villi (v) fraction and lower in the crypt (c) fraction. The villi (v) fraction is relatively devoid of lysozyme and vimentin, indicating its relative enrichment of villus epithelium. The crypt (c) fraction has the highest level of lysozyme from each genotype, and devoid of vimentin levels. This fraction still retains low levels of alkaline phosphatase. These low levels may reflect either contamination of villus enterocytes in this fraction or crypt cells that express alkaline phosphatase. Immunohistochemical studies show alkaline phosphatase localization exclusively in villi epithelial cells, extending to the base of the villus (Hauft et al., 1992). Perhaps cells at the base of the villi are collected in the crypt fraction.

To characterize the fractions lysed for RNA, RTPCR was performed using specific primers for cell type-specific transcripts. Many genes are expressed in varying gradients along the crypt-villus axis. Gradients allow for the effect of enrichment to be easily obscured by semi-quantitative analysis such as RTPCR. A few genes are exclusively expressed in the villus or the crypts. One such transcript for L-mannose binding protein (L-MBP) is exclusively expressed in a few villi tip cells (Uemura et al., 2002). Another, SM22 α , is expressed exclusively in smooth muscle cells (Solway et al., 1995). EphB3, a protein exclusively expressed in the paneth cells at the base of the crypts, was used as a marker for crypt cells (Batlle et al., 2002). RTPCR for

Alkaline phosphatase was also used as a secondary marker for villi enrichment, although its distribution is less exclusive as determined by RTPCR.

The characterization of epithelial fractions by RTPCR demonstrates the enrichment of markers in their appropriate fractions in non-transgenic fractions (Fig.9a). The same transcripts are less restricted to their appropriate fractions in TAg^{wt}-transgenic intestinal epithelia, (Fig.9a, compare lanes 3-5 to 6-8). This result may reflect either differences in fractionation efficiency of TAg^{wt}-transgenic intestines, or alterations of expression of these markers along the crypt-villus axis by T antigen expression.

The intensities of the PCR products were quantified and normalized to a loading control (Fig.9b). A statistical students' t-test was performed to determine the significance of enrichment for the markers in their appropriate fraction. In general, comparing v and c fraction enrichments gave p values between 0.1-0.2 in the non-transgenic fractions, indicating minimal significance. The p value for T antigen fraction enrichments were not even as significant, with values for alkaline phosphatase (p=0.453) and L-MBP (p=0.379) barely making the p<0.5 cut-off. However, the p value for T antigen EphB3 signal in the crypts was one of the most significant values at p=0.056. One explanation for such low significance in enrichment of transcript markers in the villi fractions of TAg^{wt} intestines may be due to the intestinal phenotype. When isolating the intestinal epithelium, the villi of TAg^{wt} intestines are more brittle as compared to non-transgenic intestines. These structures break off from the underlying mesenchyme more easily with less vigorous shaking than non-transgenic. Thus, the cells from the villi of TAg^{wt} intestines break apart more easily, perhaps causing the degradation of RNA before RNase inhibitors are added in the lysis buffer. To address this issue, inhibitors may need to be added in the release

buffer. This has not been incorporated into the protocol because the RNase inhibitors interfere with the fractionation process.

2.3.2. *WT* T antigen is expressed in the crypts and in the villi fractions, whereas *N136* and *3213* T antigens are expressed highest in the villi fractions.

The purpose of collecting epithelial fractions was to enrich for villus enterocytes, so that the effect of T antigen in these growth-arrested cells could be assessed. As described above, the villi (v) fractions are enriched for enterocytes and devoid of crypt paneth cells. T antigen protein and transcripts are expressed in these fractions of TAg^{wt} intestines (Fig 8a, lane 5). However, it is also clear that T antigen is expressed in the crypt (c) fraction at similar levels as in the villi (v). The highest levels of T antigen protein are seen in the intermediate villi/crypt (v/c) fraction (Fig.8a, lanes 5-7). These results indicate that T antigen is expressed in the crypts, in contrast to published reports demonstrating T antigen expression exclusive to the villi enterocytes by immunohistochemistry (Kim et al., 1993; Kim et al., 1994). It is possible that there are contaminating villi enterocytes in the crypts (c), as this fraction also contains low levels of alkaline phosphatase protein. However, the pattern of alkaline phosphatase protein expression is such that the highest levels are in the villi (v) fraction and the lowest levels are in the crypts (c). The pattern is clearly different for T antigen expression, with highest levels in the intermediate villi/crypt (v/c) fraction. To confirm this aberrant expression of T antigen, immunohistochemical analysis of this transgenic line was performed. Preliminary results indicate that T antigen is indeed expressed in both the crypts and the villi epithelium, with greatest levels at the villus/crypts interface (A. Rathi, University of Pittsburgh, unpublished data).

The intestinal epithelium was also fractionated from transgenic mouse intestines expressing mutants of T antigen in the villi enterocytes. *N136* (an amino-terminal 136 truncation mutant), and *3213* (a LxCxE motif mutant, incapable of binding Rb family members) show the expected pattern of protein expression along the villi/crypt axis, with greatest levels in the villi (v) and lowest or undetectable levels in the crypts (c) (Fig.8a, lanes 9-11 and 13-15). The relative levels of transgene expression are depicted in Figure 8b. It is difficult to compare levels between the amino truncation mutant, *N136*, and either *WT* T antigen or *3213* because it migrates faster in a gel and therefore must be detected by running a higher percentage polyacrylimide gel. This may result in differences in antibody interactions on the blot. The levels of *WT* T antigen are consistently greater than those for *3213* (Fig.7b, compare lanes 2 and 4). Quantification of the relative levels, normalized to β -tubulin levels, indicate that there is ten fold more *WT* T antigen than *3213* in their respective villi fractions. Although multiple lines have been generated for the transgenic expression of *3213*, none of them exceed the levels presented here. Multiple lines of transgenic mice expressing *N136* have also been generated, some expressing very high levels which are likely to be as great or greater in level to *WT* T antigen expression in villi.

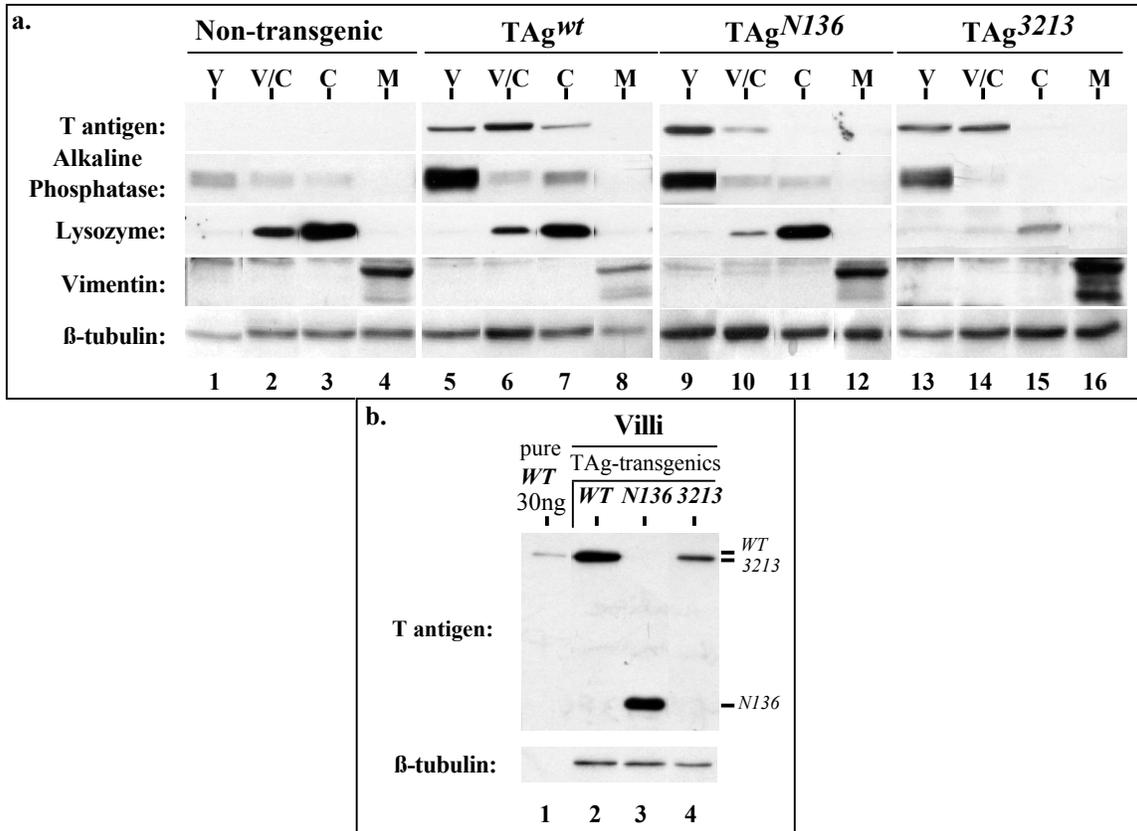


Figure 8. Molecular characterization of intestinal epithelial fractions: Enrichment of protein markers. a. Relative enrichment of expression of protein markers in appropriate fractions compared across genotypes. Non-transgenic, TAg^{wt}, TAg^{N136}, and TAg³²¹³ have similar patterns of enrichment of protein markers in their appropriate fractions. Alkaline phosphatase is enriched in villi fraction ‘v’, lysozyme is enriched in the crypt fraction ‘c’, and vimentin is enriched in the mesenchyme/muscle fraction ‘m’. β tubulin expression was used as a loading control. Relative T antigen, and mutant, expression was determined in the fractions from each genotype. **b. Relative expression of T antigen and mutants in villi from TAg-transgenics.** 30ng of purified, WT T antigen (a generous gift from P.G. Cantalupo), was used as a control to compare levels of T antigen expression in 30μg of total soluble protein extracts from villi of TAg-transgenics. Samples were electrophoresed through a 15% gel by SDS-PAGE. The membrane was probed two different times with two different antibodies against T antigen, PAb419 and PAb416 (Harlow et al., 1981).

2.3.3. Transgene transcripts are expressed in both villi and crypts, but the levels of protein are regulated post-transcriptionally

T antigen transcript expression was also analyzed in each of the transgenic fractions by RTPCR (Fig.9c, lanes 3-8). *WT* T antigen, *N136* and *3213* transcripts are all expressed at approximately equal levels in the same pattern, in both villi and crypts fractions. Taken together with the pattern of protein expression, these results indicate that the regulation of transcription and translation of these transgenes are different. Since the transgenes are driven by the same promoter sequences in the intestinal fatty-acid binding protein (fabpi) promoter, the pattern of endogenous fabpi expression was analyzed by RTPCR (Fig.8c). Fabpi transcript is also expressed equally in the villi and crypt fractions. As was the case for the protein expression pattern of the *N136* and *3213* transgenes, fabpi is expressed exclusively in the villi enterocytes. This has been reported in many reports cases fabpi expression was analyzed by immunohistochemistry (Cohn et al., 1992; Hauff et al., 1992; Kim et al., 1993).

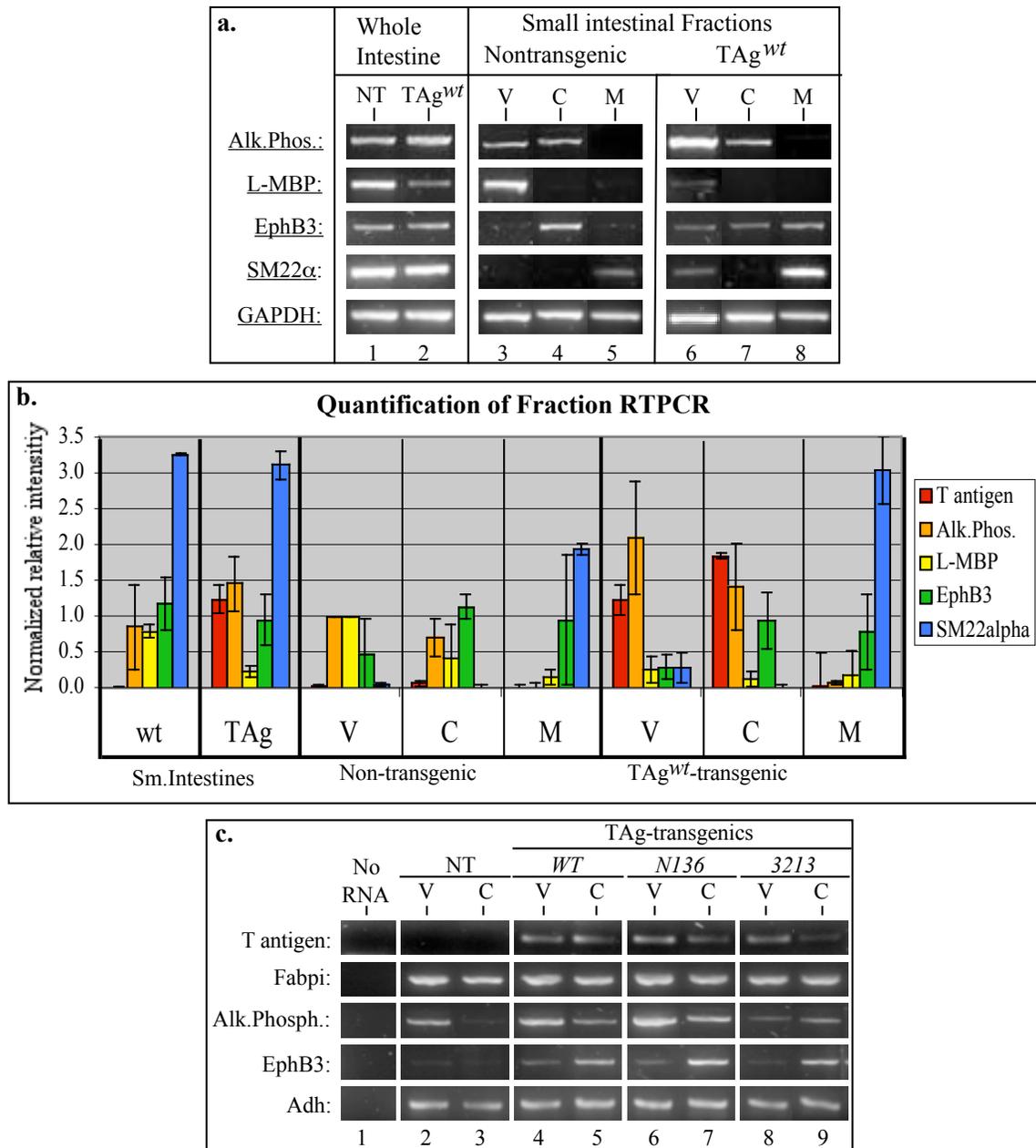


Figure 9. Molecular characterization of intestinal epithelial fractions: Enrichment of transcript markers. **a.** Relative enrichment of expression of transcripts in appropriate fractions from non-transgenic and TAg^{wt} intestines. RTPCR products specific for cell-type specific transcripts in the different epithelial compartments are enriched in appropriate fractions in non-transgenic and TAg^{wt} intestines. Alkaline phosphatase is enriched in the villi ‘v’ fraction in TAg^{wt} intestines, while L-MBP is enriched in the villi ‘v’ fraction of both genotypes. EphB3 is enriched in the ‘c’ fraction of non-transgenic, but distributed evenly throughout in the fractions

from the TAG^{wf} mouse depicted in the figure. SM22 α is enriched in the mesenchyme/muscle 'm' fraction. RTPCR of GAPDH was performed as a loading control. **b. Quantification and normalization of relative transcript levels in different intestinal epithelial fractions.** The intensity levels from the electrophoresed RTPCR products were quantified and normalized as described in material and methods. Average values are graphed with average deviations depicted as plus and minus error bars. The comparison of enrichment and statistical significance are discussed in the text. **c. Relative levels of transgene transcripts in villi and crypt fractions from TAG-transgenics.** Non-transgenic and the three TAG-transgenic mouse intestines were fractionated similarly as depicted by relative enrichment of markers. Endogenous fabpi transcript and transgene expression have relatively equal transcript levels in both villi 'v' and crypt 'c' fractions from mice of all appropriate genotypes. RTPCR of Adh5 was performed as a loading control.

2.4. DISCUSSION

At the beginning of this study, we could isolate villus epithelium separately from crypt epithelium using a scraping method and resulting in a relatively high yield of extracted protein (unpublished data, S.M. Hasso and M.T. Sáenz-Robles, University of Pittsburgh). However, we could not obtain RNA using this method (data not shown). The refinement of a non-enzymatic, chelating and shaking release method described in this chapter has allowed us to obtain high yields of intact cells from which both protein and RNA can be extracted for molecular analysis. The resulting molecular characterization indicates a complex regulation of transcript and protein expression in the intestinal epithelium along the crypt/villus axis. It is not the objective of this report to engage in a thorough investigation of this regulation, although the result of this regulation affects the expression of the transgenes used in this model system. Previous studies have reported on the exclusive expression in the villi of genes regulated by the promoter sequences (fabpi⁻¹¹⁷⁸⁻⁺²⁸) used in these transgene constructs. However, these reports presented

data on protein expression and did not assess transcripts along the villi/crypt axis (Cohn et al., 1992). Interestingly, the removal of sequences in the transgene corresponding to -277 to -185 nucleotides before the start (+1) site of the *fapbi* transcript results in inappropriate expression of the transgene protein in proliferating and non-proliferating epithelial cells of the upper crypts (Cohn et al., 1992).

There are at least five possible explanations for the differences in transgene expression patterns between the different mutants and *WT* T antigen. Since the TAg^{WT} transgene was constructed over a decade previous to the construction of the TAg^{N136} and TAg^{3213} -transgenes, one possibility is that there are slight differences in sequence between the promoter constructs in TAg^{WT} and TAg^{N136} or TAg^{3213} -transgenes. One possibility is that after multiple generations of breeding the TAg^{wt} -transgenic mice, this transgene lost these sequences, *fapbi*^{-277 to -185}, or they have become epigenetically modified in such a way to lead to expression of the transgene in the crypts, possibly due to some sort of unknown selective pressure. If this were the case, it is predicted that the future generations of the new transgenics may result in selective pressure to express the transgene in the crypts. Alternatively, differences in the transgene promoter constructs of the TAg^{N136} and TAg^{3213} -transgenes that were generated more recently compared to the original TAg^{wt} -transgene, although probably quite small, may result in different patterns of transgene expression along the crypt-villus axis. If this were true, a new TAg^{wt} -transgene constructed in a similar fashion as the other two recently constructed transgenes should show a similar expression pattern of transgene expression as the TAg^{N136} or TAg^{3213} -transgenes. A third possibility is that the high-expressing TAg^{WT} -transgenic line (line 103) we have studied incorporated the transgene in region of DNA that enhances its expression in a progenitor-like cell. If this were true, then independent lines of TAg^{WT} -transgenic mice should incorporate the

transgene in other genomic regions where this enhancement does not occur. In this case the pattern of its expression would be similar to that of the mutant T antigen transgenics. A fourth possibility is that there is something inherent to the *WT* T antigen that enhances its own transgenic expression in the crypts. If this were true, then all TAg^{*WT*}-transgenic mice would express in the same pattern. A fifth possibility is that a genetically linked gene in this line is a modifier of *WT* T antigen transgene expression, allowing for its expression in the crypts. If the transgene is genetically linked to a modifier, other transgenic lines would not likely be linked to the modifier and would therefore lose expression in the crypts. In order to distinguish between these possibilities, additional TAg^{*WT*}-transgenic mice are being generated. The expression patterns of T antigen along the crypt-villus axis will be determined and correlated to the resulting phenotypes.

Despite differences in transgene expression along the crypt/villus axis, the villi (v) fraction from each of the transgenic lines is the most enriched fraction for *WT* and mutant T antigen (and mutant) expressing enterocytes. The studies presented in the rest of this report focus primarily on samples from the villi of non-transgenic and TAg-transgenic mice.

3. T ANTIGEN-INDUCED INTESTINAL DYSPLASIA DOES NOT REQUIRE P53 INACTIVATION

3.1. INTRODUCTION

The gene for p53 is mutated in most cancers, signifying its global importance as a key target contributing to tumorigenesis (Prives and Hall, 1999). Coined “the guardian of the genome,” p53 protects cells from propagating damaged DNA by signaling damaged cells to growth arrest, correct the damage, and/or die by apoptosis (Levine, 1997; Sherr, 2002). These general protective functions are believed to be important for the survival of every cell in the organism. p53 is protective and is not normally activated in the cell unless it is damaged or stressed. Apoptosis is not necessarily an abnormal cellular activity. Some cells undergo programmed cell death during development. For example, the ectodermal cells at the distal ends of the limbs must die by apoptosis in order to form digits of the hands and feet in humans (Chen and Zhao, 1998). However, the role of apoptosis in regulating the homeostatic growth and growth arrest in the intestinal epithelium remains controversial (Potten et al., 1997).

The homeostatic regulation of the intestinal epithelium requires that cells be removed from the epithelium at the same pace as they are dividing and differentiating from the stem cells. The method of removal may be apoptosis, phagocytosis or exfoliation into the lumen and subsequent death because of removal of contact with a neighboring cell or the basement

membrane (Potten et al., 1997; Radtke and Clevers, 2005). Different epithelial cell types may be removed by distinct methods. For example, paneth cells at the base of the crypts are removed by phagocytosis by neighboring cells (Garabedian et al., 1997). Some reports suggest that the epithelial cells of the villi die by apoptosis, while other reports suggest that these cells are simply extruded into the lumen where they die either by necrosis or anoikis (Potten et al., 1997; Radtke and Clevers, 2005). The p53 pathway is functional in the crypt epithelial cells, since cells in the crypts undergo p53-mediated apoptosis upon UV or gamma irradiation (Wilson et al., 1998; Watson and Pritchard, 2000). If p53 plays an important role in regulating homeostasis in villus enterocytes, then it is predicted that T antigen expression and interaction with p53 would disrupt this homeostasis.

Transgenic expression of *WT* T antigen in villi enterocytes causes hyperplasia which progresses to dysplasia. T antigen-induced dysplasia is dependent on two things: 1) initial hyperplasia which requires an intact LxCxE motif and 2) an additional T antigen-dependent function or levels of expression. There are several additional factors that could contribute to T antigen-induced dysplasia: levels of expression above a particular threshold, the carboxy terminal 572 amino acids of T antigen (containing the p53 interaction region) and/or expression in the crypts. Lower expressing TAg^{wt}-transgenic lines developed intestinal hyperplasia that did not progress to dysplasia with age, providing evidence that expression levels play an important role in the ability of T antigen to cause the progressive phenotypes (Kim et al., 1993). Crossing these lines with transgenic mice expressing an activated ras oncogene, K-ras^{Val12}, is sufficient to recapitulate the progression to dysplasia, indicating that these two oncogenes can cooperate to cause intestinal tumorigenesis. Previous reports demonstrated that expression of the amino-terminal truncation mutant of T antigen, dl137, causes hyperplasia that does not progress to

dysplasia. Co-expression of the K-Ras^{Val12} oncogene also does not facilitate progression of TAg^{dl1137} intestines to dysplasia (Kim et al., 1994). These data provide evidence that the carboxy terminal half of T antigen plays an important role in progression to dysplasia. A known transformation function of T antigen in its carboxy terminal region is its interaction with p53. Thus, we hypothesized that T antigen inactivation of p53 in intestinal enterocytes is required for progression to dysplasia.

Several criteria must be met if T antigen interaction with p53 is required for progression to dysplasia. First, *dl1137* expression should cause an up-regulation of the p53 pathway and p53-dependent apoptosis. Second, *WT* T antigen should bind and stabilize p53, blocking the p53 pathway and p53-mediated apoptosis. Thirdly, expression of *dl1137* in a p53 null background should allow progression to dysplasia.

To test the hypothesis that T antigen interaction with p53 is required to cause progression to dysplasia in the intestine, transgenic mice expressing the T antigen mutant, *dl1137*, were generated in a p53 null background (Markovics et al., 2005). In addition, I undertook a molecular characterization of the interaction of *WT* T antigen with p53 and the status of the p53 pathway in villi epithelium.

3.2. MATERIALS AND METHODS

Production and maintenance of transgenic mice. FVB/N non-transgenic and TAg^{wt} mice were produced and maintained as described in the previous chapter. Modifications relevant to this chapter are described. Mice with the same region of the Fabpi promoter as previously described linked to SV40 TAg^{dl1137} (T antigen amino acids 1 – 121) or K-Ras^{Val12} were obtained from Jeff

Gordon (Washington University) and have been described (Kim et al., 1993; Kim et al., 1994). Fabpi-SV40 TAg^{dl1137} mice were from pedigree number 18 and Fabpi-K-Ras^{Val12} mice were from pedigree number 73. All pedigrees were maintained by crosses to non-transgenic FVB/N littermates and/or commercially obtained FVB/N mice (Taconic Labs). p53^{-/-} animals were purchased from GenPharm International (Mountain View, CA). Standard genetic crosses were performed to obtain mice with various combinations of the Fabpi-SV40 TAg^{dl1137} and Fabpi-K-Ras^{Val12} transgenes in p53^{+/+}, p53^{+/-} or p53^{-/-} background, as described in the text. For simplification purposes, animals are referred in the text as TAg^{dl1137} (Fabpi-SV40 TAg^{dl1137}), K-Ras^{Val12} (Fabpi-K-Ras^{Val12}) or their various combinations. The genotype of each mouse was determined using DNA isolated from murine tails followed by polymerase chain reaction (PCR) with protocols detailed elsewhere (Kim et al., 1993; Kim et al., 1994; Chandrasekaran et al., 1996).

Isolation of primary fibroblasts, cell culture conditions and establishment of cell lines.

Mouse Embryo Fibroblasts (MEFs) were harvested from 13.5 day old FVB embryos as described (Lindeman et al., 1998) and grown in DMEM supplemented with 10% Heat Inactivated Fetal Calf Serum, in the presence of antibiotics (penicillin/streptomycin). Primary cultures of MEFs were transfected with 5 µg of pRSVneoT using Lipofectamine Reagent (Life Technologies), according to the manufacturer's instructions. Upon selection in culture with 0.4 mg/ml of G418, transformed colonies (foci) were selected and grown and several independent cell lines were established.

Immunoblot analysis. Intestinal samples were obtained as described in the previous chapter. Conventional Western blot techniques were employed using 30µg of total protein lysates. Three antibodies were used to detect p53, each targeting different epitopes. In addition to the antibodies described in the previous chapter, appropriate dilutions of the following primary antibodies were used: p53- mouse monoclonal PAb421 (Harlow et al., 1981); p53- rabbit polyclonal FL-393 (*sc-6243*), p53- mouse monoclonal PAb240 (*sc-99*) (Santa Cruz biotechnology). MDM2- mouse monoclonal SMP14 (*sc-965*), p21- rabbit polyclonal M-19 (*sc-471*) (all from Santa Cruz Biotechnology); and anti-p19ARF-rabbit IgG (Ab80) (Novus *NB200-106*) were also used in these studies. Goat anti-mouse A2554, and goat anti-rabbit A0545 (Sigma) were used as secondary antibodies. The peroxidase reactions were developed with ECL-plus reagents according to the associated protocol (Amersham Life Sciences).

Immunoprecipitation analysis. Intestinal or mouse embryo fibroblast samples were homogenized in lysis buffer containing protease and phosphatase inhibitors and protein extract concentrations were determined as described above. 90µg of total soluble protein whole cell extracts were immunoprecipitated for T antigen using the mouse monoclonal antibody PAb419 as described (Srinivasan et al., 1997). 30µg of input lysate, 1/6th of the supernatant and total immunoprecipitates were denatured in SDS-PAGE buffer and electrophoresed through a 10% polyacrylamide gel. Immunoblot analysis was performed using standard procedures.

3.3. RESULTS

3.3.1. *WT* T antigen does not stabilize p53 in the intestine

p53 protein is present in most cells, but is maintained at barely detectable levels by its negative feedback mechanism with mdm2 (Levine, 1997; Prives and Hall, 1999). *WT* T antigen binds p53 and stabilizes it so that high levels are present in many systems, including the primary cell line, mouse embryo fibroblasts (MEFs) (Pipas and Levine, 2001). To determine if *WT* T antigen causes the stabilization of p53 in TAg^{wt} villi, immunoblot analysis was performed on extracts from villi fractions and compared to wildtype and TAg^{wt}-transformed MEFs (Fig.10). These results show that although p53 is stabilized to high steady-state levels in TAg^{wt}-transformed MEFs, it is not similarly stabilized in TAg^{wt}-transgenic villi (Fig.10, compare lanes 2 and 4). To determine if the p53 pathway is active in TAg^{wt}-transgenic villi, the levels of p53-responsive genes mdm2 and p21 were analyzed by immunoblot (Fig.10). Mdm2 is undetectable in T antigen expressing villi. In contrast to its down-regulation by T antigen expression in MEFs, p21 is up-regulated in T antigen expressing villi (Fig.10, compare lanes 2 and 4).

T antigen can activate the p53 pathway through de-repression of E2F, thereby activating p19ARF expression. p19ARF blocks mdm2 degradation of p53, causing its activation. To analyze the status of this mechanism in the intestine, p19ARF protein levels were determined by immunoblot. Figure 10 shows that p19ARF is upregulated in TAg^{wt}-transformed MEFs, but not in the intestine.

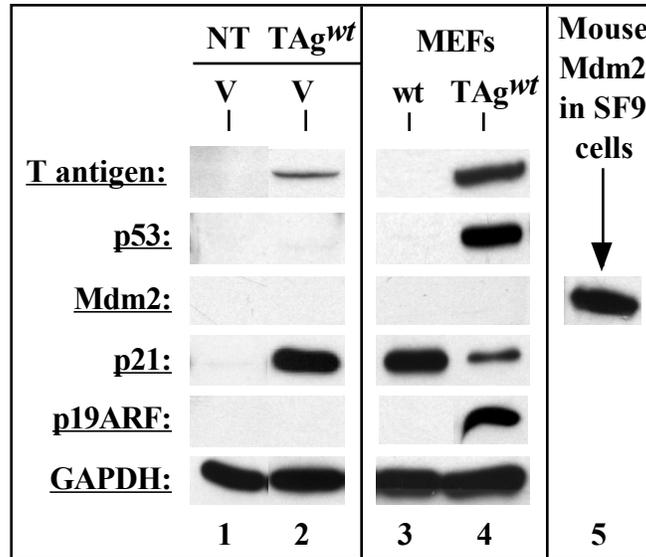


Figure 10. p53 is not stabilized in villi from TAg^{wt} mice. Equal concentrations of extracts from villi of non-transgenic (NT) and TAg^{wt} mice or wildtype (wt) and TAg^{wt}-transformed MEFs were immunoblotted for T antigen, p53, mdm2, p21 and p19ARF. Protein levels of GAPDH were determined as a loading control. A positive control for the mdm2 blot was 15µg of a baculovirus-infected mouse mdm2 over-expressing SF9 lysate prepared by J.M. Moskow.

3.3.2. WT T antigen does not bind p53 in the intestine

To determine if T antigen is bound to p53 in the intestinal epithelium, immunoprecipitation experiments were performed using specific antibodies against T antigen. Following immunoprecipitation, no detectable levels of T antigen were present in the supernatant of the immunoprecipitated extract, indicating that T antigen was quantitatively immunoprecipitated in this reaction (Fig.11, compare lanes 5 and 6). No detectable levels of p53 were present in these precipitates. This result is in contrast to the high levels of p53 co-immunoprecipitated with T antigen in the TAg^{wt}-transformed MEFs, (Fig.11, compare lanes 6 and 8). I conclude that p53 is

not stably complexed to T antigen in transgenic enterocytes as it is in other systems, such as T antigen-transformed MEFs.

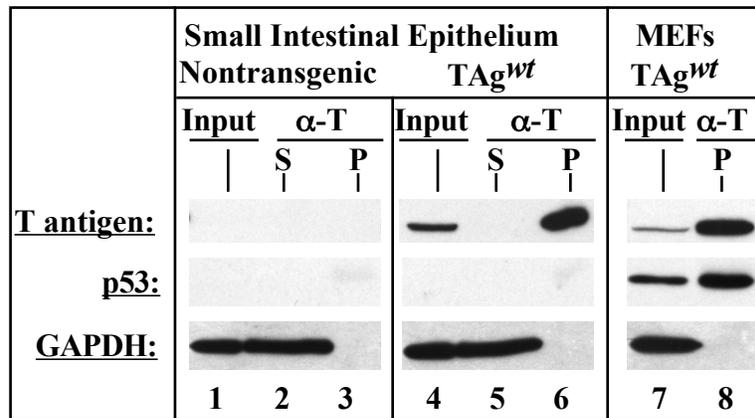


Figure 11. SV40 large T antigen is not bound to p53 in the intestinal epithelium of transgenic mice. T antigen was immunoprecipitated from total soluble protein extracts from the small intestinal epithelium of either non-transgenic or TAg^{wt}-transgenic mice or T antigen-transformed (TAg^{wt}) MEFs, as described in materials and methods. Immunoblots were performed using antibodies against T antigen and p53 as described in materials and methods. Protein levels for GAPDH were determined as a loading control. These results are representative of similar immunoprecipitations from 4 different pairs of mice or 3 different clones of TAg^{wt} MEFs. S = supernatant, P = pellet, αT = anti-T antigen antibody PAb419 (Harlow et al., 1981).

3.3.3. TAg^{dl1137} mice do not develop dysplasia in a p53 null background

To test the hypothesis that T antigen-induced progression to dysplasia in the small intestine requires inactivation of p53, mice expressing the amino-terminal truncation mutant of T antigen, dl1137 in villus enterocytes were generated in a p53 null background. These mice developed low-grade hyperplasia that does not progress to dysplasia (unpublished data, C. Coopersmith). Since transgenic expression of an oncogenic ras allele, K-ras^{Val12}, can facilitate progression to dysplasia in mice expressing low levels of *WT* T antigen, TAg^{dl1137}-transgenic mice were generated to co-express the K-ras^{Val12} transgene. These bi-transgenic mice also do not progress

beyond hyperplasia (Kim et al., 1994). Ultimately, TAg^{dl1137}/K-ras^{Val12} bi-transgenic mice were generated in a p53 null background. These mice also did not develop an intestinal phenotype that progressed beyond hyperplasia (Markovics et al., 2005).

3.4. DISCUSSION

Previously, Gordon and coworkers generated transgenic mice that express SV40 large T antigen specifically in enterocytes, a terminally differentiated cell population located in the intestinal villi. They found that wild-type T antigen induced enterocyte proliferation leading to intestinal hyperplasia evident by 6 weeks after birth that then progressed to dysplasia with age. They also found that progression to dysplasia is accelerated by the presence of an activated ras oncogene, and does not require a functional p53 (Kim et al., 1993; Coopersmith and Gordon, 1997b). In contrast, mice expressing a mutant large T antigen that is defective for Rb binding exhibited normal intestinal morphology and growth control (Chandrasekaran et al., 1996). Finally, they demonstrated that expression of a truncated T antigen consisting of the J domain and LxCxE motif induced enterocyte proliferation and hyperplasia, but mice expressing this mutant failed to progress to dysplasia even in the presence of the activated ras oncogene (Kim et al., 1994). Based on these observations, it was hypothesized that inhibition of the pRb family is required for enterocyte entry into the cell cycle and hyperplasia. These data also raised the possibility that progression to dysplasia requires a function in the carboxy-terminal portion of T antigen. Alternatively, differences in T antigen expression levels and/or localization between TAg^{wt} and TAg^{dl1137} mice may explain the lack of dysplasia in these animals.

3.4.1. T antigen induces intestinal dysplasia independent of p53 inactivation.

The observation that TAg^{dl137}, unlike TAg^{wt} mice, do not progress to dysplasia led us to hypothesize that T antigen action on p53 is required for this process. However, two lines of evidence indicate that this is not the case. First, the intestines of TAg^{dl137} mice in a p53 null background remain hyperplastic. Since this genetic combination should inactivate both the Rb and p53 pathways, the elimination of p53 in enterocytes is not sufficient to induce the progression towards intestinal dysplasia. Second, we failed to detect the presence of T antigen/p53 complexes in the intestines of transgenic mice. The absence of such complexes along with the absence of p53-stabilization or p19ARF expression in T antigen-expressing enterocytes leads us to suggest that the mechanism to activate p53 is not present in this cell type. In support of this proposition, it has been reported that p53 protein remains undetectable in villus enterocytes of normal mice, even when irradiated with gamma-rays, although p53 is detected at the base of the crypts in these irradiated mice (Coopersmith et al., 1997a). It is likely that the small amount of p53 we detect in T antigen-expressing intestine is derived from crypt progenitor cells.

Although T antigen-mediated inactivation of p53 seems to be an essential requirement for full transformation in several cell culture systems and transgenic models (Sáenz-Robles 2001; Ahuja *in press*), our unanticipated results indicate that the p53 protein may not be a key mediator of growth arrest, or even play a significant role, in normal, unstressed murine intestinal enterocytes. Perhaps p53 function as guardian of the genome is not necessary in a cell type that has only a three to five day lifespan. Rather, we speculate that p53 function is critical for genomic integrity in the intestinal stem cells, which only contribute 1-3 cells per crypt (Sancho et

al., 2004). The fact that these cells are relatively rare and that they do not express T antigen may explain our inability to detect p53 in transgenic intestine.

All three independently developed p53 nullizygous mouse strains do not exhibit spontaneous tumor formation in the small intestine, thereby supporting the lack of a role of p53 in the homeostasis of this regenerative epithelium (Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994). Additionally, the basal levels of apoptosis observed in the unstressed, homeostatic murine small intestine have been shown to be p53-independent (Merritt et al., 1994). Furthermore, previous experiments demonstrated the irrelevance of p53 in accelerating the dysplastic phenotype and in changing the apoptotic rates observed in T antigen-expressing intestinal samples (Chandrasekaran et al., 1996; Coopersmith and Gordon, 1997b). In contrast, a number of studies have implicated p53 as a critical factor in stressed tissues (Coopersmith and Gordon 1997b; Kamarova et al., 2000; Watson and Pritchard, 2000). Nevertheless, villus enterocytes do not undergo irradiation-induced apoptosis (which requires p53) even when expressing T antigen (Coopersmith et al., 1997a). These results emphasize the importance of tissue and intra-tissue cell type specificity and reveal how different mechanisms are required to control cell cycle and growth regulation in distinct cell types.

There are three possible explanations for the failure of TAg^{dl1/37} mice to progress to dysplasia in a p53 null background. First, T antigen may target a protein other than p53 to effect dysplasia. Several reports have indicated the existence of T antigen transforming activities other than p53-binding, mapping to the carboxy-terminal region (Thompson et al., 1990; Kierstead and Tevethia, 1993; Dickmanns et al., 1994; Sachsenmeier and Pipas, 2001; Beachy et al., 2002). Second, T antigen action on p53 may not be equivalent to a total loss of p53 function. Perhaps the T antigen/p53 complex exerts a function necessary for dysplasia. For example, it has

recently been shown that mutant p53 proteins that are found in some cancers have a gain-of-function phenotype (Olive et al., 2004; Lang et al., 2004). However, this hypothesis is disfavored by our failure to detect complexes of p53 and wild-type T antigen in intestine. A final possibility is that TAg^{dl1137} mice fail to progress to dysplasia because the steady-state levels of this mutant protein are lower than wild-type T antigen. While this remains a formal possibility we think that it is unlikely. Recently we have generated several new founder lines expressing a T antigen mutant similar to dl1137. These lines show a range of T antigen expression levels but, thus far, none have progressed to dysplasia (data not shown).

In at least one other transgenic model system, T antigen-induced tumorigenesis is independent of its action on p53. Expression of a truncated T antigen in pancreatic acinar cells leads to carcinoma (Tevethia et al., 1997). However, these results are in clear contrast to T antigen expression in the choroid plexus where inactivation of p53 plays a critical role in tumor expansion. In this case, T antigen induces quick tumor formation and kills the animals, while dl1137 facilitates dysplasia and tumor formation, but at markedly slower rate (Chen and Van Dyke, 1991; Sáenz-Robles et al., 1994). This delayed rate of tumor growth is abrogated when dl1137 is expressed in a p53 null background, which eliminates p53-dependent apoptosis (Symonds et al., 1994). This indicates the relevance of p53 as a critical regulator of tumorigenesis in the choroid plexus.

3.4.2. T antigen expressing intestines contain high levels of p21

SV40 T antigen binding to p53 results in its stabilization and inactivation (Pipas and Levine, 2001). As a consequence, activation of genes normally regulated by p53 (e.g. p21) is impaired. Accordingly, upon p53 stabilization by T antigen in primary MEFs, we observed a reduction in

the amount of p21 protein. In clear contrast, augmented levels of p21 upon expression of T antigen in the intestine were readily observed. This increase in p21 levels occurs largely through increased transcription of the p21 gene (P.G. Cantalupo et al., unpublished data).

T antigen efficiently induces enterocyte proliferation and a dramatic expansion of intestinal villi. Thus, it was surprising to find that T antigen expressing enterocytes contain abundant levels of the cell-cycle inhibitor, p21. It is unclear how enterocyte proliferation proceeds in the presence of these high p21 levels. Furthermore, the mechanism by which T antigen induces increased p21 transcription in intestine is unknown. The lack of p53 stabilization and the concomitant upregulation of p21 suggest that regulatory mechanisms other than those mediated by p53 are responsible for this observed increase in p21 product. In fact, p21 expression is controlled by several factors other than p53 (Macleod et al., 1995), and the p21 protein has been found to be expressed in the intestinal differentiation zone comprised of the upper crypts and lower villi portions, where it may be a marker of differentiating enterocytes (Gartel et al., 1998; van der Wetering et al., 2002). However, intestinal cell differentiation does not seem to be perturbed by T antigen expression (Hauft et al., 1992). Thus, at present the significance of increased p21 levels and the mechanism by which this increase is achieved are unclear. Perhaps T antigen expression results in the expansion of the intestinal differentiation zone, thereby increasing the number of enterocytes expressing p21.

4. T ANTIGEN-INDUCED INTESTINAL HYPERPLASIA REQUIRES DISRUPTION OF RB/E2F COMPLEXES AND UPREGULATES E2F2 AND E2F3A ACTIVITY

4.1. INTRODUCTION

Several different signaling pathways regulate development, differentiation and homeostasis in the small intestinal epithelium. Wnt/ β catenin signaling is important in the formation and maintenance of proliferation in the crypt department, while BMP/TGF β signaling is important for villus formation and maintenance of growth arrest (Sancho et al., 2004). Other pathways regulate specific differentiation of epithelial cell lineages. The role of the Rb/E2F pathway downstream of these signaling pathways, however, has not been fully described in regulating growth control in the intestinal epithelium. If any of the Rb family members play an important role in regulating growth arrest in villus enterocytes, then T antigen expression and interaction with the Rb family member(s) would be predicted to disrupt this growth arrest and induce these cells to enter S phase, independent of the status of upstream signaling pathways.

It was previously shown that expression of *WT* T antigen in villus enterocytes causes these cells to enter S phase resulting in intestinal hyperplasia. This hyperplasia is dependent on an intact LxCxE motif in T antigen, as mice expressing a T antigen mutant in this motif, *3213*, are normal (Kim et al., 1994; Chandrasekaran et al., 1996). These results suggest that T antigen binding the Rb family is required to cause intestinal hyperplasia. Expression of an amino-

terminal truncation mutant of T antigen, *dl1137* or *N136*, containing the J domain and the LxCxE motif, also causes intestinal hyperplasia, supporting the hypothesis. In addition, these results suggest that expression of the first 121 amino acids (*dl1137*) of T antigen is sufficient to cause hyperplasia. This led to the hypothesis that T antigen causes cell cycle entry through chaperone-mediated disruption of Rb/E2F complexes and activation of E2F-responsive gene transcription, thus causing hyperplasia.

Several criteria must be met to verify these hypotheses. First, *WT* T antigen must bind Rb family member(s) in the intestinal epithelium, while *3213* must not. Secondly, Rb/E2F complexes must be disrupted in *WT* and *N136* expressing villi enterocytes, but not in *3213* expressing cells. Thirdly, the “Rb-free” E2Fs must be associated with E2F-responsive promoters, causing their transcriptional up-regulation in the villi of TAg^{wt} and TAg^{N136}, but not in TAg³²¹³ or non-transgenic mice.

To test these predictions, molecular analyses of the Rb and E2F family members were performed from intestinal villi of non-transgenic and TAg-transgenic mice. The levels and activities of each individual Rb and E2F family members were determined, revealing specificity amongst family members in regulating normal growth arrest and abnormal growth in villi enterocytes.

4.2. MATERIALS AND METHODS

Production and maintenance of transgenic mice. FVB/N non-transgenic, TAg^{wt}, TAg^{N136}, and TAg³²¹³ mice were produced and maintained as described in the previous chapter. Modifications relevant to this chapter are described. For analysis of transgenic mice expressing T antigen in

villus enterocytes in an E2F2 null background, TAg^{wt};E2F2^{-/-} mice were generated in collaboration with Gustavo Leone (Ohio State University). E2F2^{-/-} mice were generated as previously described (Leone et al., 2001). Since the E2F2^{-/-} mice are maintained in the C57B/L6 strain, TAg^{wt} mice were bred into this line over several generations. Standard genetic crosses were performed to generate E2F2^{+/+}, E2F2^{-/-}, TAg^{wt}, and TAg^{wt}/E2F2^{-/-} mice or their various combinations. The genotype of each mouse was determined using DNA isolated from murine tails followed by polymerase chain reaction (PCR) with protocols detailed elsewhere (Kim et al., 1993; Kim et al., 1994; Chandrasekaran et al., 1996; Leone et al., 2001).

Isolation of primary fibroblasts, cell culture conditions and establishment of cell lines.

Mouse Embryo Fibroblasts (MEFs) were harvested, transfected and grown as described in the previous chapter.

Subcellular fractionation. Epithelial fractions were collected as described in chapter 2. Nuclear and cytoplasmic fractionation of these epithelial fractions was performed as described, with modifications (Leone et al., 1998). Before swelling in hypotonic buffer, the epithelial tissue was disaggregated by 10-20 strokes of dounce homogenization using pestle A (“loose” pestle) on ice.

Immunoblot analysis. Intestinal samples were obtained as described in the previous chapter. Conventional Western blot techniques were employed using 30μg of total protein lysates. In addition to the antibodies described in the previous chapter, appropriate dilutions of the following primary antibodies were used: a mixture of mouse monoclonal antibodies against pRb-mouse IgG (G-3245) (*14001A*)(BD-Pharmingen); and pRb-mouse IgG₁ (IF8) (*sc-102*), anti-

p130-rabbit IgG (C-20) (*sc-317*), anti-p107-rabbit IgG (C-18) (*sc-318*), anti-E2F1-rabbit IgG (C-20) (*sc-193*), anti-E2F2-mouse IgG₁ (TFE25) (*sc-9967*), (*sc-633*), anti-E2F3-rabbit IgG (C-18) (*sc-878*), anti-E2F4-rabbit IgG (A-20) (*sc-1082*), and anti-histone H3-rabbit IgG (FL-136) (*sc-10809*) (all from Santa Cruz Biotechnologies). Goat anti-mouse A2554, and goat anti-rabbit A0545 (Sigma) were used as secondary antibodies. The peroxidase reactions were developed with ECL-plus reagents according to the associated protocol (Amersham Life Sciences).

Immunoprecipitation analysis. Immunoprecipitation (IP) for T antigen was performed as described in the previous chapter, with one modification. Instead of performing the IP in the buffer described, the extracts were diluted in TBS (10mM Tris, pH 7.5, 100mM NaCl, 1mM EDTA, 0.1% Tween 20) plus 4mM EDTA, 0.5% NP40 and fresh protease/phosphatase inhibitors. 30µg of input lysate, 1/6th of the supernatant and total immunoprecipitates were denatured in SDS-PAGE buffer and electrophoresed through 8%, 10% or 15% polyacrylamide gels. Immunoblot analysis was performed using standard procedures.

Phosphatase assay. Whole cell extracts were prepared as described in the previous chapter. 100µg of villi extracts from non-transgenic and TAg^{wt} mice were subjected to phosphatase assays using alkaline phosphatase or PP2A as described (Shenolikar et al., 1996). More precisely, 30U of calf intestinal alkaline phosphatase (CIP) (BioLabs *M0290S*) and 0.5U of PP2A (*P-6993*) (Sigma) were used per reaction, while 1µmol of microcystin-LR (*EI-193-0500*) (BIOMOL) was used to inhibit the PP2A reaction. 1/3 of each reaction was denatured and subjected to SDS-PAGE through 10% polyacrylimide gels. Immunoblot analysis was performed according to standard procedure using antibodies as described above.

E2F electrophoretic mobility shift assay (EMSA). Equal amounts of top and bottom ssDNA strands of oligonucleotides containing wildtype (wt) E2F binding sites (5' – ATTTAAGTTTCGCGCCCTTTCTCAA – 3') or mutant (mt) E2F binding site (5' – ATTTAAGTTTCGATCCCTTTCTCAA – 3') were annealed in TE buffer. 500ng/28pmol of the wt double-stranded oligonucleotide was then end-labeled with ³²P-ATP γ as described, with modifications (Sullivan et al., 2000). To purify the end-labeled oligonucleotide from unlabeled ³²P-ATP γ , the oligonucleotide was subjected to centrifugation through the “Centri-spin™-20” columns as per the manufacturer’s protocol (Princeton Separations). Intestinal or mouse embryo fibroblast samples were homogenized in lysis buffer containing protease and phosphatase inhibitors and protein extract concentrations were determined as described above. 30 μ g of extracts plus or minus antibodies were diluted in gelshift buffer (10mM Hepes KOH, pH 7.9, 20mM KCl, 3mM MgCl₂, 0.5mM EGTA, 0.5mM DTT, 0.5mM PMSF, 0.05%NP40), plus an additional 100mM DTT, 1.5mg/mL BSA, 50 μ g/mL salmon sperm DNA, 10% glycerol and incubated with 2 μ g of labeled probe for 30 minutes at room temperature. Unlabeled wt and mt oligonucleotides were added at a 100-fold molar excess of labeled probe for competitor assays. Antibodies used for supershift assays were a mixture of mouse monoclonal antibodies against pRb-mouse IgG₁ (IF8) (*sc-102*)(Santa Cruz Biotechnologies, Inc.) and pRb-mouse IgG (G-3245) (14001A)(BD-Pharmingen); anti-p130-rabbit IgG (C-20) (*sc-317*), anti-p107-rabbit IgG (C-18) (*sc-318*) or anti-p107-mouse IgG₁ (SD9) (*sc-250*), anti-E2F1-mouse IgG_{2a} (KH95) (*sc-251*) or anti-E2F1-rabbit IgG (C-20) (*sc-193*), anti-E2F2-mouse IgG₁ (TFE25) (*sc-9967*) or anti-E2F2-rabbit IgG (C-20) (*sc-633*), anti-E2F3a-rabbit IgG (N-20) (*sc-879*), anti-E2F3-rabbit IgG (C-18) (*sc-878*), anti-E2F4-rabbit IgG (A-20) (*sc-1082*) and anti-E2F5-rabbit IgG (H-111) (*sc-1699*) (all

from Santa Cruz Biotechnologies, Inc.). The samples were then subjected to gel electrophoresis through a non-denaturing polyacrylimide gel (4% bis-acrylamide, 0.25X TBE, 0.1% ammonium persulfate (APS), 0.001X TEMED) in 0.25X TBE running buffer at 4°C for approximately 4-6 hours. The gel was then dried for approximately 2 hours and exposed to film for various lengths of time at -80°C.

RT-PCR Analysis. Whole intestine or intestinal fractions were collected, lysed and homogenized in buffer containing an RNase inhibitor, guanidine isothiocyanate, and the total RNA was extracted using the RNeasy kit (Qiagen). 1µg of RNA was reverse transcribed into cDNA using the Superscript First-Strand Synthesis kit (Gibco BRL). The cDNAs were amplified with the polymerase chain reaction (PCR) using primers specific for Adh5 as described in chapter 2, E2F1 (5' - TTGCCTGTCTGTTTGCTGAGCC/5' - CGGAGATTTTCACACCTTCCCTG), E2F2 (5' - TTCGCTTTACACGCAGACGG/5' - AATGAACTTCTTGGTCAGGAGCC), E2F3a (5' - AGCCTCTACACCACGCCACAAG/5' - ATCCAGGACCCCATCAGGAGAC), E2F3b (5' - TTACAGCAGCAGGCAAAGCG/5' - GAACTTCTTGGTGAGCAGACCG), E2F4 (5' - CCAAGAATCCTCTCCTCCAAGC/5' - GCACAGACACCTTCACTCTCGTCC), RB (5' - TCACACAACCCAGCAGTGCG/5' - CTATCCGAGCGCTCCTGTTC), p107 (5' - TGGATTATTGAAGTTCTC/5' - CTGATCCAAATGCCTATC), p130 (5' - TTGGGACTCTGTCTCGGTGTCTAAG/5' - AATGCGTCATGCTCCAGAACACCAG), p19ARF (5' - TGGACCAGGTGATGATGATGG/5' - AAGAAAAAGGCGGGCTGAGG), TK1 (5' - GCTTTCGGCAGCATCTTGAAC/5' - CCCTCAGTTGGCAGAGTTGTATTG), and DHFR (5'

– TGGTTTGGATAGTCGGAGGCAG/5' – GGGGAGCAGAGAACTTGAAAGC). RTPCR primers for b-myb and Cyclin E1 were previously described (Wells et al., 2000a).

Exponential amplifications of PCR products were generally obtained as follows: 2' at 94°C; a series of 25 cycles between 94°C for 30", 55°C (annealing temperature) for 30", and 72°C for 30", and a final extension step of 7' at 72°C. The annealing temperatures and product sizes for each reaction are as followed: 60°C, 420bp for E2F1, 60°C, 289bp for E2F2, 60°C, 309bp for E2F3a, 56°C, 189bp for E2F3b, 59°C, 495bp for E2F4, 56°C, 270bp for RB, 57°C, 499bp for p130, 50°C with 1' extension, 936bp for p107, 60.2°C, 415bp for p19ARF, 59°C, 312bp for TK1, 55°C, 261bp for DHFR, 58°C, 350bp for b-myb, and 55°C, 608bp for Cyclin E1.

Chromatin immunoprecipitation (ChIP): Collection of cross-linked villus epithelial cells.

Mice were sacrificed, intestines dissected, measured and cleaned as described in chapter 2. Three pairs of mice were treated with 0.3% formaldehyde in PBS before fractionation, while the other three pairs of mice were treated after fractionation and combination of all 'v' fractions. For the method of cross-linking before fractionation, each intestinal section was placed in 10mL of 0.3% formaldehyde in PBS and incubated for 15min. at room temperature while rotating on a Clay Adams™ Nutator (Becton Dickinson 421105) under a ventilation hood. 1M glycine was then added to a final concentration of 0.125M to stop the cross-linking reaction. Each section was then removed from the tube and placed in a 50mL falcon tube with the appropriate concentration of EDTA and 1mM DTT in PBS to begin the fractionation. Fractionation then proceeded as described in chapter 2 with one modification – the incubation times were approximately doubled to account for the cross-linking and increasing difficulty of release. For the method of cross-linking after fractionation, the collected villus (v) fractions were centrifuged for 5min. at 4°C at

1200 rpm(300g) on a desktop swinging-bucket Sorvall RT600B then resuspended in 10mL of 0.3% formaldehyde in PBS and incubated for 15min. at room temperature while rotating on the Nutator under a ventilation hood. 1M Glycine was then added to a final concentration of 0.125M to stop the cross-linking reaction.

ChIP: Extraction and shearing of chromatin. After collection of cross-linked villi, the villi were then centrifuged for 5min. at 4°C at 1200rpm(300g) and resuspended in 1-2mL of PBS plus protease/phosphatase inhibitor. The tissue was then disaggregated by 10-20 strokes in a dounce homogenizer using the pestle A (“loose” pestle) on ice. Again, the villi were pelleted by centrifugation at 2000g (~3100rpm on desktop Sorvall) for 5min. at 4°C. Villi cells were then swollen in 5-10volumes of RSB buffer (10mM Tris, pH 7.4/10mM NaCl/3mM MgCl₂) plus protease/phosphatase inhibitors on ice for 15min. To assist in release of nuclei, swelled villus cells were subjected to 10-20 strokes of dounce homogenization using a pestle B (“tight” pestle) on ice. The nuclei were then pelleted by centrifugation at 2000g (~3100rpm on desktop sorvall) for 5min. at 4°C. The nuclei were then quickly rinsed with 5 volumes of RSB buffer, re-pelleted and resuspended in 1-2 volumes (at least 500µL total) of FA lysis buffer (50mM Hepes, pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) plus protease/phosphatase inhibitors and incubated on ice for 15min. After this incubation, a Kontes™ Pellet Pestle Motor (Fisher K749540-000) was used to assist in nuclei lysis on ice. Chromatin was sheared by an empirically determined number of sonications, cycling between 20” sonication and 20” rest on ice. This resulted in an average length of 500-1000bp of DNA fragments. Membranes were pelleted by centrifugation at 4°C at 14000rpm for 15min. Supernatants were then decanted and used for immunoprecipitation. The concentration of chromatin was determined by Bradford assay as described (Bradford et al., 1976).

ChIP: Immunoprecipitation and DNA extraction. Immunoprecipitation using antibodies against Rb and E2F family members was performed on 1.5mg of chromatin per reaction as described for steps 8 through 14 of the protocol from the Farnham lab (University of California at Davis), from the preclearing step to the washing step (<http://genomecenter.ucdavis.edu/farnham/farnham/protocols/tissues.html>). The antibodies used were: normal rabbit IgG (Sigma *I-5006*); anti-acetyl Histone H4-rabbit IgG (Upstate *06-598*); anti-E2F1-rabbit IgG (C-20) (*sc-193*); anti-E2F2-rabbit IgG (C-20) (*sc-633*); anti-E2F3a-rabbit IgG (N-20) (*sc-879*); anti-E2F3-rabbit IgG (C-18) (*sc-878*); anti-E2F4-rabbit IgG (A-20) (*sc-1082*); anti-pRb-rabbit IgG (M-15) (*sc-1538*); anti-p130-rabbit IgG (C-20) (*sc-317*); anti-p107-rabbit IgG (C-18) (*sc-318*) (all from Santa Cruz Biotechnologies, Inc.) Two serial immunoprecipitations (IPs) were performed, each incubating with primary antibodies for ≥ 4 hours at 4°C, and incubating with secondary antibodies for 15 min. at room temperature. Precipitates from both IPs were combined after the initial DNA extraction after proteinase K treatment. DNA extraction was performed as described, from steps 15 to 21 (<http://genomecenter.ucdavis.edu/farnham/farnham/protocols/tissues.html>). DNA was then purified by phenol/chloroform extraction as described (Budelier and Schorr, 2002). DNA from precipitates was resuspended in 50 μ L TE, while DNA from input/IgG supernatant was resuspended in 300 μ L TE.

ChIP: Promoter PCR. 2 μ L (or 1/25 of precipitates, 1/150 of inputs) of sample DNA was subjected to PCR using specific primers for promoter regions spanning E2F binding sites of the following genes: dihydrofolate reductase (DHFR), thymidine kinase 1 (TK1), b-myb, p19ARF, p107, E2F1, E2F2, and E2F3a. The promoter of alcohol dehydrogenase 5 (Adh5) was used as a control since it does not contain E2F binding sites and is not E2F-regulated. The ChIP primer

sequences are: pE2F1 (5' – CAACCAATGCTCGCCATC, 5' – CGCCAAATCCTTTTTGCC), pE2F2 (5' – CCTTTAAGCCACTAAGCTCCTG, 5' – TCTAGTTCCCGTCCCTTGC), pE2F3a (5' – CCAATGGAAAACAGCCAGTG, 5' – GACAATGAATGAAGGGATCGG), pAdh5 (5' – ACCCTGACTGGAGACAGACTATGG, 5' – GTGGACCAACTATGTGGAGACAGAG). Gene promoters are abbreviated by a 'p' in front of the gene, such as "pE2F1". For proteins that have a 'p' at the beginning of their name, the initial 'p' is for the promoter and the second 'P' is capitalized for the gene name. The ChIP primer sequences for pDHFR, pTK1, and pbmyb were previously described (Wells et al., 2000a). The ChIP primer sequences for pP19ARF and pP107 were also previously described (Aslanian et al., 2004).

Exponential amplifications of PCR products were generally obtained as follows: 2' at 94°C, a series of 25-30 cycles of 94°C for 30", 55°C (annealing temperature) for 30", and 72°C for 30", and a final extension step of 7' at 72°C. The annealing temperatures and product sizes for each reaction are as followed: 64°C, 399bp for pDHFR; 60°C, 390bp for pTK1; 63°C, 413bp for pbmyb; °C, bp for pP19ARF; 59°C, 246bp for pP107; 57°C, 103bp for pE2F1; 53°C, 246bp for pE2F2; 56°C, 197bp for pE2F3a; 54°C, 254bp for pAdh5.

Quantification of ChIP PCR products. PCR products of expected molecular weight, as determined by agarose gel electrophoresis using GelStar® nucleic acid stain (Cambrex), were digitally photographed and analyzed by NIH Image v1.63, as previously described in chapter 2. Measurements of band intensities, using equal area measurements per band, was generated for each sample and surrounding background. For normalization, each sample-background measurement was subtracted from the foreground ($S = b-f$) then this difference obtained from the negative control (IgG) ($C = b-f$, should be 0) was subtracted from each sample ($S_{norm} = S-C$). To

control for each PCR reaction and gel electrophoresis, each normalized value was divided by the normalized value for its input sample, separate for non-transgenic (NTin) or TAg^{wt} (Tin) ($S' = S_{\text{norm}}/\text{NTin}_{\text{norm}}$) or ($S' = S_{\text{norm}}/\text{Tin}_{\text{norm}}$). To determine the relative sample intensity value (S_{RV}) for comparison of each PCR reaction, each value obtained after this previous control was divided by the value of that sample obtained for the previous control from the control reaction, Adh5 ($S_{\text{RV}} = S'_{\text{rxnX}}/S'_{\text{Adh5}}$). These values were graphed using the Excel software from Microsoft Office X for Macintosh.

4.3 RESULTS

4.3.1 T antigen alters specific Rb family member levels in intestinal villi

To initiate the investigation into how T antigen affects the Rb/E2F pathway in intestinal enterocytes, the steady state levels of the Rb family members were determined by immunoblot (Fig.12a). To determine the specificity of the antibodies, expression of these proteins was compared in wildtype (+/+) or pRb, p130 or p107 null (-/-) intestinal extracts (Fig.11a, compare lanes 1 and 2). The antibodies for pRb and p107 specifically detect the intended target proteins. The antibody for p130 detects two migrating species, one that is p130, as determined by its absence in the p130 null extract (indicated with an arrow) and another non-specific band, which migrates more slowly. The non-specific band is a cross-reactive species in the intestinal epithelium, but is not present in extracts from MEFs (data not shown). The villi contain pRb in both hypo and hyperphosphorylated forms. Abundant amounts of p130 are detected as well. However, enterocytes contain very low levels of p107. These data fit the current model for the

levels of pRb family proteins in growth-arrested cells. Expression of T antigen does not perturb the levels or phosphorylation status of pRb in intestinal villi. However, p107 levels are increased while p130 levels are decreased in *WT* T antigen expressing villi (Fig.12a, compare lanes 3 and 4). These data are also consistent with the current model as these cells are no longer growth arrested but are cycling. The increase in p107 is observed at the transcript level by semi-quantitative RTPCR. However, p130 transcript levels are consistently decreased in TAg^{wt} and TAg^{N136} villi (Fig.12b, compare lanes 2 and 3). Quantification of the down-regulation of p130 protein and transcript revealed a difference at a p value < 0.1 (Fig.12c). Although these statistical results are on the cusp of significance, these results may suggest that the down-regulation of p130 protein by expression of T antigen is predominantly post-transcriptional. As reported by the DeCaprio lab, T antigen can target p130 for degradation (Stubdal et al., 1997). Our data supports a hypothesis that T antigen is targeting p130 for degradation in villi enterocytes. To further investigate this hypothesis, several additional experiments could be performed. First, for a better quantification of the relative transcript levels of p130 in TAg^{wt}-transgenic villi as compared to non-transgenic villi, real-time RTPCR experiments could be performed. Additionally, a titration of protein extracts could be immunoblotted for a more quantitative comparison of p130 steady-state protein levels from villi extracts of TAg^{wt}-transgenic and non-transgenic mice. Furthermore, to test if p130 protein is targeted for proteasomal degradation in villus enterocytes of TAg^{wt}-transgenic mice, fresh protein extracts could be obtained from TAg^{wt}-transgenic mice with and without proteasome inhibitors. If p130 protein is stabilized, this result would indicate that p130 steady-state protein levels are down-regulated by T antigen through a proteasome-dependent effect. If p130 protein is not stabilized in the presence of proteasomal inhibitors, this may indicate that either p130 steady-state protein levels are down-regulated by T antigen through

degradation by other proteases or that the translation of p130 transcripts is inhibited by T antigen. Alternatively, p130 gene transcription may be down-regulated by T antigen, causing the subsequent down-regulation of steady-state protein levels.

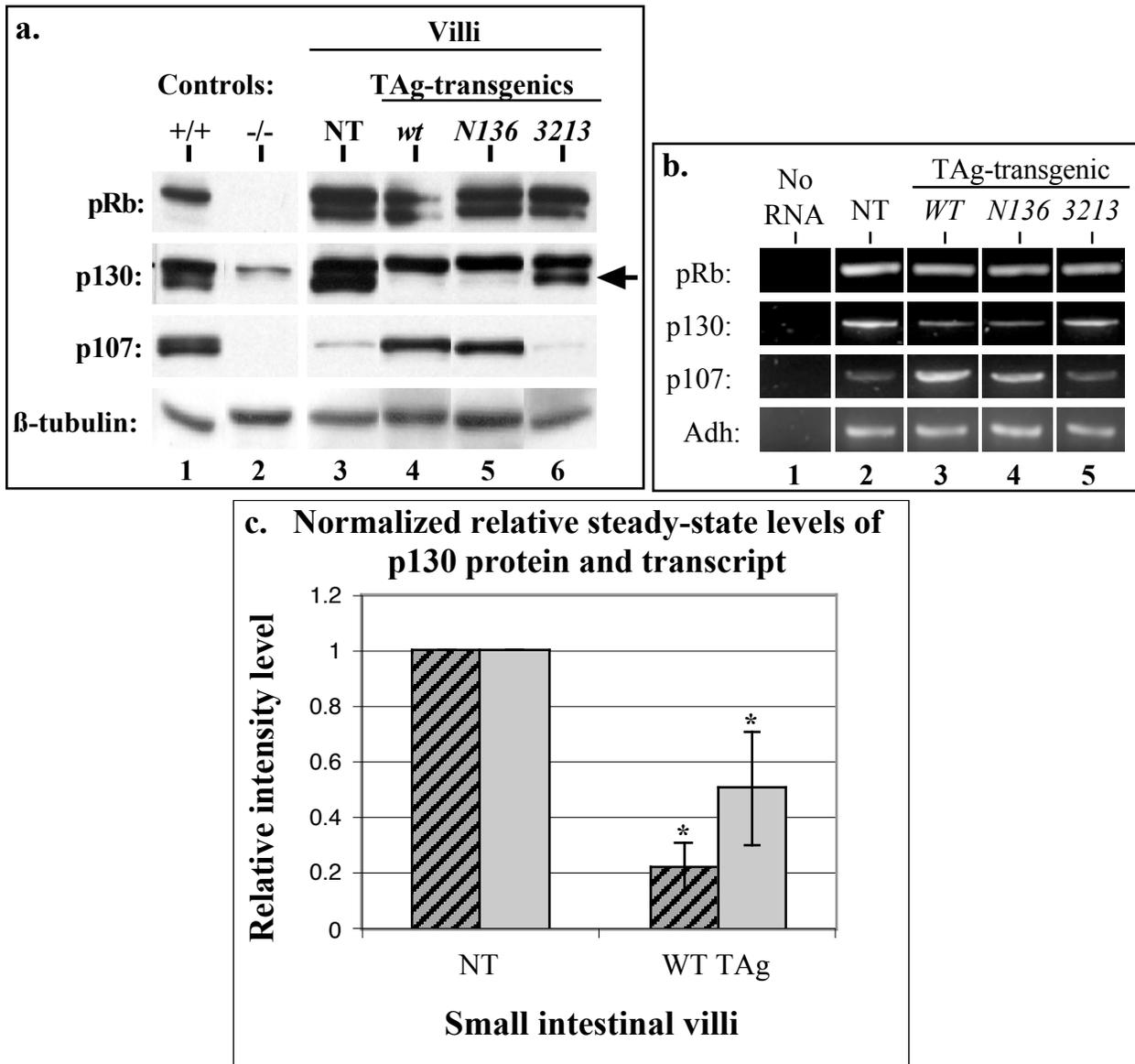


Figure 12. Expression of T antigen alters specific Rb family protein and transcript levels in villi, dependent on an intact LxCxE motif. a. Rb family steady-state protein levels in non-transgenic and TAg-transgenic villi samples. Equal concentrations of total soluble protein extracts from villi of non-transgenic and TAg-transgenic mice as well as wildtype (+/+) and knock-out (-/-) intestinal controls, as described in text, were

subjected to immunoblots for pRb, p130 and p107, as described in the materials and methods. Protein levels for β tubulin were determined as a loading control. **b. Rb family steady-state transcript levels in non-transgenic and TAg-transgenic villi samples.** Equal amounts of cDNAs reverse-transcribed from total RNA extracts from villi of non-transgenic and TAg-transgenic mice were subjected to 25 cycles of PCR using specific primers for pRb, p130 and p107, as described in materials and methods. Transcript levels of Adh5 were determined as a loading control. **c. Quantification and normalization of p130 steady-state protein and transcript levels in villi from non-transgenic and TAg^{wt} mice.** Quantification of protein and transcript levels were calculated by quantifying intensities of specific electrophoresed bands from immunoblot or RTPCR, normalized to the loading controls and relative to levels in non-transgenic villi, set to 1. Statistical analysis was performed using the student's t-test.  = protein levels.  = transcript levels. Error bars indicate \pm average deviations. * comparison of p130 protein and transcript levels in TAg^{wt}-transgenic villi, p value < 0.1.

4.3.2 T antigen binds all three Rb family members, dependent on an LxCxE motif, in intestinal villi.

Transgenic mice that express the T antigen mutant in the LxCxE motif, *3213*, do not show an intestinal phenotype. It is therefore hypothesized that T antigen binding to the Rb family via the LxCxE motif is required to induce hyperplasia. Alternatively, there could be a different cellular protein that T antigen interacts with through the LxCxE motif that is required for hyperplasia. To distinguish between these possibilities, immunoprecipitation experiments using antibodies specifically against T antigen were performed and co-precipitating Rb family members detected by immunoblot assay. As predicted, wild-type T antigen (*WT*) binds all three Rb family members in the intestinal epithelium, while *3213* does not (Fig.13, compare lanes 3 and 9). Since there is ten fold more *WT* T antigen than *3213* in the same amount of intestinal epithelial extract, immunoprecipitation for T antigen at normalized levels of *WT* and *3213* was performed. Again,

there was no evidence of co-immunoprecipitated Rb family members with 3213 (data not shown). These data indicate that T antigen interacts with the Rb family members through its LxCxE motif and that this interaction correlates to hyperplasia.

Mice expressing the amino-terminal truncation mutant, *N136*, in villus enterocytes develop hyperplasia. *N136* retains an intact LxCxE motif and is capable of disrupting Rb/E2F complexes as demonstrated *in vitro* (unpublished results, C.S. Sullivan, University of Pittsburgh, 2000). Therefore, it is not surprising that *N136* binds pRb in the intestinal epithelium (Fig.13, lane 6). In contrast, immunoprecipitation for *N136* did not result in detectable levels of co-immunoprecipitated p130 or p107 (Fig.13, lane 16). These results suggest that stable complex formation with p130 and p107 requires the remaining 582 amino acids of the carboxy half of T antigen. These results also suggest that T antigen binding to and action on pRb in villus enterocytes may be sufficient to cause intestinal hyperplasia. Alternatively, there may be other functions in the first 136 amino acids of T antigen that are also required to cause hyperplasia.

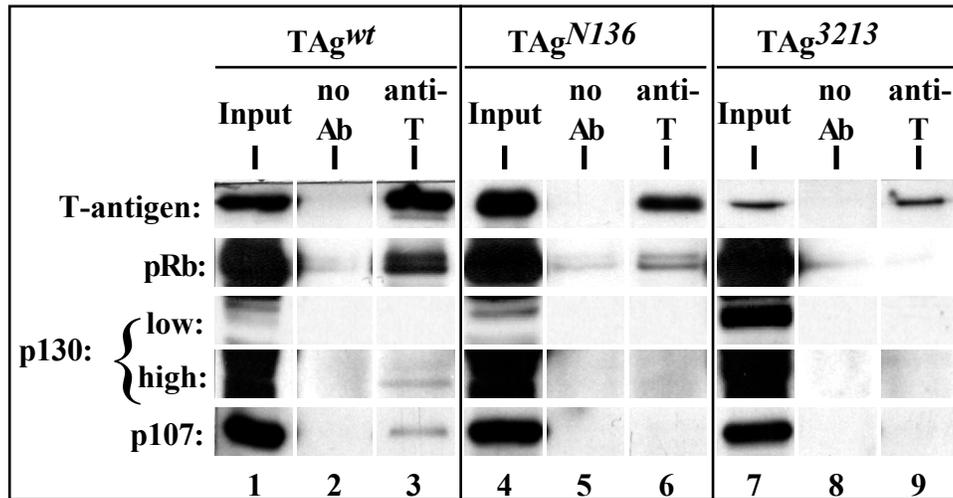


Figure 13. T antigen binds pRb, p107 and p130 in the intestinal epithelium of transgenic mice, dependent on an intact LxCxE motif. Immunoprecipitation for T antigen from equal concentrations of total intestinal epithelial cell extracts from TAg^{wt}, TAg^{N136} and TAg³²¹³ mice, immunoblotting for T antigen, pRb, p130

(at “low” and “high” exposures of the film) and p107, as described in materials and methods. Half the inputs were loaded.

4.3.3 T antigen disrupts p130/E2F and induces pRb/E2F DNA binding complexes in intestinal villi

To determine the activity of the Rb/E2Fs, DNA binding assays and chromatin immunoprecipitation experiments were performed on non-transgenic and TAg-transgenic villi extracts. *In vitro* binding assays indicate that each E2F binds the same consensus sequence with similar affinity (Chittenden et al., 1991; Ouellett et al., 1992). E2F1-5 DNA binding complexes contain at least one E2F and one DP (1 or 2), but can also contain pRb family members or larger complexes. To detect the E2F DNA binding activity, electrophoretic mobility shift assays were performed on extracts using a radiolabeled oligonucleotide probe containing E2F consensus sequences. Three migrating species (bands ‘c’, ‘g’ and ‘j’) are apparent in non-transgenic (NT) and TAg³²¹³-transgenic villi (Fig.14a, lanes 1 and 4). There are 8 migrating species (bands ‘a’, ‘b’, and ‘d’ through ‘i’) in TAg^{wt} and TAg^{N136}-transgenic villi (Fig.14a, lanes 2 and 3). Notably, there are only two co-migrating species between the NT and TAg^{wt} extracts, bands ‘g’ and ‘i’. Although equal amounts of extracts were loaded, these species are consistently more intense in TAg^{wt}-transgenic villi extracts compared to non-transgenic, while the other species vary slightly in intensity between different TAg^{wt}-transgenic villi extracts (Fig.14a, compare lanes 1 and 2).

The specificity of the E2F EMSA complexes was determined by competition experiments using unlabeled wildtype or single-site mutant probe. All bands described here were competed with 100 fold molar excess of unlabeled wildtype probe to labeled probe, but not competed with the same concentration of mutant E2F-site probe (Fig.14c). The identification of the different

complexes was determined by supershifting the migrating species using specific antibodies against different E2Fs and Rb family members. These species were confirmed by comparison to E2F over-expressing extracts or extracts null for various Rb and E2F family members. Antibody specificity was also determined by the ability to supershift complexes in wild-type extracts but not in extracts that are depleted for the specific E2F or Rb family member or an extract that over-expresses Rb and E2F family members other than the protein that the antibody recognizes.

The model of T antigen action on Rb/E2F complexes predicts the presence of p130/E2F4 DNA binding complexes in the growth-arrested cells of the non-transgenic villi and their absence in TAg^{wt}-transgenic villi. In addition, the model predicts that the cycling cells in TAg^{wt}-transgenic villi contain E2F1, E2F2, and E2F3a DNA binding complexes free of pRb. Band 'c' in non-transgenic villi extracts supershifts with antibodies against p130 (Fig.14b, compare lanes 1 and 4). Although this same migrating species, 'c', is present at low levels in the p130 null intestinal extract, antibodies against p130 fail to supershift this or any other species in this extract, as expected, demonstrating antibody specificity (Fig.14c, compare lanes 6 and 7). TAg^{wt}-transgenic villi extracts do not contain this p130/E2F DNA binding activity (Fig.14b, compare lanes 5 and 8). This result is consistent with the immunoblot result that shows a decrease in p130 protein levels in these extracts.

In contrast, TAg^{wt}-transgenic villi extracts contain band 'a', which supershifts with antibodies against p107 (Fig.14b, compare lanes 5 and 7). Villi extracts from non-transgenic mice do not contain a co-migrating species 'a'. However, antibodies against p107 supershift a portion of band 'c' in non-transgenic villi (Fig.14b, compare lanes 1 and 3). This activity may be due to the presence of some p107/E2F DNA binding activity or cross-reactivity of the antibody. The antibody is somewhat cross-reactive as it supershifts a small amount of band 'c' in p107 null

intestinal extracts (Fig.14b, compare lanes 9 and 11). These results indicate that T antigen up-regulates a p107/E2F DNA binding complex in villi enterocytes that is different than the p107/E2F DNA binding complex in non-transgenic villi enterocytes.

TAg^{wl}-transgenic villi extracts contain E2F EMSA band 'e', which is not present in non-transgenic villi extracts, that supershifts with antibodies against pRb (Fig.14b, compare lanes 5 and 6). pRb null intestinal extracts do not contain the co-migrating species, 'e', and antibodies against pRb do not supershift any species in pRb null intestinal extracts (Fig.14b, compare lanes 4 and 5). However, species 'e' is not present in the wildtype pRb extract, either (Fig.14b, lane 3). Expression of pRb/E2F1/DP1 via baculovirus vectors in SF9 extracts subjected to EMSA resulted in two migrating species, including band 'e'. This band supershifted with antibodies against pRb (Fig.14b, compare lanes 1 and 2). These results indicate that T antigen expression induces a pRb/E2F DNA binding activity in villus enterocytes.

It is also important to note that antibodies against all of the three Rb family members were unable to supershift E2F EMSA complexes 'b', 'd', and 'f'-'i', indicating that these complexes are all Rb-free E2F binding complexes (Fig.14b). Alternatively, Rb family members may be bound in these complexes, but the antibody cannot supershift them due to epitope inaccessibility. However, the faster migrating species, 'g'-'i', are not likely to contain Rb family members as they co-migrate with over-expressing E2F DNA binding complexes that are Rb-free (Fig.14d, lane 1 and 2; Fig.17a, lane 8 and 11; data not shown).

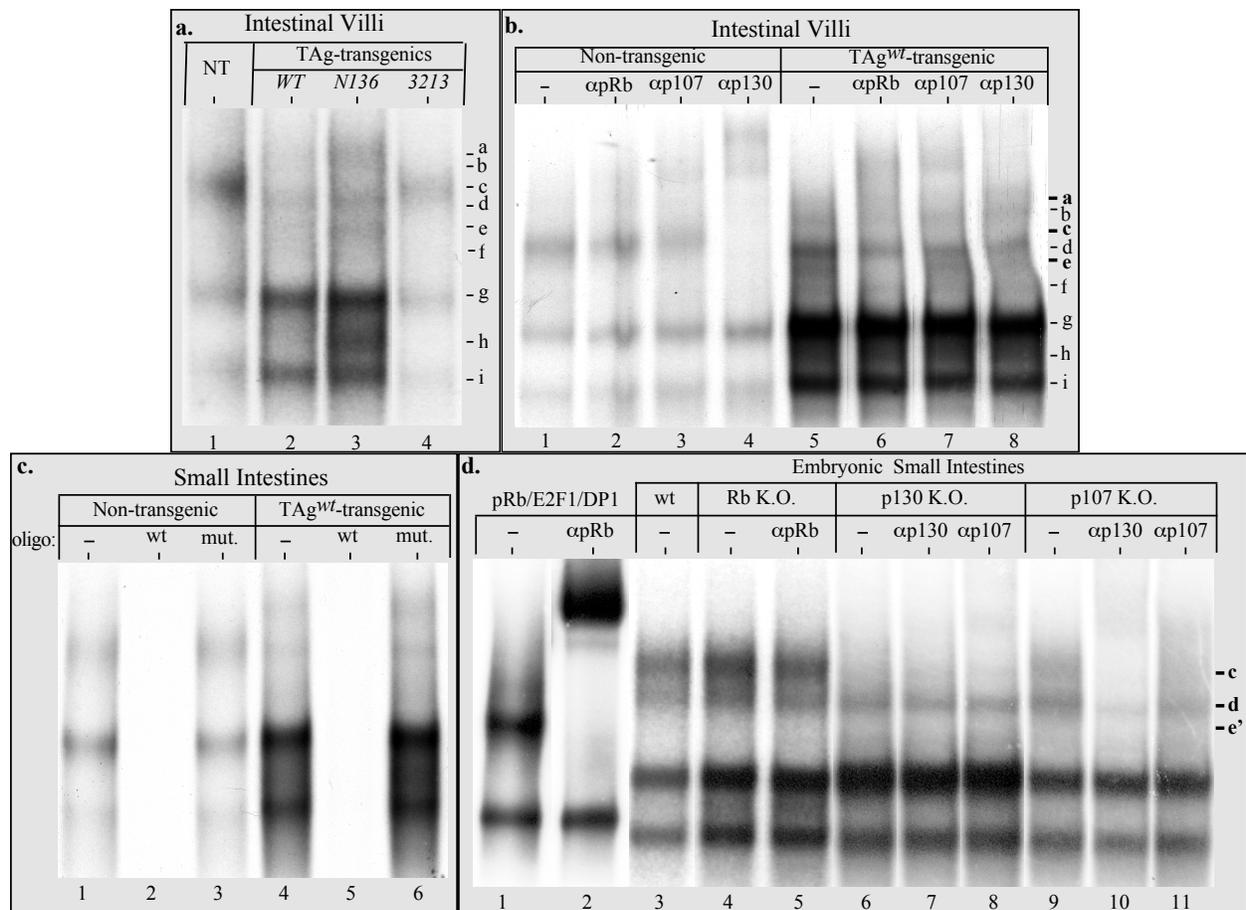


Figure 14. T antigen disrupts p130/E2F and induces pRb/E2F DNA binding complexes in villi, dependent on an intact LxCxE motif. a. E2F DNA binding complexes in non-transgenic and TAG-transgenic villi. Equal concentrations of total soluble protein extracts from villi of non-transgenic (NT) and TAG-transgenic mice were subjected to E2F EMSA analysis as described in materials and methods. NT and TAG³²¹³ intestinal villi contain species ‘c’, ‘g’ and ‘i’. TAG^{wt} and TAG^{N136} intestinal villi contain species ‘a’, ‘b’, ‘d’, ‘e’, ‘f’, ‘g’, ‘h’, and ‘i’. **b. Pocket protein/E2F DNA binding complexes in non-transgenic and TAG-transgenic villi.** Equal concentrations of total soluble protein extracts from villi of NT and TAG^{wt} mice were subjected to E2F EMSA analysis with antibody supershifts as described in materials and methods. Species ‘c’ supershifts with antibodies to p130 in NT villi. Species ‘a’ supershifts with antibodies against p107 and species ‘e’ supershifts with antibodies against pRb in TAG^{wt} villi. **c. Competition experiments reveal the specificity of E2F DNA binding complexes in E2F EMSAs.** Equal concentrations of total soluble protein extracts from NT and TAG^{wt} mouse small intestines were subjected

to E2F EMSA analysis with 100-fold excess unlabeled oligonucleotide competitors as described in materials and methods. All species are competed when wildtype (wt) competitor is added, while no species are competed when mutant (mt) competitor is added in both NT and TAg^{wt} extracts. **d. Rb family antibody supershift controls reveal antibody specificity.** 10µg of triple-infected baculovirus-pRb, -E2F1 and -DP1 over-expressing SF9 lysates and 30µg of wildtype (wt) and Rb family knock-out E18.5 embryonic intestinal extracts were subjected to E2F EMSA analysis with antibody supershifts as described in materials and methods. Species 'c' supershifts with antibodies against p130 and p107, depending on the extract, while species 'd' also supershifts with antibodies against p130 and species 'e' supershifts with antibodies against pRb.

4.3.4 T antigen decreases the number of post-translational forms of E2F4 and increases its nuclear localization in villus enterocytes

Each DNA binding complex detected by E2F EMSA contains an E2F/DP heterodimer. E2F is the limiting factor that determines the DNA binding activity of E2F/DP since DPs are expressed at high levels throughout the cell cycle. Therefore, to characterize the E2Fs driving these activities, E2F levels were determined by immunoblot in villi extracts from non-transgenic and TAg-transgenic mice (Fig.15). The specificity of the antibodies for each E2F was determined by comparison of wildtype extracts to extracts over-expressing E2F or from E2F null MEFs (Fig.15a, compare lanes 1 and 2). Nuclear extracts from S phase synchronized wildtype and E2F1 or E2F3 null MEFs were used as controls for E2F1 and E2F3. 293 kidney cells infected with an adenovirus vector that expresses E2F2, or mock infected 293 cells were used as controls for E2F2. E2F4^{+/-} or E2F4^{-/-} neonatal mouse colon extracts were used as controls for E2F4.

These results indicate that the migrating species recognized by the antibodies are specific to their appropriate E2F.

Immunoblot experiments showed that non-transgenic villi contain four specific migrating species of E2F4 'a'-'d' (Fig.15b, lane 1). Species 'b'-'d' collapsed to a faster migrating species 'e' when these extracts were treated with alkaline phosphatase (Fig.15c, compare lanes 1 and 2). No changes in the migration of these bands were seen when extracts were treated with the serine/threonine-specific phosphatase, PP2A (Fig.15c, compare lanes 1 and 3). This indicates that these species 'b', 'c', and 'd' are different phosphorylated forms of E2F4, while species 'a' is most-likely another post-translationally modified form of E2F4. TAg^{wt}-transgenic villi extracts contain two E2F4-specific species that co-migrate with 'c' and 'd' (Fig.15a, compare lanes 3 and 4; Fig.14c, compare lanes 1 and 4; Fig.14d, compare lanes 1 and 4). These two species also collapse to species 'e' when the extract is treated with alkaline phosphatase but not PP2A (Fig.15c, compare lanes 4, 5 and 6). The overall levels of E2F4 do not appear to change between non-transgenic and TAg^{wt}-transgenic villi extracts. These results indicate that T antigen expression alters the phosphorylated forms of E2F4 in villus enterocytes.

The significance of the alteration of E2F4 phosphorylated forms is unclear. However, reports indicate that the phosphorylation of E2F4 affects its nuclear/cytoplasmic localization (Olgiate et al., 1999; Deschênes et al., 2004). To determine if T antigen alters E2F4 subcellular localization, subcellular fractions of villi cells from non-transgenic and TAg^{wt}-transgenic mice were analyzed for E2F4 levels by immunoblot (Fig.15c). The nuclear and cytoplasmic fractions were first characterized by co-localization of either histone H3, a nuclear marker, or β tubulin, a cytoplasmic marker. While the whole cell extracts (W.C.E) contain both β tubulin and histone H3, the nuclear extracts (N.E.) are absent of β tubulin and contain high levels of histone H3,

indicating the enrichment in nuclear proteins (Fig.15c, compare lanes 1 and 3, 4 and 6). The cytoplasmic (cyto.) fraction contains both β tubulin and histone H3, suggesting that either some histone H3 is localized in the cytoplasm or that the cytoplasmic fraction contains contaminating nuclear proteins (Fig.15c, compare lanes 1 and 2, 4 and 5). All four migrating species in non-transgenic villi localize to both the cytoplasmic and nuclear fractions, although species 'c' is enriched in the nuclear extract (Fig.15c, compare lanes 2 and 3). The two E2F4 species, 'c' and 'd', in TAg^{wf}-transgenic villi are enriched in the nuclear extract (Fig.15c, compare lanes 5 and 6). These results indicate that T antigen expression causes an increase of E2F4 in the nucleus of villus enterocytes.

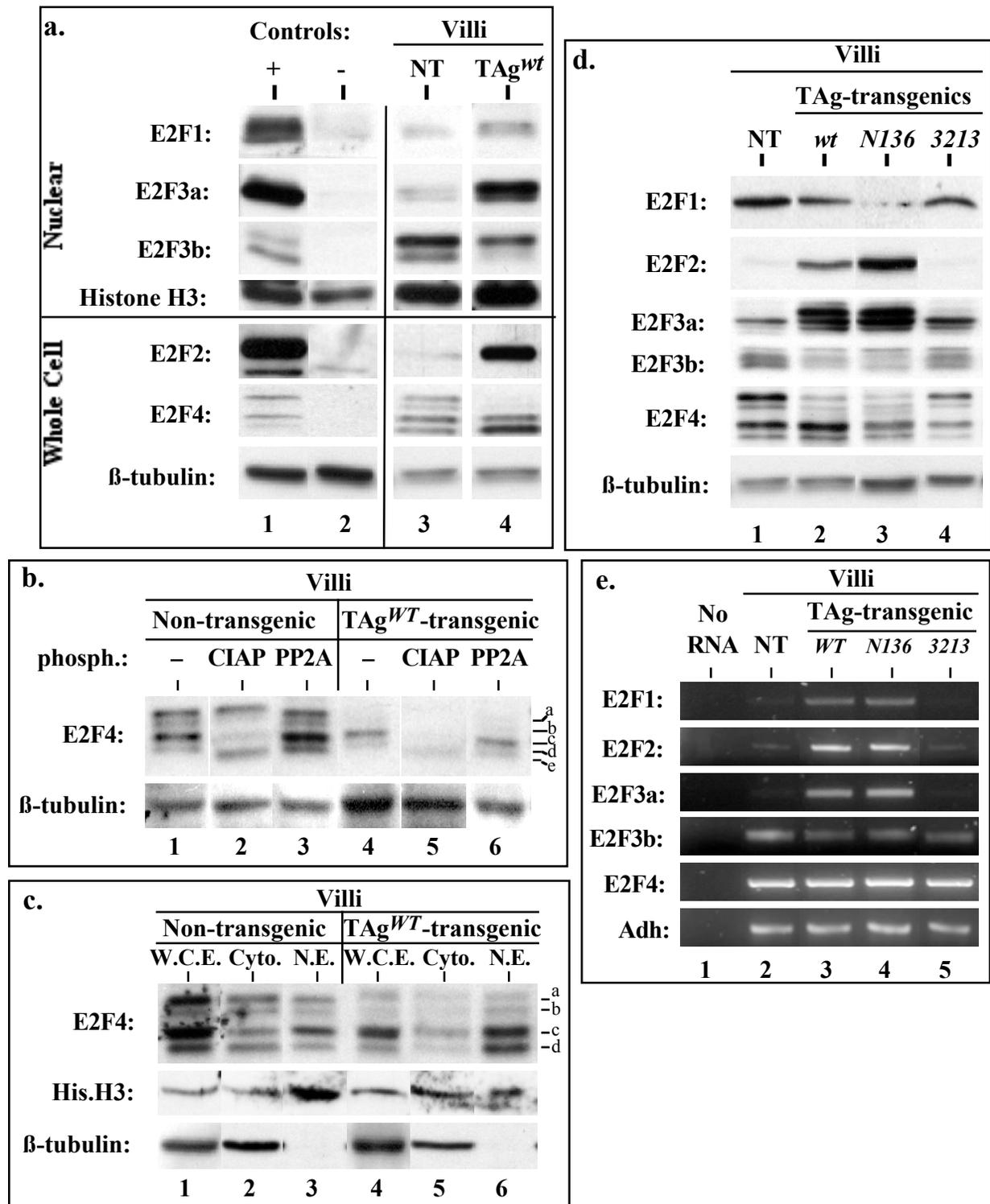


Figure 15. Expression of T antigen alters specific E2F protein and transcript levels, alters E2F4 post-translational forms and up-regulates E2F4 nuclear localization. a. E2F1-4 steady-state protein levels in non-transgenic and TAg^{WT} intestinal villi. 30μg of whole cell extracts or nuclear extracts from villi of non-transgenic or

TAg^{wt} mice were subjected to immunoblot analysis. The controls for E2F1 were nuclear extracts from S phase synchronized wild type MEFs (+) or E2F1^{-/-} MEFs (-). The controls for E2F3 were nuclear extracts from S phase synchronized wild type MEFs (+) or E2F3^{-/-} MEFs (-). Histone H3 was immunoblotted as a loading control for the nuclear extracts. Controls for E2F2 were 15µg of whole cell extracts from E2F2 over-expressing 293 cells (+) and non-overexpressing 293 cells (-). The controls for E2F4 were colon extracts from E2F4^{+/-} mice (+) or colon extracts from E2F4^{-/-} mice (-). **b. Phosphatase treatment of E2F4 in non-transgenic and TAg^{wt} intestinal villi.** 30µg of calf-intestinal alkaline phosphatase (CIP) or PP2A treated villi extracts from non-transgenic or TAg^{wt} mice were subjected to immunoblot analysis. β tubulin was probed as a loading control. There are four E2F4-reactive species ('a'-'d'). 'b'-'d' collapse to species 'e' when treated with CIP, but not PP2A. **c. Relative nuclear and cytoplasmic localization of E2F4 in non-transgenic and TAg^{wt} intestinal villi.** 30µg of whole cell extracts, cytoplasmic or nuclear extracts from villi of non-transgenic or TAg^{wt} mice were subjected to immunoblot analysis and probed for E2F4. Histone H3 was probed as a loading control for nuclear extracts while β-tubulin was probed as a loading control for cytoplasmic extracts. **d. E2F1-4 steady-state protein levels in non-transgenic and TAg-transgenic intestinal villi.** 30µg of whole cell extracts from villi of non-transgenic, TAg^{wt}, TAg^{N136} and TAg³²¹³ mice were subjected to immunoblot analysis for E2F1-4. β tubulin was probed as a loading control. **e. E2F1-4 steady-state transcript levels in non-transgenic and TAg-transgenic intestinal villi.** RNA from villi of non-transgenic, TAg^{wt}, TAg^{N136} and TAg³²¹³ mice was subjected to RTPCR analysis using primers specific for E2F1-4 transcripts. The products were of the appropriate molecular weights and within exponential range of 25 cycles of PCR.

4.3.5. T antigen up-regulates E2F2 and E2F3a expression in villi enterocytes.

E2F1, E2F2 and E2F3 are expressed in the normal intestinal epithelium. The steady-state levels of E2F2 and E2F3a are greater in the crypts than the villi, whereas E2F1 and E2F3b levels are equal in both fractions (data not shown). The levels of E2F2 and E2F3a are low in non-transgenic villi while E2F3b levels are relatively high (Fig.15a, lane 3). T antigen causes an increase in E2F2 and E2F3a steady-state levels in the villi, and a moderate reduction in E2F3b levels (Fig.15a, compare lanes 3 and 4). E2F1 levels vary between different villi extracts

prepared from TAg^{wt}-transgenic mice, indicating either no change in E2F1 levels or a slight increase as compared to non-transgenic villi (Fig.15a, compare lanes 3 and 4; Fig.15d, compare lanes 1 and 2). Increases in E2F1, E2F2 and E2F3a are detected at the transcript level, although the decrease in E2F3b is not. There is no change in E2F4 mRNA levels (Fig.15e, compare lanes 2 and 3). *N136* expression causes a similar increase in E2F2 and E2F3a protein and transcript levels, whereas *3213* expression does not, (Fig.15d and e). These results indicate that T antigen interaction with the pRb family is required and the first 136 amino acids are sufficient to cause these effects on E2Fs 1-3a levels and on E2F4 post-translational modifications.

4.3.6. T antigen up-regulates Rb-free E2F4 and E2F5 DNA binding activity in intestinal villi.

Next, E2F EMSAs were performed using antibodies against different E2Fs to supershift the DNA binding complexes. Since p130 preferentially binds E2F4 and E2F5, it is predicted that p130 is bound to the E2F probe via E2F4 or E2F5 (Hijmans et al., 1995; Vairo et al., 1995). Therefore, to identify the E2F with which p130 is complexed in the E2F EMSA, antibodies against E2F4 and E2F5 were used for supershift analysis. The specificity of these antibodies was determined by comparing the E2F EMSA pattern from an E2F4 null colon extract to TAg^{wt}-transgenic villi extract, supershifted for E2F4, or the E2F EMSA pattern from an E2F5 null small intestinal extract to the TAg^{wt}-transgenic villi extract, supershifted for E2F5 (Fig.16a, compare lanes 1 and 4, 2 and 5). These data confirm that the supershifted band is absent in the respective null extract. The antibodies do not cross-react with other E2Fs as indicated by their not supershifting other characterized bands, and by the inability of the antibody against E2F4 to

supershift overexpressing E2F2 or E2F1 extracts (Fig.16b). These results indicate that the antibodies used are specific for their respective proteins in EMSA experiments.

The addition of antibodies against E2F4 or E2F5 did not supershift the p130-containing band 'c' in non-transgenic villi, although they did supershift bands 'g' and 'j', respectively (Fig.16c, compare lanes 1 and 2, 1 and 3). It remains possible that band 'c' contains E2F4 or E2F5, but cannot be supershifted due to epitope inaccessibility when bound to p130 and DNA. Bands 'g' and 'j' also supershift with antibodies to E2F4 and E2F5, respectively, in TAg^{wt} villi extracts (Fig.16c, compare lanes 4 and 5, 4 and 6). In addition, antibodies to E2F4 supershift band 'd' in the TAg^{wt} villi extract (Fig.16c, lane 5). In summary, Rb-free E2F4 and E2F5 DNA binding complexes are present in both villi extracts, increasing in levels in TAg^{wt}-transgenic villi. These data support the hypothesis that T antigen disrupts p130/E2F DNA binding complexes and increases Rb-free DNA binding complexes in the intestine, dependent on interaction with the pRb family.

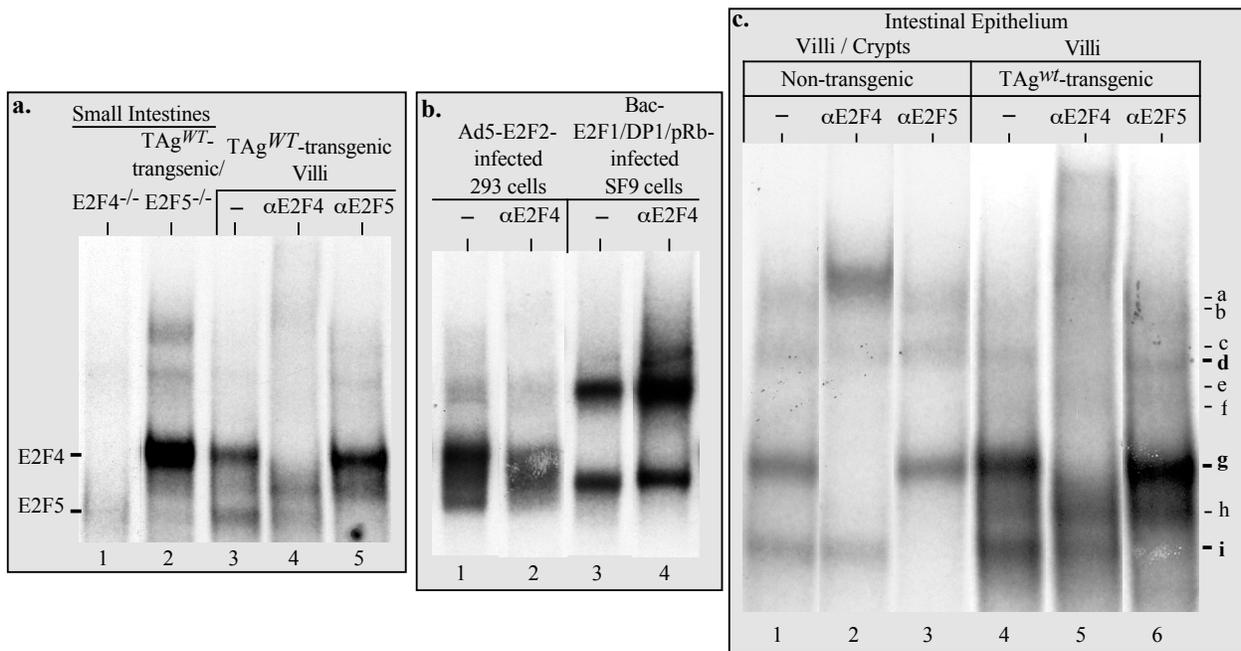


Figure 16. T antigen expression up-regulates E2F4 and E2F5 DNA binding activity. a. Confirmation of E2F4 and E2F5 DNA binding complexes in E2F EMSAs. 30µg of whole cell extracts from TAg^{wt} villi or control intestinal samples were subjected to E2F EMSA analysis. 1.6µg of E2F4 (A-20) and E2F5 (H-111) antibodies were incubated with the samples for supershift analysis. **b. E2F4 antibody specificity.** 15µg of E2F2 or E2F1 over-expressing extracts were subjected to E2F EMSA and supershift with antibodies against E2F4 (A-20). These antibodies do not supershift E2F2 or E2F1 DNA binding complexes, demonstrating the antibody as not cross-reactive. **c. E2F4 and E2F5 DNA binding activity in non-transgenic and TAg^{wt} intestinal villi.** 30µg of whole cell extracts from non-transgenic or TAg^{wt} villi were subjected to E2F EMSA analysis with E2F4 and E2F5 antibody supershifts. Antibodies to E2F4 supershifts species 'g' in both extracts, while antibodies to E2F5 supershifts species 'i' in both extracts. In addition, Antibodies to E2F4 supershift species 'd' in TAg^{wt} villi extracts. α = antibody.

4.3.7. T antigen induces E2F2 and E2F3a DNA binding activity in intestinal villi.

In addition to up-regulating Rb-free E2F4 and E2F5 DNA binding activity, villi extracts from TAg^{wt}-transgenic mice contain several other E2F DNA binding complexes that are absent in non-transgenic villi. These include bands 'b', 'e', 'f' and 'h'. To identify the E2Fs in these complexes, E2F EMSAs were performed using antibodies against E2F1, E2F2 and E2F3 (Fig.17). The specificity of the antibodies for E2F1 and E2F2 were tested by supershifting appropriate species in over-expressing extracts (Fig.17a, compare lanes 8 and 11 or compare lanes 5 and 7, respectively). The specificity of the antibody for E2F3 was demonstrated by supershifting species in a wildtype extract, but not in E2F3 null extracts (Fig.17b). The cross-reactivities of the antibodies were analyzed by attempting to supershift species in mock or E2F over-expressed extract. For instance, antibodies against E2F2 do not supershift any species in mock-infected 293 extracts or E2F1-over-expressing extracts (Fig.17a, compare lanes 1 and 4, or compare lanes 8 and 10). These data indicate that these antibodies are specific for their

appropriate proteins. It is also important to note that two antibodies were used to detect E2F3, one that can detect both E2F3a and E2F3b and one that detects only E2F3a. The antibody for E2F3a is known to have a lower affinity (Aslanian et al., 2004). Using both antibodies, a species that co-migrates with the E2F2 and E2F1 super-shifting species is supershifted in mock-infected 293 cell extracts and therefore is labeled 'E2F3a' (Fig.17a, compare lanes 1, 2 and 3). The antibody against both forms also supershifts two species in E2F1 null MEFs, a co-migrating species as before, 'E2F3a', and a faster migrating species, labeled 'E2F3b' (Fig.17b, compare lanes 1 and 2). As both species are absent in E2F3 null MEFs, they are both specific to E2F3 and their migration corresponds to previous reports (Gaubatz et al., 2000). Together, these pieces of evidence suggest that the components of these complexes have been correctly identified.

No species were supershifted with E2F1 antibodies in non-transgenic or TAg^{wt}-transgenic intestinal extracts, even though the same antibodies were able to supershift the control (Fig.17c). Antibodies against E2F2 supershifted species 'e' and 'h' in both TAg^{wt} and TAg^{N136} villi extracts, but not in TAg³²¹³ or non-transgenic villi extracts (Fig.17d, compare lanes 1 and 2, 5 and 6; data not shown). Since species 'e' also supershifted with antibodies to pRb, this complex contains pRb and E2F2. Antibodies against the pRb family members fail to supershift band 'h', therefore it is a pRb-free E2F2 DNA binding complex. Notably, another band remains after supershifting for E2F2 that co-migrated with band 'h', labeled 'h''. This band is obscured before supershifting for E2F2. After supershifting species 'h' with antibodies to E2F2 in TAg^{N136}-transgenic villi extracts, the addition of antibodies against E2F3 supershifted the species 'h'', (Fig. 17d, compare lanes 6 and 7). Nothing supershifts in non-transgenic villi extracts using an E2F3a-specific antibody. However, the E2F3a-specific antibody does supershift band 'h'' in TAg^{wt} and TAg^{N136}-transgenic villi extracts (data not shown). The migration of this species and

supershift data suggest that species 'h' contains pRb-free E2F2 and species 'h'' contains pRb-free E2F3a DNA binding complexes. Addition of antibodies against E2F3 also supershifted an elusive species 'j' in non-transgenic villi extracts (Fig.17d, compare lanes 1 and 3, 4). This species 'j' is not readily identifiable in all E2F EMSAs, however it co-migrates with the E2F3b species, suggesting that non-transgenic villi contain a pRb-free E2F3b DNA binding complex. In conclusion, T antigen expression causes the induction of pRb/E2F2, pRb-free E2F2 and pRb-free E2F3a DNA binding activity. Again, these effects by T antigen require interaction with the pRb family and the first 136 amino acids are sufficient (Fig.17; data not shown).

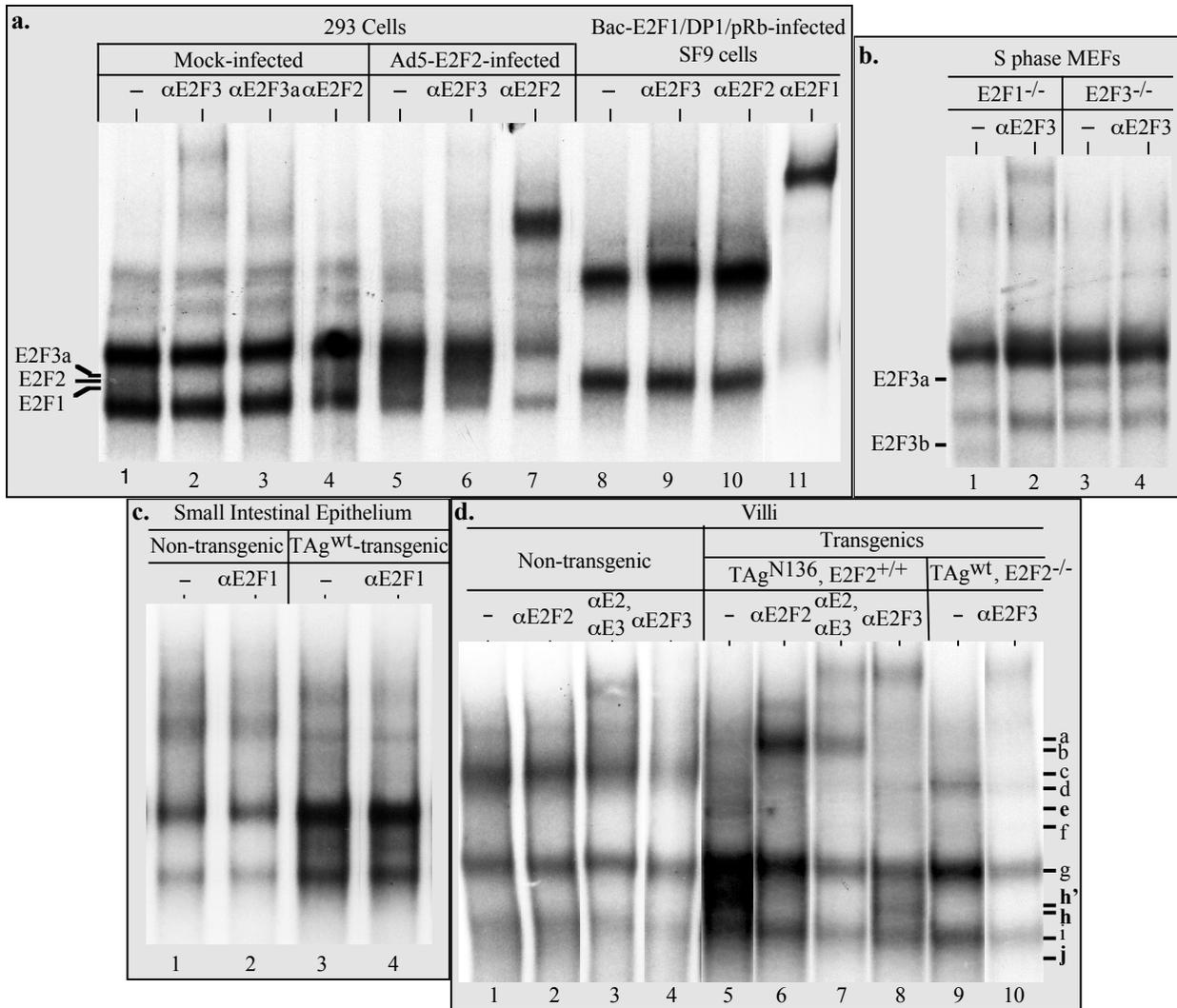


Figure 17. T antigen expression induced E2F2 and E2F3a DNA binding activity in intestinal villi. a. E2F1, 2, 3a and 3 antibody specificities. 15µg of over-expressing E2F2, E2F1 or mock extracts were subjected to E2F EMSA analysis and E2F1, E2F2, and E2F3 antibody supershifts. Rb-free complexes of all three E2Fs run in a similar region. However, the antibodies do not cross react. **b. E2F3 antibody specificity showing migration of E2F3a and E2F3b DNA binding complexes.** 30µg of nuclear extracts from S phase synchronized E2F1^{-/-} or E2F3^{-/-} MEFs were subjected to E2F EMSA. The migration of E2F3a and E2F3b in E2F1^{-/-} MEFs coincides with the reported migration of these species. Antibodies against E2F3 fail to supershift any species in the E2F3^{-/-} MEFs, demonstrating their specificity. **c. Neither non-transgenic or TAg^{wt} intestinal epithelia contain detectable E2F1 DNA binding complexes.** Although the same antibody against E2F1 (KH95) supershifts E2F1 in Fig.17a, lane 11, this same antibody fails to supershift any species in epithelial extracts from non-transgenic or TAg^{wt} mice. **d. E2F2**

and E2F3a DNA binding complexes are induced in TAg^{wt} and TAg^{N136} intestinal villi. Antibodies against E2F2 (TFE25) and E2F3 (C-18) were used to supershift E2F DNA binding species in villi extracts from non-transgenic, TAg^{N136}, TAg^{wt} (data not shown), and TAg^{wt};E2F2^{-/-} mice. Antibodies against E2F2 fail to supershift any species in non-transgenic or TAg^{wt};E2F2^{-/-} villi (Fig.23c, data not shown). However, antibodies against E2F2 supershifts species 'h' in TAg^{wt} and TAg^{N136} villi extracts. In addition, E2F3 supershifts the co-migrating species, 'h', in TAg^{wt} and TAg^{N136} villi extracts. Antibodies to E2F3 supershift species 'j' in non-transgenic villi extracts. α = antibody.

4.3.8. T antigen up-regulates E2F-responsive gene transcripts in the intestinal villi.

The chaperone model of T antigen action on Rb/E2F complexes states that when T antigen binds Rb family members, it disrupts Rb/E2F complexes, freeing E2F to transcribe S phase genes (Sullivan et al., 2000; Imperiale, 2001). Therefore, this model predicts that in villus enterocytes T antigen should cause the up-regulation of E2F responsive gene transcription, and that this up-regulation should be dependent on T antigen binding to the pRb family members through the LxCxE motif. To determine the state of the E2F-responsive gene transcription, several well-characterized E2F-responsive genes were chosen for analysis of the steady-state levels of their mRNAs via RTPCR. These genes included: *thymidine kinase 1* (TK1), *dihydrofolate reductase* (DHFR), *cyclin E*, *b myb*, and *p19ARF*. Each transcript is up-regulated in *WT* T antigen and *N136* expressing villi extracts, but not in *3213* expressing villi extracts (Fig.18a). The study was expanded by using a mouse cDNA microarray to compare relative changes in global transcript levels between non-transgenic and TAg^{wt}-transgenic villi (data not shown). 32 out of 38 stringently characterized E2F-regulated genes found on the microarray platform are up-regulated in TAg^{wt}-transgenic villi (Table 2; P.G. Cantalupo et al., unpublished data). These results indicate that E2F responsive genes are upregulated in TAg-transgenic villi, dependent on T

antigen interaction with the pRb family. Furthermore, expression of the first 136 amino acids of T antigen is sufficient for this up-regulation.

p19ARF steady-state transcript levels are up-regulated in TAg^{wt} or TAg^{N136}-transgenic villi (Fig.18a). However, p19ARF protein levels are below detection in TAg^{wt} or TAg^{N136}-transgenic villi, although p19ARF is present in TAg^{wt}-transformed MEFs (Fig.18b). These data suggest that the first 136 amino acids of T antigen are capable of post-transcriptional regulation of p19ARF. As previously demonstrated in chapter 3, p21 is induced by *WT* T antigen expression in villi enterocytes. Here, I show that *N136* expression also induces p21 expression, but *3213* expression does not (Fig.18b). These data suggest that expression of the first 136 amino acids of T antigen is sufficient to induce p21 expression. Furthermore, p21 up-regulation by T antigen is dependent on Rb binding.

Table 2. T antigen regulation of E2F-responsive genes in villi by microarray analysis. Genes were characterized as E2F-regulated by cluster analysis of results from nine independent reports of microarray analyses determining E2F-regulated genes (Ishida et al., 2001; Müller et al., 2001; DeGregori, 2002; Ren et al., 2002; Stevaux and Dyson, 2002b; Wells et al., 2002; Black et al., 2003; Polager and Ginsberg, 2003; Young et al., 2003). From this cluster analysis, 51 genes were identified, 38 of which were present on the microarray platform used in these studies (unpublished data, P.G. Cantalupo, University of Pittsburgh). Of these 38, 32 sequences had p values of confidence at $p < 0.05$ in either microarrays comparing villi fractionated as described in Materials and Methods and/or enterocytes isolated from villi by laser capture microscopy (LCM) (unpublished data, W.H. Whitehead, Vanderbilt University). Average ratios of four biological replicates indicate fold changes in TAG^{wf}-transgenic villi as compared to non-transgenic villi. All interpretable E2F-responsive transcripts are up-regulated by T antigen. [Values marked in red have low confidence with $p \geq 0.05$.]

Name	Accession No.	Villi		Villi-LCM	
		Avg. Ratio	P value	Avg. Ratio	P value
DNA primase, p49 subunit	AI324469	7.85	0.0031	14.98	0.0211
uracil-DNA glycosylase	W83782	7.15	0.0111	12.40	0.0275
public domain EST	AA636924	6.90	0.0006	7.31	0.0083
MCM5	AA162741	6.84	0.0137	10.97	0.0251
thymidine kinase 1 (TK1)	AA545691	6.31	0.0007	11.35	0.0119
cdc2 homolog A (<i>S. pombe</i>)	AW210382	6.29	0.0063	12.33	0.0097
DNA ligase I, ATP-dep.	AA684487	6.20	0.0010	4.67	0.0006
Ki67	AA671317	5.92	0.0036	13.78	0.0290
MCM4	AW210222	5.60	0.0040	8.78	0.0338
MCM7	W54667	5.48	0.0004	7.27	0.0329
DNA topoisomerase II α	AA063958	5.43	0.0027	9.51	0.0274
MCM	AI645415	5.29	0.0003	8.13	0.0386
cyclin E	AA437868	4.39	0.0026	7.94	0.0033
cyclin A2	AA469716	4.36	0.0006	4.51	0.0403
MCM2	AA792000	3.81	0.0005	4.68	0.0134
flap structure specific endonuclease 1	A89311	3.77	0.0017	4.14	0.0482
serine/threonine kinase 5	AA470284	3.73	0.0039	8.48	0.0172
H2A histone family, member z	AA606684	3.34	0.0021	4.42	0.0158
activator of S phase kinase	AA067144	3.07	0.0024	4.86	0.0001
DNA polymerase α 2, 68kDa subunit	AA072666	3.06	0.0006	2.20	0.0374
EZH2 homolog (<i>Drosophila</i>)	AA445767	3.04	0.0005	4.20	0.0169
DP1 (E2F dimerization partner)	AA517509	2.68	0.0025	2.55	0.0047
p107 (Rb family member)	AA738977	2.64	0.0043	3.05	0.0110
cyclin B1, related sequence 1	AA450523	2.25	0.0051	5.05	0.0038
cyclin B2	AA671428	2.14	0.0399	5.63	0.0118
MCM6	AA016759	5.92	0.0036	-	-
leukemia-associated gene	AA265396	4.99	0.0106	9.46	0.0695
thymidylate synthase	AI323028	4.87	0.0019	10.08	0.0673
cdc25 homolog A (<i>S. cerevisiae</i>)	AA064214	1.36	0.0141	1.50	0.0526
RIKEN cDNA 5031412I06 gene	AA671302	2.30	0.0653	2.47	0.0444
kinesin family member C1	AA003218	1.50	0.6701	9.90	0.0109
Rad51 homolog (<i>S. cerevisiae</i>)	AI592821	-	-	14.29	0.0193

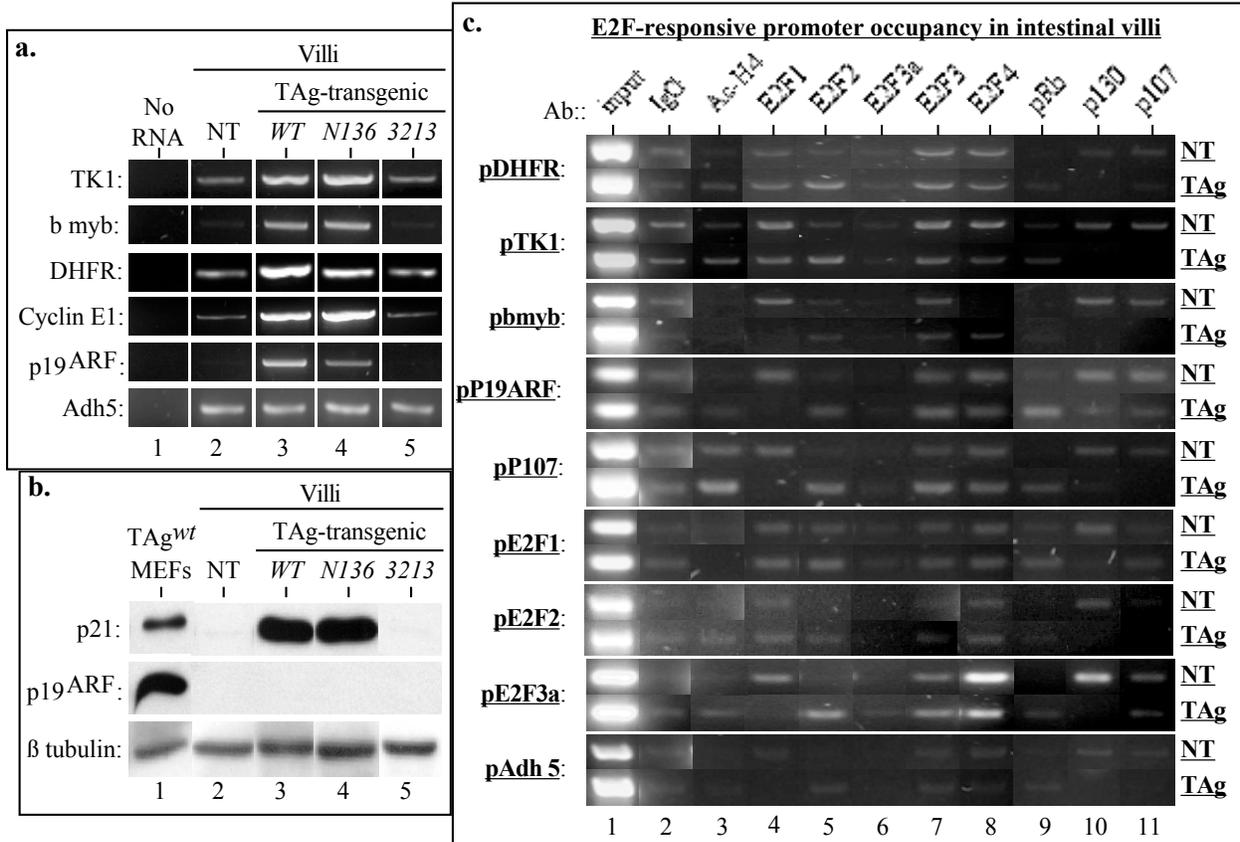


Figure 18. E2F-responsive gene transcripts are up-regulated by expression of T antigen and their promoters are bound different E2F complexes. a. Steady-state transcript levels of E2F-responsive genes in villi from non-transgenic and TAG-transgenic mouse intestines. E2F-responsive gene transcripts are upregulated in TAG^{wt} and TAG^{N136} intestinal villi as compared to non-transgenic villi, but not in TAG³²¹³ villi. **b. p19ARF protein is undetectable in intestinal villi while T antigen induces p21 protein expression dependent on Rb binding.** 30µg of villi extracts from non-transgenic and TAG^{wt}, TAG^{N136}, and TAG³²¹³ mice were subjected to immunoblot analysis for p19ARF or p21. 30µg of whole cell extracts from TAG^{wt}-transformed MEFs were ran for comparison. β tubulin was probed as a loading control. **c. E2F-responsive gene promoter occupancy as determined by ChIP analysis.** ChIPs using normal rabbit IgG or antibodies against acetyl-Histone H4, E2F1, E2F2, E2F3a, E2F3, E2F4, pRb, p130, or p107 in non-transgenic and TAG^{wt} villi were performed as described in materials and methods. E2F-responsive promoter (p) regions were subjected to PCR amplification for: pDHFR, pTK1, pbmyb, pP19ARF, pP107, pE2F1, pE2F2, pE2F3a, and alcohol dehydrogenase 5 (pAdh5) as a control. 1.5 mg

of chromatin from intestinal epithelium from each mouse was precipitated, 1/25 of the precipitated, extracted DNA was subjected to between 25-30 cycles of PCR for each promoter region, 1/150 of the no antibody supernatant DNA was used as 'input'.

4.3.9. Up-regulated E2F-responsive gene promoters are preferentially occupied by E2F2, E2F3a and p130-free E2F4.

The E2F DNA binding complexes as determined by EMSA may or may not correlate to occupation of endogenous E2F-responsive promoters. We expect that repressed E2F-responsive gene promoters will be preferentially occupied by p130, E2F4 and/or E2F5 in non-transgenic villi, whereas activated E2F-responsive gene promoters will be preferentially occupied by E2F2 and/or E2F3a, while absent for any of the Rb family members in TAg^{wt}-transgenic villi. To determine the biological relevance of the E2F DNA binding complexes revealed by EMSA, chromatin immunoprecipitation experiments were performed on villi chromatin using specific antibodies against Rb and E2F family members. PCR primers that amplify genomic regions of several E2F-responsive gene promoters containing E2F sites were used for PCR to analyze promoter occupancy. These promoters included: pDHFR, pTK1, pbmyb, pP19ARF, pP107, pE2F1, pE2F2 and pE2F3a. The promoter of the *aldehyde dehydrogenase 5* was used as a negative control as it does not contain E2F binding sites and is not responsive to E2F regulation. Antibodies against acetylated histone H4 were used as a positive control for active promoters whose transcriptional activation requires the acetylation of histone H4. Normal rabbit IgG was used as a negative control.

Chromatin immunoprecipitation involves many variable processes; it starts with cross-linking of protein to DNA, then shearing the DNA into appropriate-sized fragments,

immunoprecipitating the protein of interest, isolating the co-precipitated DNA fragment, and results in the amplification of a small region of DNA by PCR. Ultimately, the resulting data from this method can only be appropriately interpreted if each technical step proceeds as ideally as possible. The amount of cross-linking affects the result in such a way that insufficient cross-linking would prevent the co-precipitation of the promoter DNA with the immunoprecipitated protein of interest, thus resulting in the lack of PCR products above background for the amplified promoter regions. However, excess cross-linking may cause the proteins to cross-link to even very transiently interacting proteins to form very large higher-order complexes, resulting in the masking of any epitope, blocking any accessibility to the antibody. Excess cross-linking can also lead to high background due to “stickiness” of the chromatin from excess formation of labile Schiff bases. Therefore, concentrations of formaldehyde between 0.1% and 1% were used to empirically determine the appropriate amount of formaldehyde to use for cross-linking of total epithelium from non-transgenic and TAg^{wt}-transgenic mouse intestines. I determined that 0.3% formaldehyde resulted in the least amount of background while allowing for PCR amplification of products above this background level (data not shown).

The DNA fragment sizes produced by sonic shearing of the chromatin affects the ChIP results in such a way that large fragments well above 1kb will increase the non-specificity of co-precipitated promoter regions with the protein of interest. Large fragments of DNA could also result in high background since DNA is also “sticky” due to the highly charged phosphate backbone. However, DNA fragment sizes below 200bp would be approximately the same size or small than the PCR products that are predicted to be amplified, thus resulting in the absence of products in the PCR reactions, or stochastic product formation if the fragment serendipitously contained the DNA region to be amplified. To determine the appropriate number of sonications

to use for shearing of the chromatin, shearing efficiency was determined prior to immunoprecipitation by extracting DNA from a small aliquot of sheared chromatin and subjecting it to agarose electrophoresis to determine if the sheared DNA fragments are predominately between 1kb and 500bp. Typically, the ideal result was a smear of DNA observed at a peak around 500 - 750bp (data not shown.) To determine that the PCR product could be amplified from the DNA fragments if co-precipitated, PCR for each DNA region was performed on a fraction of the input DNA (non-precipitated). If the PCR did not produce a product, additional primers were designed that spanned a nearby region on the promoter of interest, but adjusted either 5' or 3' of the original product region. For example, additional sets of PCR primers were designed for the p107 promoter before settling on the set described in Materials and Methods, while three sets of PCR primers were designed for the cyclin E1 promoter, none of which produced a product and are not included in this report (data not shown).

The most difficult technique to refine in the entire chromatin immunoprecipitation method is the immunoprecipitation step. The accuracy of this step depends entirely on the ability of the antibody to specifically immunoprecipitate the protein of interest. To determine the efficiency of immunoprecipitation, the chromatin immunoprecipitated proteins would be subjected to SDS-PAGE and immunoblotted for the protein of interest. I attempted to immunoblot the ChIPed proteins, but was unable to interpret the results due to the incredibly high background of non-specific reactions of the secondary antibody with the protein-A insoluble secondary used for precipitation (data not shown.) However, there are no other reports using the same antibodies for ChIP that demonstrate the efficient immunoprecipitation of the protein of interest by immunoblot. Furthermore, the immunoprecipitated protein could be at undetectable levels, while amplification of the co-precipitated region by PCR could still produce

a detectable product. To determine the specificity of the antibody immunoprecipitation for the protein of interest, comparison of ChIP results in extracts from the appropriate gene knock-out mice would ideally be compared to ChIP results in extracts from wildtype mice. However, I was unable to perform these comparisons. Therefore, these results must be taken in the context that these controls were not performed.

Figure 18c presents the raw data of the chromatin immunoprecipitation experiments for Rb and E2F family members and PCR amplification of co-precipitated E2F-regulated promoters. Several promoter PCR reactions show products in the IgG control, suggesting high background of co-precipitation of that DNA region (Fig.18c, lane 2). To normalize for the background levels and the control promoter, the PCR products were quantified, the background subtracted, and the results normalized for each PCR reaction and to the pAdh5 negative control. The results are presented in both graphical form for each promoter and by models depicting the complexes present at each promoter in non-transgenic and TAg^{wt}-transgenic villi (Fig.19). Two preliminary observations can be reached from these data: 1) Promoter occupancy by p130 or p107 is exclusive to non-transgenic villi. 2) Promoter occupancy by E2F2 or pRb is exclusive to TAg^{wt}-transgenic villi.

The analyzed promoters are classified into three groups: 1) classic E2F-regulated genes (Fig.19a), 2) negative feedback-regulated genes (Fig.19b), and 3) positive feedback-regulated genes (Fig.19c). The classic E2F-regulated genes include DHFR, TK1 and b-myb. E2F3 and E2F4 are bound to pDHFR in non-transgenic villi. T antigen expression increases the pDHFR occupancy by E2F3 and E2F4, and induces its occupation by E2F1 and E2F2. pTK1 is occupied by E2F4 and p107, but also contains E2F1 and E2F3. T antigen expression de-represses pTK1 by relinquishing p107 occupancy, reduces the occupancy by E2F1, E2F3 and E2F4, while inducing

E2F2 occupancy. pbmyb is occupied by both a repressive complex, p130/E2F4, and E2F1 in non-transgenic villi. T antigen expression de-represses pbmyb by releasing p130 from the promoter, down-regulating E2F4, and inducing E2F3 and pRb occupancy at the promoter.

The negative feedback-regulated genes include p19ARF and p107. The p19ARF promoter is maintained in a repressive state in non-transgenic villi by a p130/E2F4 repressive complex. This promoter is also occupied by E2F1 in non-transgenic villi. T antigen expression causes the disruption of p130 from the promoter, resulting in the de-repression of the p19ARF promoter. The amount of E2F4 bound to this promoter is decreased in T antigen expressing enterocytes, while there is an increase in E2F3 and pRb occupation.

The positive feedback-regulated genes include E2F1, E2F2 and E2F3a. These promoters are each occupied by a repressive p130/E2F4 complex in non-transgenic villi. Expression of T antigen causes the disruption of p130 from these promoters, causing their de-repression, and increases either E2F2 or E2F3 occupation.

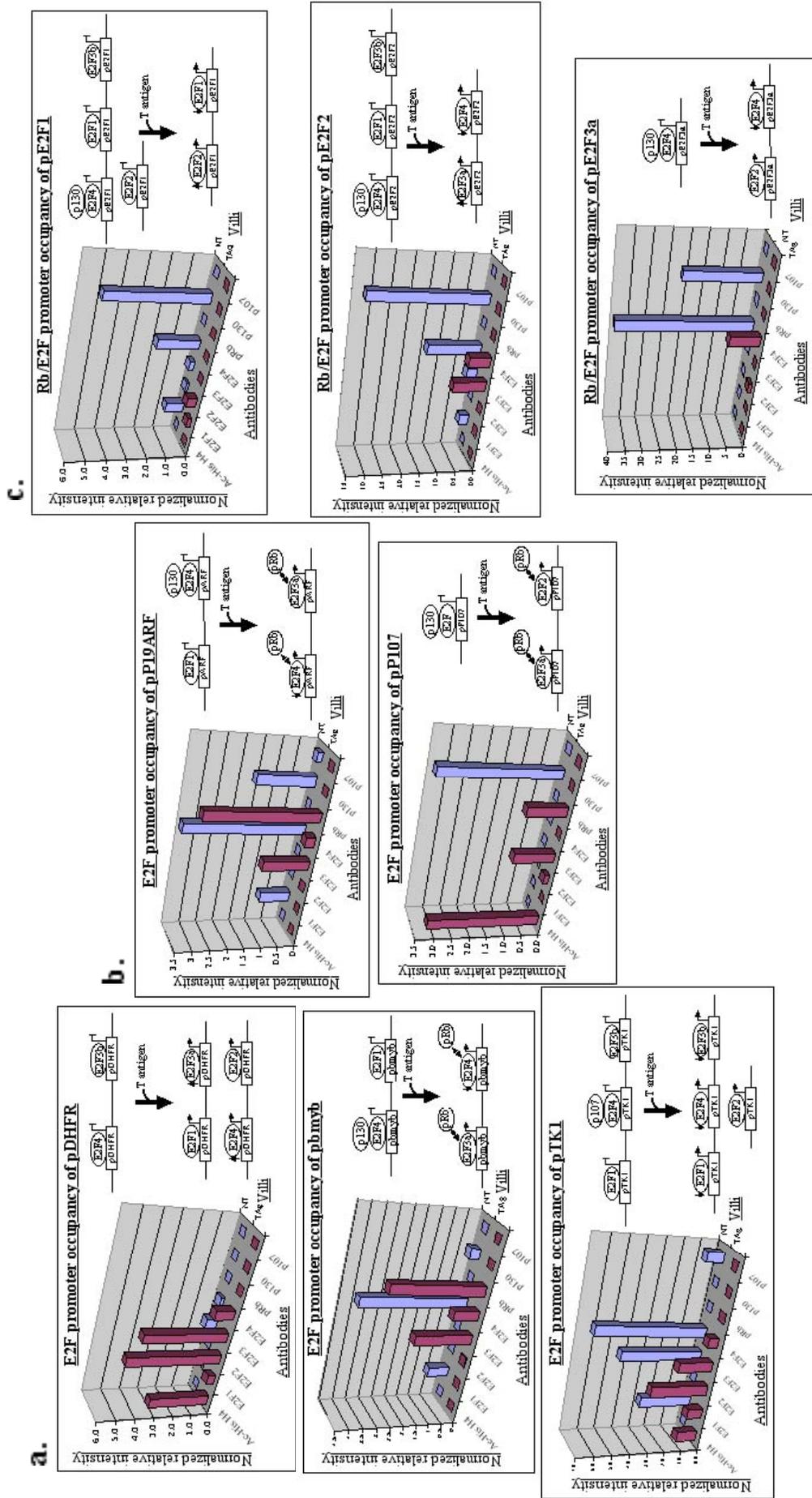


Figure 19. Quantification and normalization of E2F-responsive promoter occupancy by Rb and E2F family members in non-transgenic and TAg^{wt} intestinal villi with summary models. Quantification and normalization of promoter PCR products from the CHIP samples was performed as described in materials and methods. For each promoter, a model of E2F and Rb occupancy at that promoter in non-transgenic or TAg^{wt} villi is presented. The promoters are presented in groups as described in the text. **a. Promoter occupancy of classic E2F-responsive genes. b. Promoter occupancy of negative feedback regulator genes. c. Promoter occupancy of ‘activator’ E2Fs 1-3a genes.**

4.3.10. T antigen regulates E2F and p130 levels independent of E2F2.

The observed increase in E2F2 activity led to the hypothesis that E2F2 plays an important role in T antigen-mediated hyperplasia. To test this hypothesis, TAg^{wt}-transgenic mice were generated in an E2F2 null background. Preliminary histological results are unclear, but appear to indicate that hyperplasia can still occur in the absence of E2F2 (data not shown). To determine if the loss of E2F2 affects the levels of other E2F and Rb family members, immunoblot analysis was performed using villi extracts from wildtype, E2F2 null, TAg^{wt}-transgenic, and TAg^{wt}-transgenic mice in an E2F2 null background (TAg^{wt}, E2F2^{-/-}) (Fig.20a). Loss of E2F2 does not alter the levels of E2F1 in villi, even in the presence of *WT* T antigen (Fig.20a, compare lanes 1 and 2, 3 and 4). Loss of E2F2 results in a moderate increase in E2F3a steady-state protein levels (Fig.20a, compare lanes 1, 2 and 3). Loss of E2F2 does seem to reduce the levels of up-regulated E2F3a by expression of T antigen (Fig.20a, compare lanes 3 and 4). These data suggest that E2F2 may be involved in both positive and negative regulation of E2F3a in intestinal villi. p130 levels are not altered by loss of E2F2, even in the presence of T antigen (Fig.20a). The levels of other Rb and E2F family members remain to be determined in E2F2 null mouse intestines.

Preliminary studies of E2F DNA binding activity in E2F2 null mouse intestinal villi were performed to assess if loss of E2F2 alters other E2F family member activities. The E2F EMSA pattern for both wildtype and E2F2 null intestinal villi extracts contain species 'c', 'g' and 'i'. In addition, E2F2 null extracts contain species 'a', 'a'', 'd' and 'f' (Fig.120b, compare lanes 1 and 3). The E2F EMSA banding pattern is similar for TAg^{wt}, TAg^{N136} and TAg^{wt}/E2F2^{-/-} villi, except TAg^{wt}/E2F2^{-/-} extracts lack species 'e' and most of 'h', as expected since these bands contain E2F2 (Fig.17d, compare lanes 5 and 9; Fig.20b, compare lanes 5 and 7). Although the banding pattern is similar, the intensity of all species vary between villi samples from TAg^{wt}/E2F2^{-/-} mice (Fig.20b and c, compare lane b7 and c5). TAg^{wt}/E2F2^{-/-} villi extracts retain the E2F3a-supershifting band 'h'' (Fig.17d, compare lanes 9 and 10). There are no other E2F DNA binding species that are evident in villi extracts from TAg^{wt}/E2F2^{-/-} mice as compared to TAg^{wt}-transgenic mice. These data suggest that loss of E2F2 may not alter the E2F DNA binding activity in T antigen expressing villi enterocytes. However, a thorough analysis of TAg^{wt}-transgenic mice in an E2F2 null background will have to be performed to confirm these preliminary results.

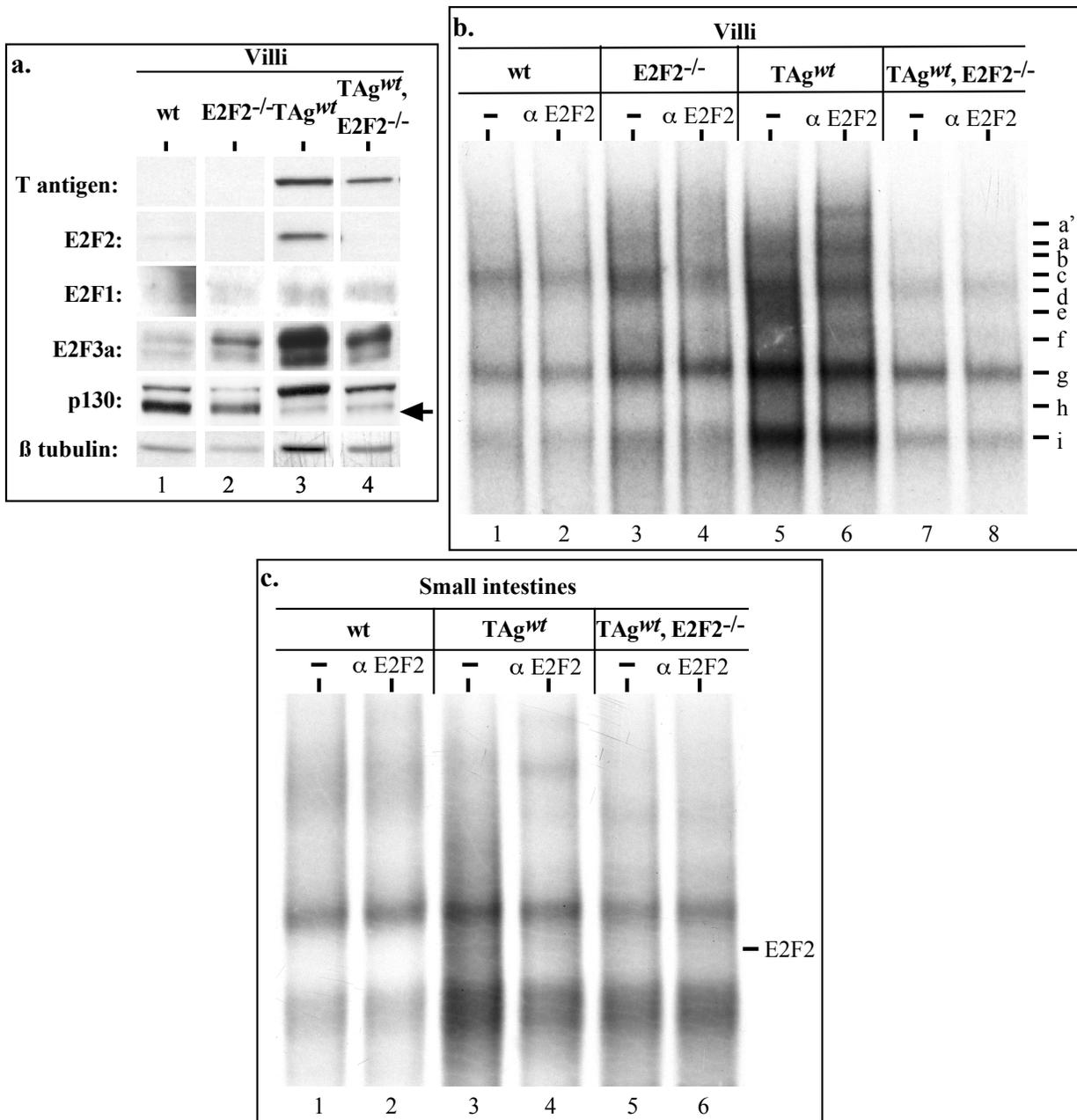


Figure 20. Loss of E2F2 does not alter T antigen-induced changes in E2F levels and DNA binding complexes. a. Loss of E2F2 causes upregulation of E2F3a, but does not alter T antigen-induced down-regulation of p130. 30µg of villi extracts from wildtype, E2F2^{-/-}, TAg^{wt}, and TAg^{wt};E2F2^{-/-} mice were subjected to immunoblot analysis for T antigen, E2F2, E2F1, E2F3a and p130. **b. Loss of E2F2 does not affect E2F DNA binding complexes altered by T antigen expression.** 30µg of villi extracts from wildtype, E2F2^{-/-}, TAg^{wt}, and TAg^{wt};E2F2^{-/-} mice were subjected to E2F EMSA analysis. Antibodies against E2F2 supershift species 'd' in TAg^{wt} villi extracts but not in any of the other extracts. E2F EMSA activity is reduced in this TAg^{wt};E2F2^{-/-} villi extract as

compared to its sibling TAg^{wt} villi extract. **c. Loss of E2F2 has variable effects on levels of E2F DNA binding activity induced by T antigen expression.** These intestinal extracts demonstrate the variability in E2F EMSA activity intensities between different TAg^{wt};E2F2^{-/-} intestinal extracts. Here, the intestinal extract of TAg^{wt};E2F2^{-/-} mice is comparable in E2F EMSA overall banding intensities as to its sibling TAg^{wt} intestinal extract after supershift with antibodies against E2F2 (lane 4). α = antibody.

4.4. DISCUSSION

The results presented here demonstrate that T antigen binds the three Rb family members, pRb, p107 and p130, in the intestinal epithelium, dependent on an intact LxCxE motif, consistent with *in vitro* results for the proteins. T antigen affects the three pocket proteins differently; it causes a decrease in p130 steady-state protein levels and an increase in p107 levels, dependent on interaction with the Rb family. T antigen disrupts p130/E2F complexes but induces pRb/E2F2 complexes. p130 is associated with many E2F-responsive promoters in non-transgenic villi, whereas it is not associated with these promoters in TAg^{wt}-transgenic villi. In contrast, pRb is preferentially associated with several E2F-responsive promoters in TAg^{wt}-transgenic villi as compared to non-transgenic villi. This unexpected result suggests that T antigen does not disrupt all Rb/E2F complexes in villus enterocytes, either due to T antigen functional specificity for each of the pocket proteins or due to inefficiency. These results also suggest that pRb association with a promoter is not sufficient to cause its repression. In support, chromatin immunoprecipitation experiments using antibodies against Rb and E2F family members in synchronized MEFs demonstrated that several promoters are occupied by Rb family members during G₁ and/or S phase of the cell cycle, when they are highly expressed (Wells et al., 2000a). In fact, pRb

expression may even activate transcription from a promoter, as supported by data demonstrating the activation of the cyclin D1 promoter by pRb expression (Watanabe et al., 1998).

4.4.1. T antigen functionally distinguishes between Rb family members in villus enterocytes.

The specificity of T antigen's effect on the Rb family members leads to an important question – must T antigen target all the Rb family proteins to cause hyperplasia, or is T antigen's effect on one Rb more important than on another? If so, which pocket protein is the most important for T antigen to interact with in order to induce hyperplasia? To address this, the intestines from mice containing genetic disruptions for the different pocket proteins were analyzed. Neither p130 or p107 null mice develop an intestinal phenotype, whereas the double null mice are perinatal lethal in a pure background such as FvB, the background of the TAg-transgenic mice. Rb null mice die before intestinal development. The generation of mice with homozygous, conditional, floxed *RB* alleles, as well as a Cre recombinase transgene driven by the *Mox2* promoter, which drives expression in the embryo proper but not the extra-embryonic tissue, allows these embryos to live longer, approximately until birth (de Bruin et al., 2003b). Therefore, the intestinal phenotypes of these embryos can be assessed. Preliminary results suggest that loss of pRb in these embryos is sufficient to cause ectopic S phase entry in intestinal villi (unpublished results, M.T. Sáenz-Robles, University of Pittsburgh). However, not all villus cells enter S phase, indicating that a specific epithelial lineage may be deregulated by loss of pRb.

If the down-regulation of p130 is primarily at the protein level, this result would then fit a model that T antigen binds p130 and targets it for degradation. The amino-terminal truncation mutant of T antigen, *N136*, is sufficient to cause these effects. Recent reports demonstrate that T

antigen interacts with an E3 ubiquitin ligase, Cul7, between amino acids 69-83, an interaction that is necessary for transformation (Ali et al., 2004; Kasper et al., 2005). These findings suggest a possible mechanism by which the first 136 amino acids of T antigen are sufficient to cause the degradation of p130 through interactions with Cul7 and p130. This may be an alternative mechanism for T antigen action on the Rb/E2F pathway other than the chaperone model. However, a recent study suggests that both *WT* T antigen and a Cul7-binding mutant cause the dephosphorylation of p130, suggesting that T antigen-induced degradation of p130 is independent of Cul7 binding. Thus, the mechanism by which T antigen specifically targets p130 for degradation remains to be determined. If T antigen is indeed targeting p130 for degradation in villus enterocytes, disruption of this mechanism in the intestines of transgenic mice would be a method by which to determine its requirement for T antigen-induced hyperplasia.

Although the interaction with Cul7 is not required for T antigen to target p130, this interaction may nonetheless be important for T antigen-induced neoplasia in the intestine. Loss of Cul7 rescued the transformation defect of the Cul7-binding mutant, suggesting that T antigen disrupts Cul7 function to cause transformation and that Cul7 acts a tumor suppressor in the presence of T antigen (Kasper et al., 2005). Future studies will have to be performed to clarify whether or not Cul7 is present in enterocytes and if T antigen is bound to it in transgenic mice. If there is an interaction, studies with transgenic mice expressing a mutation of T antigen in this Cul7-interaction region will be necessary to elucidate the significance of this interaction.

4.4.2. T antigen up-regulates E2F1 and p19ARF transcripts, but not proteins

The up-regulation of p107 transcript levels fits a model that T antigen causes the up-regulation of E2F-responsive genes. In fact, data presented in this report also supports this model, as all the

E2F-responsive gene transcripts analyzed are increased in TAg^{wt}-transgenic villi and correlate with the ability to cause hyperplasia. Activating E2Fs, E2F1, E2F2 and E2F3a transcripts are up-regulated by T antigen expression. However, only E2F2 and E2F3a are up-regulated at the protein level and have increased DNA binding activities. This lack of E2F1 protein and activity in TAg^{wt}-transgenic villi is supported by a previous report that also presented a lack of E2F1 protein up-regulation in TAg^{wt}-transgenic intestines (Chandrasekaran et al., 1996). Similarly, p19ARF is up-regulated at the transcript level, but not at the protein level. These results suggest that T antigen regulates specific genes post-transcriptionally in villus enterocytes. One method to regulate genes post-transcriptionally is by microRNA-mediated interference of specific transcript translation (Gebauer and Hentze, 2004). Recently, SV40 was demonstrated to encode microRNAs that are involved in evading host immune defenses (Sullivan et al., 2005). Although this microRNA is transcribed from the late promoter of SV40 and is not likely to play a role in these transgenic mice, these results indicate that SV40 has evolved the capabilities to use this method of post-transcriptional regulation. Therefore, T antigen may use a similar method in villus enterocytes to prevent p19ARF or E2F1 translation. Alternatively, T antigen may target newly synthesized p19ARF and E2F1 proteins for degradation. The variable results for E2F1 protein levels support this latter hypothesis, since the amount of degradation might vary between samples.

Preventing p19ARF translation is beneficial to SV40 as its expression can cause growth arrest and/or apoptosis. Therefore, T antigen may have evolved a mechanism to block p19ARF translation. However, blocking E2F1 synthesis is not as clearly beneficial to the virus, as its over-expression can cause cells to enter S phase. In fact, in the choroid plexus and the lens of the eye, T antigen causes ectopic proliferation in part through E2F1 (Pan et al., 1998). In the lens,

over-expression of E2F1 is sufficient to cause proliferation (Chen et al., 2000a). However, in the intestinal epithelium, over-expression of E2F1 is not sufficient to cause hyperplasia (Chandrasekaran et al., 1996). In a recent report, E2F1 over-expression suppresses ras-induced skin carcinogenesis in transgenic mice (Russell et al., 2005). These results suggest tissue-type specificity in E2F1 oncogenic activity. E2F1 is also known to have tumor suppressive activity through its ability to cause apoptosis. Since E2F1 can cause both p53-dependent and independent apoptosis, this tumor suppressive function of E2F1 may play a role in villus enterocytes despite the finding that p53 may not (Markovics et al., 2005). In fact, E2F1 occupies several E2F-responsive promoters in non-transgenic villus enterocytes when these promoters are repressed. However, this repression is pRb-independent as these same promoters are not occupied by pRb in non-transgenic villi. In support of this notion, a report demonstrated that E2F1 represses the cyclin D1 promoter through interaction with SP1, while over-expression of pRb activates the promoter (Watanabe et al., 1998). Therefore, in light of these results, blocking E2F1 may indeed be beneficial for SV40 in certain circumstances, and thus be a specific function of T antigen.

T antigen expression causes the up-regulation of p21 at both the transcript and protein level (this report and unpublished results, P.G. Cantalupo, et al., University of Pittsburgh). This up-regulation is dependent on T antigen binding to Rb family members, suggesting that Rb may regulate p21 expression. In fact, two reports suggest that Rb can regulate the p21 promoter through both E2F-dependent and independent means (Gartel et al., 1998; Carreira et al., 2005). One report states that there are two non-canonical E2F binding sites in the p21 promoter and that E2F1 and E2F3 transactivate transcription from them, while an Rb-binding protein, HBP1 (HMG-box protein 1) represses E2F-activated expression (Gartel et al., 1998). Another report demonstrates the pRb can also bind Mit1 to activate transcription from the p21 promoter in

melanoblasts (Carreira et al., 2005). To distinguish between these possibilities, chromatin immunoprecipitation using antibodies against pRb, E2Fs, Mit1 and HBP1 could be performed in non-transgenic and TAg^{wt}-transgenic villi to determine their occupation on the p21 promoter. Alternatively, p21 up-regulation may be regulated by another mechanism that is activated during T antigen-induced intestinal hyperplasia.

4.4.3. T antigen disrupts repressive p130/E2F complexes, up-regulates E2F2 and E2F3a levels and activities while activating the E2F pathway

According to the chaperone model of T antigen action on the Rb/E2F pathway, T antigen binds Rb family members in Rb/E2F complexes, recruits the molecular chaperone, Hsc70, stimulates its ATPase activity, and then Hsc70 rearranges and disrupts these complexes, releasing “Rb-free” E2F. Evidence from this report supports this model in villus enterocytes. There are p130/E2F DNA binding complexes in the growth-arrested cells of non-transgenic villi. T antigen expression disrupts this p130/E2F complex, concomitant with p130 down-regulation, and an increase in Rb-free E2F4 and Rb-free E2F5 DNA binding complexes. To further test this model, transgenic mice expressing a mutant of T antigen in the J domain, *D44N*, in the intestinal epithelium are being generated.

There are several forms of E2F4 in the non-transgenic villi, three of which are phosphorylated. T antigen expression in villus enterocytes alters the proportions of these forms such that two of the phosphorylated forms are enriched. It is unclear if these forms enriched in T antigen expressing villi are more or less phosphorylated than the other forms. Conflicting reports in the field suggest the unphosphorylated E2F4 correlates with an increase in p130/E2F4 DNA binding complexes in post-confluent Caco-2 cells or that unphosphorylated E2F4 correlates with

nuclear localization and increased E2F-responsive promoter activation and proliferation in human intestinal epithelial cells (HIEC) (Ding et al., 2000; Deschênes et al., 2004). Since HIECs are derived from human fetal small intestinal epithelium, whereas Caco-2 cells are derived from a human colon carcinoma and serendipitously appear to transdifferentiate into small intestinal enterocytes upon confluence in culture, the results from studies in the HIEC cell system might be more relevant to the normal regulation of E2F4 in intestinal epithelial cells. In agreement with the results in HIECs, the hypophosphorylated E2F4 forms in T antigen expressing villi are enriched in nuclear extracts. These results support a model in which T antigen either prevents the export of E2F4 from the nucleus, or causes its import. The preliminary chromatin immunoprecipitation results suggest that T antigen maintains E2F4 as de-repressed at the promoters of several up-regulated E2F-responsive genes, also in agreement with the previous report that nuclear E2F4 correlates with proliferation and active E2F-responsive gene transcription (Deschênes et al., 2004).

T antigen causes an increase in E2F2 and E2F3a steady-state protein and transcript levels. This effect by T antigen is concomitant with an induction of pRb-E2F2, Rb-free E2F2 and Rb-free E2F3a DNA binding activity, dependent on T antigen interaction with the Rb family. Analysis of transcript changes caused by T antigen expression in villus enterocytes determined that E2F-responsive genes are up-regulated, indicating the T antigen activates the E2F pathway in these cells. The disruption of p130/E2F complexes and the induction of E2F2 and E2F3a complexes are predicted to cause the activation of the E2F pathway in TAg^{wt}. To determine the endogenous activities of these complexes in villus enterocytes, I analyzed the *in vivo* promoter occupancies of these complexes in intestinal villi. A discussion of E2F-responsive promoter studies is presented first.

4.4.4. E2F-responsive promoter studies

Microarray studies have identified a multitude of transcripts that appear to be regulated by E2F over-expression or deletion (Ishida et al., 2001; Müller et al., 2001; DeGregori, 2002; Ren et al., 2002; Stevaux and Dyson, 2002b; Wells et al., 2002; Black et al., 2003; Polager and Ginsberg, 2003; Young et al., 2003). However, interpretation of these data is complicated by the different methods used and thus it is often difficult to interpret direct or indirect regulation of these transcripts by E2F. Therefore, in this discussion, only certain E2F-responsive gene promoters will be discussed that have been analyzed to identify the *cis*-acting DNA elements that regulate them.

The E2F DNA binding site was initially identified by the regulation of the Adenovirus E2 promoter (Yee et al., 1987). It was then discovered that certain cellular genes that are up-regulated by E1A also contain a similar site in their promoters, such as the promoter for dihydrofolate reductase (*dhfr*) (Hiebert et al., 1991). DNA foot printing and sequence analysis of the *dhfr* promoter revealed both SP1 and E2F binding sites, and that the E2F site is required for efficient transcription in transient transfection reporter assays in established cell lines (Blake and Azizkhan, 1989). Later studies using low passage, primary cells revealed that the deletion of the E2F site on the *dhfr* promoter actually caused an increase in transcription, indicating a requirement for this element for repression of the promoter activity (Jensen et al., 1997). Synchronizing the cells, the authors were able to determine that the E2F site is primarily used as a repressive element during G₀/G₁, while unnecessary for late G₁/S phase transcription. These studies also revealed a more critical requirement for the SP1 sites, as deletion of them decreased transcription regardless of cell cycle stage. Later studies supported a cooperative role between SP1 and E2F for induction of transcription from the *dhfr* promoter that depended on the SP1 site

in an osteosarcoma cell line (Park et al., 2003). *RB* is mutated in osteosarcoma, therefore these data must be considered with that in mind. These experiments also show that HDAC is a potent inhibitor of SP1 transactivation of the *dhfr* promoter, which cannot be overcome by expression of E2F/DP (Park et al., 2003).

Other E2F-responsive gene promoters that have been analyzed for occupancy throughout the cell cycle reveal differences in promoter regulation. Using DNA footprinting analysis, it was shown that the b-myb promoter and the thymidine kinase 1 (TK1) promoter contain E2F binding sites that are occupied in G₀ (Zwicker et al., 1996; Tommasi and Pfeifer, 1997). The single E2F binding site on the TK1 promoter is occupied throughout the cell cycle, with changes in DNA binding complexes that occur at different stages (Tommasi et al., 1997). In contrast, the E2F site on the b-myb promoter is occupied in G₀ and early G₁, but is then unoccupied by late G₁ and S phases (Zwicker et al., 1996). These data clearly reveal differences in promoter regulation by E2F sites.

A logical explanation for the differences in the occupancies of the b-myb and TK1 promoters is that Rb/E2F complexes are bound to the promoters during G₀, repressing their transactivation, and the b-myb promoter is activated by de-repression of these complexes, whereas TK1 is activated by both de-repression by release of the Rb/E2F complex and replacement by another, “activating” E2F, on the promoter. This mechanism of regulation is further supported by studies of the *dhfr* promoter. During G₀ and G₁, p130/E2F4/DP1 complexes occupy the *dhfr* promoter in Chinese hamster ovary cells as assessed by EMSA (Tommasi et al., 1997; Wells et al., 1997). As cells move into late G₁, Rb-free E2F4/DP1 complexes occupy the TK1 promoter. By S phase, as the TK1 transcript increases, another, unidentified Rb-free E2F/DP1 DNA binding complex increases, possibly containing E2F5 since it had not yet been

identified. These data also suggest that the E2F element in the *dhfr* promoter regulates transcription similarly as the E2F element in the b myb promoter, mainly as a repressive element in G₀ leading to transactivation by de-repression in late G₁ and S phases.

Cyclin E also contains an E2F site in its promoter that is required for activation at S phase (Botz et al., 1996). These experiments demonstrated that E2F4 could induce promoter transcription, an induction that was augmented by the additional expression of DP1. Although normally described as a “repressive” E2F, E2F4 has repetitively been shown to be able to have transactivation capabilities, just as E2F1-3, albeit at a lower level unless a DP is co-expressed (DeGregori et al., 1997).

Interestingly, many of these E2F-responsive promoters contain overlapping E2F sites, such as the promoters of *thymidine kinase* and *dhfr* (Wells et al., 1997). Detailed analysis of each site in the *dhfr* promoter revealed differences in affinity of the sites for specific E2F DNA binding complexes. It is suggested that two E2F complexes could be bound to this site at the same time, as indicated by DNA foot printing analysis revealing both sites to be occupied at the same time. This may reveal a mechanism of how different E2F complexes cooperate with each other or even pass on the regulation of a promoter from one E2F complex to another throughout the cell cycle.

There are two basic mechanisms that have been proposed for promoter regulation by E2F; one is through repression by p130/E2F4/DP or p130/E2F5/DP complexes, and de-repression allowing for transcription, the other is through recruitment of activating E2F/DP complexes, (E2F1, E2F2, or E2F3a), and their subsequent transactivation of transcription. Several promoters have overlapping E2F binding sites, possibly as a mechanism of switching E2F complexes (repressive and active). Recent experiments demonstrate the repression of certain

genes when E2F1 is over-expressed (Young et al., 2004). In these studies, this repression was alleviated in an Rb KO background, thereby demonstrating that the promoters of these genes - *Histone H2B*, *cyclin B*, *ATF2* and *fas* - are regulated by pRb/E2F1 repression and de-repression.

Together with studies on Rb and E2F family proteins, these studies suggest that there are several modes of Rb-mediated transcriptional repression and E2F-mediated transactivation. Each promoter can be regulated by one or more of these mechanisms. Studies of *in vivo* promoter occupancy of Rb and E2F family members were performed to elucidate which Rb and E2F family members are at specific promoters throughout different phases of the cell cycle (Takahashi et al., 2000; Wells et al., 2000a). Intriguingly, results from these studies suggest another level of specificity for promoter regulation – the cell type or degree of its transformation.

Two different labs undertook the task of discerning the *in vivo* promoter occupancy of individual Rb and E2F family members throughout different stages of the cell cycle (Takahashi et al., 2000; Wells et al., 2000a). They presented results from different sets of E2F-responsive promoters, with the promoter for the *b-myb* gene in common. The results from one lab provide evidence to support the classic model of Rb/E2F function throughout the cell cycle – p130/E2F4 complexes occupy the promoters of E2F1, p107, cyclin A and *cdc6* in G₀ and early G₁, while the “activating” E2Fs (E2F1-3) occupy the promoters of E2F1, p107, *cdc6* and *b-myb* in late G₁ and in G₁/S phase for the promoters of E2F1 and p107 (Takahashi et al., 2000). The results from the second report do not generally support the classical model. All promoters examined by this group, such as for *b-myb*, *dhfr*, *tk1*, *cdc2*, and *cyclin E*, were occupied by p130 and E2F4 in G₀, in accordance with the model (Wells et al., 2000a). However, the promoter for *cyclin E* was also occupied by E2F1, E2F2 and pRb at G₀. The promoter for *b-myb* was only occupied during G₀ and G₁ by p130, p107 and E2F4. As discussed previously, several studies have implicated that

the *b-myb* promoter is regulated by repression and de-repression, and that the E2F-binding site is unoccupied in S phase, during its highest expression. The main difference between these two studies is the cells that were analyzed. The study that supports hypotheses predicted by the classic model was performed on T98G cells – a glioblastoma cell line, whereas the other study was performed on primary MEFs. The greater amount of “activating” E2F promoter occupancy in the T98G cells may reflect their state of transformation, whereas the greater amount of E2F4 occupancy on promoters throughout the cell cycle in MEFs may more accurately reflect the normal occupancy of these promoters in embryonic fibroblasts.

4.4.5. *In vivo* occupancy of up-regulated E2F-responsive promoters in intestinal villi

In this study, I attempted to elucidate the occupancies of the different Rb and E2F family members on several up-regulated E2F-responsive promoters in villi from either non-transgenic or TAg^{wf}-transgenic intestines. Unfortunately, the chromatin immunoprecipitation (ChIP) results presented in this report are inconclusive on their own to determine the specificity of promoter regulation by different Rb and E2F proteins in intestinal villi. However, in conjunction with the other molecular data, several suggestive interpretations could be made which are presented in this discussion. However, it is important to note that these results require reproduction as well as better quantification if they are to be presented as solid lines of evidence. Two alternative methods could be used for more accurate quantification of co-precipitated promoters: RTPCR using radioactively labeled nucleotides (i.e, dATP α ^{32P}) so that fewer cycles could be performed or real-time RTPCR.

Preliminary ChIP results suggest that E2F2 is bound to specific E2F-responsive promoters in TAg^{wf}-transgenic villi but not non-transgenic villi, indicating biological

significance to their DNA binding activity as observed by EMSA. The E2F3 antibody that recognizes both E2F3a and b forms co-immunoprecipitates specific E2F-responsive promoters in both non-transgenic and TAg^{wt}-transgenic villi. Because of their relative protein levels, it is hypothesized that the E2F3 form preferentially immunoprecipitated in non-transgenic villi is E2F3b, while the E2F3 form preferentially immunoprecipitated in TAg^{wt}-transgenic villi is E2F3a. However, certain promoters may be specifically regulated by one form of E2F3 and not the other, thereby complicating the interpretation of the results. For simplicity, it is hypothesized that the E2F3 form preferentially bound to a promoter in non-transgenic villi is E2F3b, while the form preferentially bound to a promoter in TAg^{wt}-transgenic villi is E2F3a. However, the actual form of E2F3 bound to each promoter in the villi of each mouse will remain unresolved until a better antibody for each specific form is generated.

Repressive p130/E2F4 complexes appear to preferentially associate with the promoters of the activating E2Fs in normal villi. T antigen causes the de-repression of these promoters, resulting in their transcriptional up-regulation. Interestingly, E2F2 and E2F3a each seem to be bound to the promoter of the other, but not to their own promoters in TAg^{wt}-transgenic villi, suggesting that they play a role in regulating each other, but not in their own auto-regulation. In fact, this regulation may both be positive in T antigen expressing villi and negative in normal villi since preliminary results indicate that loss of E2F2 causes the up-regulation of E2F3a.

According to these preliminary ChIP results, E2F2 and E2F3 are not associated with the promoters of all the same genes, although they may both associate with the promoters of *DHFR*, *TK1*, *p107* and *E2F1*. These results suggest that for some genes the loss of E2F2 or E2F3 can compensate for the other E2F during gene regulation, whereas for other genes, such as *b myb*, *p19ARF*, *E2F2* or *E2F3a*, the loss of one cannot necessarily compensate for the other in villi

enterocytes. However, since mice with germ-line homozygous deletions in E2F2 do not develop a gross intestinal phenotype, compensation between E2Fs is a plausible possibility (Table 1). Preliminary results also indicate that T antigen is capable of causing hyperplasia in the absence of E2F2, also supporting a compensatory mechanism between E2Fs. These results suggest that E2F3a may be required either alone or with E2F2 for T antigen-induced intestinal hyperplasia.

4.4.6. E2F2 may not be required for T antigen-induced hyperplasia

If loss of E2F2 activity is compensated by an increase in E2F1 or E2F3a activity, it is not readily apparent by immunoblot or E2F EMSA analysis. These preliminary data suggest that E2F2 is not necessary for T antigen to cause hyperplasia in the intestine. Possibly, E2F2 is just a secondary effect of a pRb-deregulated cell cycle in the intestine and is not a cause of it. In this scenario, an alternative E2F, E2F3a possibly, is responsible for activating the cell cycle and causing hyperplasia. Alternatively, both E2F3a and E2F2 are necessary for T antigen to cause hyperplasia. Therefore, while TAg^{wt}/E2F2^{-/-} mice continue to be analyzed, TAg^{wt}-transgenic mice are being crossed into E2F3a null and an E2F2/E2F3a double-knock-out background for analysis.

In conclusion, T antigen up-regulates the E2F pathway in two ways: 1) causing the de-repression of p130/E2F complexes and 2) inducing Rb-free E2F2 and E2F3a complexes. Perhaps T antigen causes these effects by first binding p130 and signaling it for degradation, thus causing the de-repression of p130/E2F regulated genes, such as E2F2 and E2F3a. These E2Fs, then would initially be up-regulated modestly, but then synergistically up-regulated by their positive feedback regulation of each other. Thus, E2F-responsive promoters would both be de-repressed and activated to cause cells to enter the cell cycle and proliferate. Since p53-mediated apoptosis

is not triggered in these cells, they would hyperproliferate causing intestinal hyperplasia. To further support the sequential mechanism suggested in this model, a timeline of effects of T antigen expression in villus enterocytes is necessary.

5. T ANTIGEN AFFECTS THE RB/E2F PATHWAY EARLY IN INTESTINAL DEVELOPMENT

5.1 INTRODUCTION

T antigen induces hyperplasia by affecting the Rb/E2F pathway through changes in the levels of specific Rb family members, disruption of repressive p130/E2F complexes, induction of activating E2Fs, E2F2 and E2F3a, and the consequent up-regulation of E2F-responsive gene transcription. It is not likely that all of these changes occur at the same time. To elucidate the mechanism of how T antigen causes these effects, a timeline is under construction. It is important to discern the time point during intestinal development when T antigen protein is first detected. A brief discussion of intestinal development will describe the importance of the different developmental time points.

In the developing embryo, the three germ layers are evident by the end of gastrulation. The innermost layer, the endoderm, closes into a tube and becomes the immature gut. The development of the intestinal epithelium begins in a wave along the proximal to distal axis of this tube (Traber and Wu, 1995; Hauck et al., 2005; Stainier, 2005). In the mouse, the initial squamous epithelium, consisting of multiple layers of cells, proliferates, and extends to form a more orderly pseudostratified epithelium by embryonic day 15.5 (E15.5) as depicted in Figure 21. Villus structures begin to form with the convergence and extension of cells into a single

contiguous layer of columnar epithelium. By birth the proliferating cells are restricted to these ‘intervillus’ regions, which are the precursors to crypts. In humans, the maturation of the intestinal epithelium occurs *in utero*. However, in the mouse this maturation occurs between birth and by the end of weaning at about one month of age. At this time, the crypts appear fully formed and paneth cells are apparent at their bases (P28.5).

With such distinct changes in proliferation and growth arrest throughout the development of the intestinal epithelium, it seems plausible that there would be changes in the Rb/E2F pathway that regulate these different phases of proliferation. However, a thorough study of the status of the Rb/E2F pathway throughout intestinal development has not been performed. There is one report that investigated the levels of specific E2F transcripts throughout embryonic development in the intestinal epithelium by *in situ* hybridization (Dagnino et al., 1997). In this report, which preceded the discovery of E2F6, E2F7 or E2F8, the authors note that the levels of E2F1 and E2F3 do not change throughout intestinal development and remain relatively low throughout the epithelium. DP-1 levels were also present in the developing intestinal epithelium at relatively high levels and do not change throughout development. E2F2 and E2F4 were first detected in the pseudostratified epithelium of the intestine at E14.5 and are restricted to the intervillus epithelium and base of the villus by late embryogenesis, while E2F5 levels were only detected at the top of the villus at the end of embryogenesis. These results suggest a role for E2F2 and E2F4 in regulating proliferation in the developing intestinal epithelium, while E2F5 plays a role in maintaining growth arrest in the maturing villus epithelium. Another report investigated the levels of Rb family members during embryonic development (Jiang et al., 1997). In this report, the authors show the predominant presence of p107 throughout the proliferating cells in the intestinal epithelium. Together, these reports suggest that E2F2 is the “activating”

E2F and p107/E2F4 is the repressive E2F complex that are the accelerator and breaks, respectively, that drive proliferation in the intervillus region of the developing intestinal epithelium.

Although these previous reports show expression levels, they do not fully characterize the activity of these Rb and E2F proteins in the developing intestinal epithelium. Therefore, an investigation into the changes caused by T antigen expression on the activities of Rb/E2F complexes might elucidate the normal activities of the Rb/E2F complexes during development. For these studies, immunoblot and EMSA analyses were performed for Rb and E2F family members using intestinal extracts from different time points during intestinal development from non-transgenic and TAg^{wf}-transgenic mouse embryos and pups.

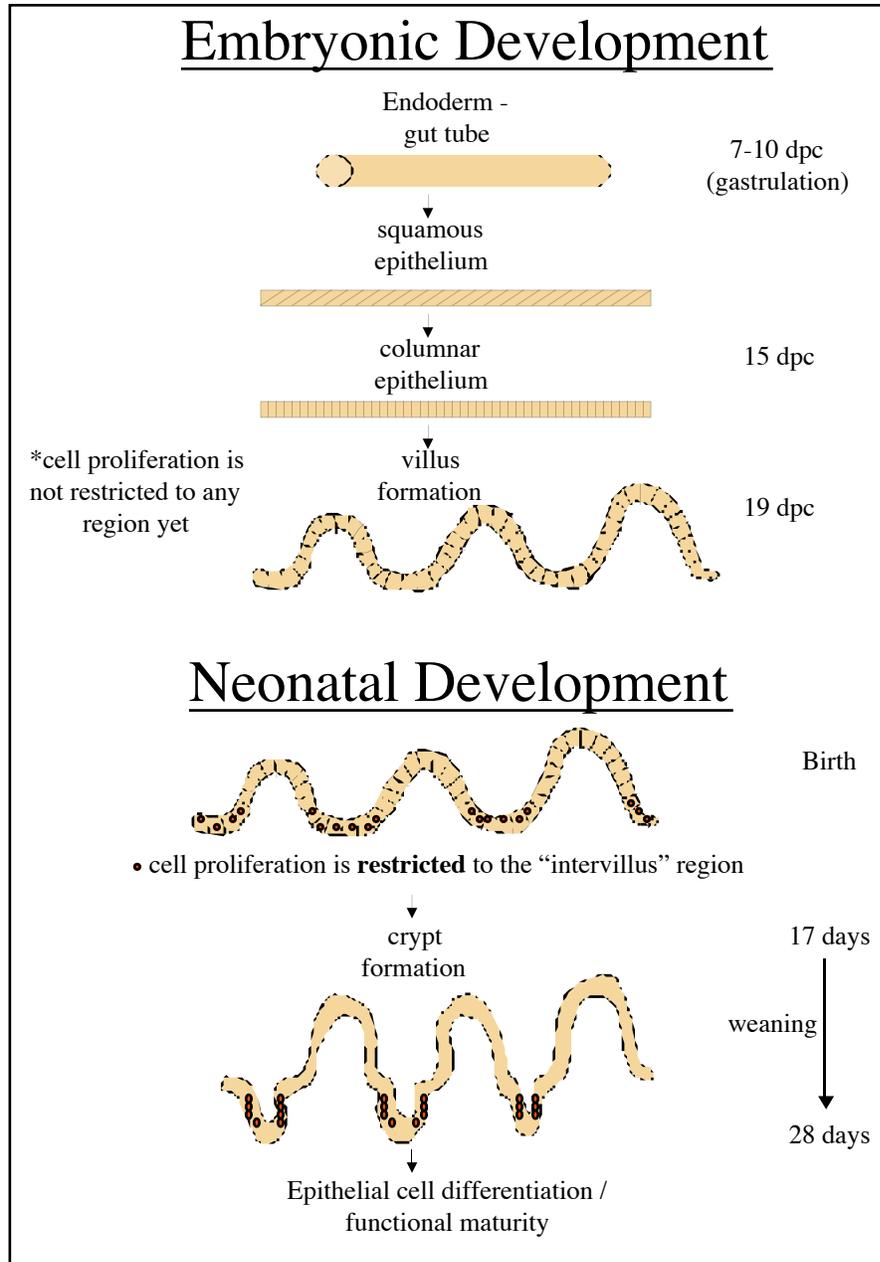


Figure 21. Localization of cell proliferation during mouse intestinal development. This is a schematic diagram summarizing the cellular localization of proliferation during intestinal epithelial development. The dividing cells are located in all cells in the squamous epithelium of the early developing intestine (E15.5.), but are restricted to the intervillus region as villi structures are formed by birth (red-dotted cells). Mature crypts are formed by the end of weaning (P21.5) in the developing mouse intestine.

5.2. MATERIALS AND METHODS

Production and maintenance of transgenic mice. FVB/N non-transgenic and TAg^{wt} mice were generated and maintained as described in chapter 2.

Immunoblot analysis. Embryos from indicated time points were harvested, small intestines dissected under a dissecting microscope in PBS and each individual intestine was immediately lysed for total soluble proteins as described in chapter 2. The remaining embryonic tissues were retained for genotyping as described in chapter 2. Conventional Western blot techniques were employed using 30µg of total protein lysates as described in the previous chapter.

E2F electromobility shift assay (EMSA). Small intestinal extracts from embryos were obtained as described above. 30µg of lysates were subjected to E2F EMSA analysis as described in the previous chapter.

5.3. RESULTS

5.3.1 T antigen is expressed and begins its effects on the Rb/E2F pathway in the early developing intestine

The onset of T antigen expression in the transgenic lines examined in these studies has not been previously established. In the high T antigen expressing TAg^{wt}-transgenic line used throughout these studies, line 103, T antigen is expressed at the earliest detectable time point for intestinal

development, embryonic day 15.5 (E15.5) (Fig.22, lane 2). In both non-transgenic and TAg^{wt}-transgenic E15.5 intestines, E2F2 protein is readily detected, with no increase in the TAg^{wt} extract (Fig.22, compare lanes 1 and 2). By the next time point, E17.5, E2F2 protein is increased in TAg^{wt} intestines compared to extracts from non-transgenic intestines or extracts from E15.5 TAg^{wt} intestines (Fig.22, compare lanes 3 and 4, 2 and 4). By birth, E2F2 is barely detectable in non-transgenic intestines, although maintained at an increased level in TAg^{wt} intestines. This trend continues through weaning and becomes most dramatic by adult, around 2.5 months of age. E2F3a and E2F3b proteins are also detected throughout embryonic intestinal development in both non-transgenic and TAg^{wt} extracts (Fig.22, lanes 1-4). By birth, however, E2F3a protein decreases in non-transgenic intestines while it is maintained at the same level as previous time points in TAg^{wt}-transgenic intestines. Higher levels are present in the adult (Fig.22, compare lanes 5-7). p130 protein levels also share this trend, high in both non-transgenic and TAg^{wt}-transgenic intestines throughout embryonic development. p130 levels remain high in non-transgenic intestines at birth, but begin to decrease in TAg^{wt} intestines at that time, with a much greater effect by adult (Fig.22, compare lanes 5-7). These results suggest that the first alteration in Rb/E2F levels by T antigen expression is an increase in E2F2 levels by E17.5 in the developing intestine, which is then followed by a decrease in p130 levels. T antigen maintains E2F3a levels throughout intestinal development, whereas E2F3a levels are decreased in non-transgenic intestines at birth.

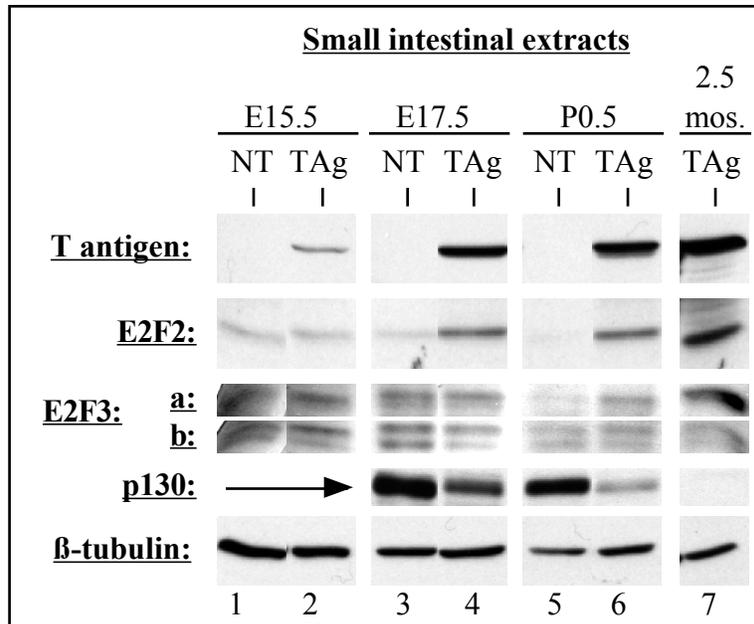


Figure 22. T antigen is expressed early during intestinal development and causes sequential changes in E2F2, E2F3a and p130 levels. 30µg of small intestinal extracts from non-transgenic and TAg^{wt} embryos and pups were subjected to immunoblot analysis for T antigen, E2F2, E2F3 and p130. β-tubulin was probed as a loading control.

5.3.2. E2F3a has DNA binding activity in the early intestine that is up-regulated by T antigen in E18.5 intestines while E2F2 DNA binding activity is induced by T antigen at birth

To investigate the activities of the Rb/E2F complexes throughout intestinal development, E2F EMSA analysis was performed on non-transgenic and TAg^{wt}-transgenic embryonic and neonatal intestines. There are five migratory species in non-transgenic and TAg^{wt}-transgenic intestines at E15.5, 't', 'u', 'x'-'z' (Fig.23a, compare lanes 1 and 2). T antigen does not alter the banding pattern by E2F EMSA analysis at this time point, E15.5. In E2F EMSAs of non-transgenic E15.5 intestines, species 'x' was supershifted with antibodies against E2F4, while species 'z' was

supershifted with antibodies against E2F5 (data not shown). This was expected since these species co-migrate with E2F4- and E2F5-containing complexes in adult intestinal tissues. To assess the Rb/E2F complexes at this time point, supershift analysis was performed on non-transgenic E15.5 intestines using antibodies against pRb and p107. The majority of both species 't' and 'u' were supershifted with antibodies against p107, while no supershifts were detected with antibodies against pRb (Fig.23b). To identify the E2F complex in species 'y', antibodies against E2F2 and E2F3 were used for supershift analysis. Antibodies against E2F3 appeared to supershift species 'y' in non-transgenic E15.5 intestines, while antibodies against E2F2 did not detectably supershift any species (Fig.23c, compare lanes 1-3). These results suggest that in both non-transgenic and TAg^{wf}-transgenic E15.5 intestines, p107/E2F, E2F4, E2F5 and E2F3a DNA binding complexes are present. However, these same supershifts as well as supershift analysis for p130 need to be performed on TAg^{wf}-transgenic E15.5 intestines for confirmation.

By the next time point, E18.5, species 'y' is decreased in non-transgenic intestines and it is undetectable by birth (P0.5) (Fig.23a, compare lanes 1, 3 and 5). T antigen expression maintains this species 'y' and induces E2F DNA binding species such as 'v', and 'w' by birth (Fig.23a, compare lanes 2, 4 and 6). To investigate the onset of E2F2 DNA binding activity in TAg^{wf}-transgenic intestines during development, supershift analysis was performed using antibodies against E2F2 on TAg^{wf}-transgenic intestines. E2F2 DNA binding activity first appears detectable in the intestines of TAg^{wf}-transgenic neonates, as antibodies against E2F2 supershift species 'v', while remaining undetectable in non-transgenic intestines (Fig.23c, compare lanes 6-12). These effects continue and are most robust in the adult intestine where antibodies against E2F2 supershift species 'y', when hyperplasia is evident (Fig.23c, compare lanes 9-16). These results suggest that at the earliest analyzed time point of intestinal development, E2F3a DNA

binding activity is evident in both non-transgenic and TAg^{wt} intestines, while this activity decreases by birth. T antigen expression up-regulates E2F2 DNA binding activity by birth. E2F3a DNA binding activity is also maintained by T antigen in E18.5 intestines. T antigen is predicted to maintain E2F3a activity throughout intestinal development as the protein levels are maintained and the adult TAg^{wt}-transgenic intestine also contains E2F3a DNA binding activity. However, this prediction remains to be confirmed by supershift analysis at each developmental time point.

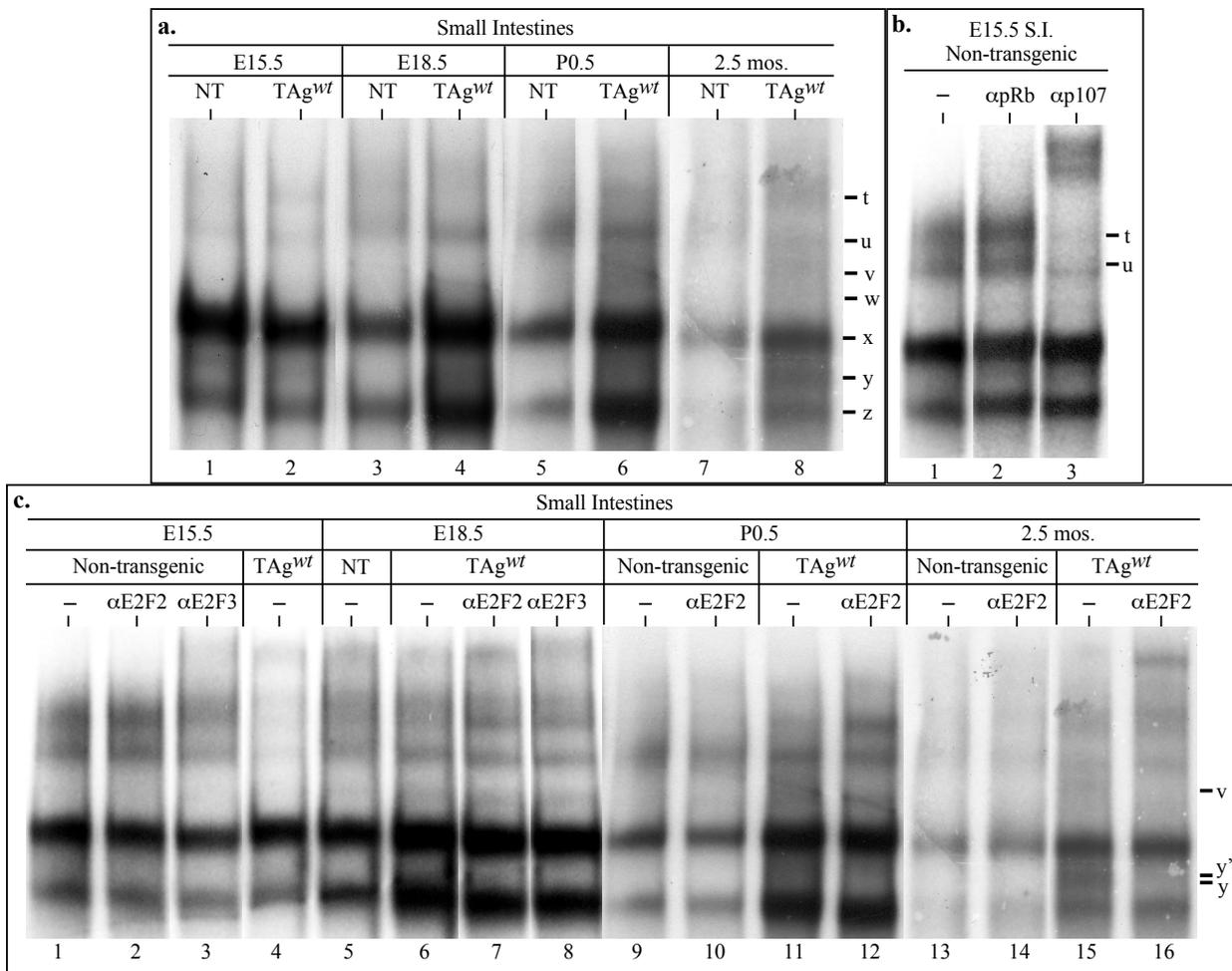


Figure 23. E2F3a DNA binding activity is present in early developing intestines and E2F2 DNA binding activity is induced in T antigen-expressing intestines by birth. **a.** E2F EMSA DNA binding complexes during intestinal development in non-transgenic and TAg^{wt} small intestines. 30μg of small intestinal extracts

from non-transgenic or TAg^{wt} embryos, pups and adult mice were subjected to E2F EMSA analysis. There is not a significant difference in E2F EMSA patterns between non-transgenic and TAg^{wt} intestinal extracts from E15.5 embryos. By E18.5, however, T antigen expression causes the up-regulation of species 'x', 'y', and 'z' intensities and induces species 'v'. **b. Non-transgenic E15.5 small intestines have mostly p107/E2F DNA binding complexes.** 30µg of small intestinal extracts from E15.5 non-transgenic embryos was subjected to E2F EMSA and supershift analysis using antibodies against pRb and p107. Species 't' and most of species 'u' supershift with antibodies against p107, but not with antibodies against pRb. **c. E2F2 and E2F3a DNA binding complexes in non-transgenic and TAg^{wt} small intestines throughout development.** 30µg of small intestinal extracts from non-transgenic or TAg^{wt} embryos, pups and adult mice were subjected to E2F EMSA and supershift analysis using antibodies against E2F2. Non-transgenic small intestinal extracts from E15.5 embryos and TAg^{wt} small intestinal extracts from E18.5 embryos were also subjected to E2F EMSA supershift analysis using antibodies against E2F3. Species 'y' supershifts with antibodies against E2F3 in non-transgenic E15.5 and TAg^{wt} E18.5 small intestinal extracts. Species 'y'' supershifts with antibodies against E2F2 in TAg^{wt} P0.5 intestinal extracts, while both species 'y' and 'y'' supershift with antibodies against E2F2 in TAg^{wt} adult intestinal extracts. α = antibody.

5.4. DISCUSSION

Intestinal development is controlled by many factors, initiating from signaling cascades like the Wnt/β catenin pathway, FGF signaling, Notch and TGFβ pathways (Sancho et al., 2004; Hauck et al., 2005). However, they all must converge downstream on the pathways that regulate the cell cycle, such as the Rb/E2F pathway. These studies investigated the levels and activities of specific Rb and E2F family members throughout intestinal development. The results presented here support previous results describing the presence of p107, E2F2, E2F3 and E2F4 transcripts throughout embryonic development.

T antigen is expressed early in the developing intestine (E15.5), but does not alter Rb and E2F levels and activities until E18.5, when the levels of E2F2 are up-regulated. Since E2F3a protein and activity is present in non-transgenic and TAg^{wf}-transgenic intestines at E15.5, and does not change in levels until birth in non-transgenic intestines, one hypothesis is that E2F2 expression is regulated by E2F3a in the developing intestine. This hypothesis suggests a model that as the intestinal epithelium develops villi, E2F2 becomes restricted to the intervillus region by the repression of its promoter in villus cells in part through a pRb/E2F3a complex, reducing the overall levels of E2F2 in non-transgenic intestines (Fig.24). T antigen expression in villus enterocytes, however, disrupts pRb/E2F3a complexes, avoiding the repression of E2F2, thus up-regulating its overall expression. E2F3a is down-regulated by birth, possibly due to the presence of p130/E2F5 or p130/E2F4 complexes on its promoter in villus cells. T antigen down-regulates p130 levels, disrupting these repressive complexes and avoiding the repression of the E2F3a promoter, thereby maintaining the levels of E2F3a.

These data suggest that E2F3a is the key E2F that regulates proliferation of the developing intestinal epithelium. However, this study is incomplete. The levels of the other Rb and E2F family members remain to be elucidated and the DNA binding activities of all Rb and E2F family members will also have to be confirmed in both non-transgenic and TAg^{wf}-transgenic developing intestines. Ideally, chromatin immunoprecipitation experiments would be performed using antibodies against the Rb and E2F family members to demonstrate the endogenous promoter occupation of these transcription factors in the developing intestine. However, the protocol for chromatin immunoprecipitation will have to be modified for use on whole intestinal tissues as the current protocol necessitates removal of the epithelium from the underlying mesenchyme, a procedure that cannot currently be undertaken with embryonic or neonatal

intestines. Targeted gene knock-outs of E2F3a and other Rb and E2F family members in the intestinal epithelium might also be informative.

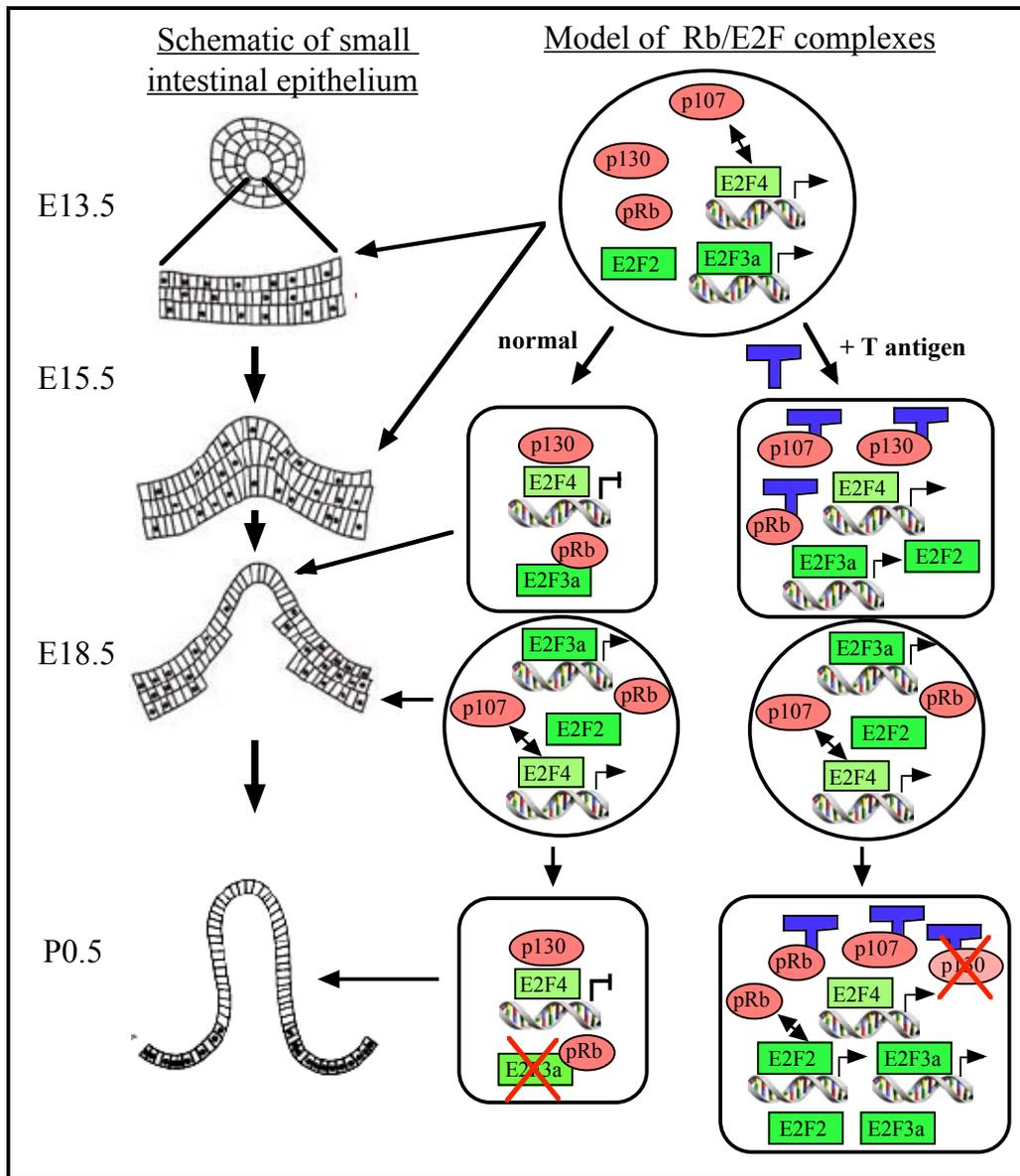


Figure 24. Model of T antigen effects on Rb/E2F complexes throughout intestinal development. In the early developing intestine, E15.5, both non-transgenic and TAG^{wt}-transgenic small intestines contain p107, E2F4, E2F5 and E2F3a DNA binding activity, and also express E2F2 protein. As the villus structures develop, repressive p130/E2F4 complexes increase in newly formed villi of non-transgenic mice, while E2F3a DNA binding activity and E2F2 protein levels become restricted to the intervillus regions in non-transgenic, although E2F3a protein levels are maintained in the villi. T antigen binds p130, de-repressing E2F responsive promoters, allowing for the

maintenance of E2F2 levels in the villi. T antigen also binds pRb, blocking its repression of E2F3a, thus allowing the maintenance of E2F3a DNA binding activity in villi. By birth, E2F3a levels and activity are restricted to the intervillus region, while p130/E2F4 complexes maintain growth suppression in villi in non-transgenic mice. T antigen down-regulates p130 levels in villi, maintaining the de-repression of E2F4. T antigen also maintains E2F3a DNA binding activity and induces E2F2 DNA binding activity, both in a complex with or free of pRb, in villi by virtue of a positive-feedback loop between these E2Fs.

6. CONCLUSIONS AND DISCUSSION

6.1. MODEL OF T ANTIGEN-INDUCED HYPERPLASIA/DYSPLASIA IN THE INTESTINAL EPITHELIUM

The results from these studies suggest that different Rb and E2F family members play distinct roles in maintaining growth-arrest and regulating normal and abnormal proliferation in the intestinal epithelium. These data also indicate that p53 does not play a significant role in maintaining homeostasis in the enterocyte lineage of the intestinal epithelium. This surprising result may indicate that p53 functions mainly in cells with long life spans or with greater potency, such as stem cells.

A compelling model of Rb/E2F activity in the normal and T antigen-induced hyperplastic intestinal epithelium can be compiled from these data and current data in the literature (Fig.25). In the developing intestine, E2F3 (both a and b), E2F2 and E2F4 all play roles in regulating the cell cycle. The main regulators at this time are E2F3 and E2F4. E2F3a actively drives the cells through proliferation, resulting in rapid turnover during development. Since E2F2 does not contain detectable DNA binding activity in the normal developing intestine, it may behave as a surveillance factor to monitor appropriate levels of proliferation. As the proliferation attenuates and becomes restricted to the intervillus region by birth, pRb represses E2F3a and E2F2. At this time, E2F4 becomes the main regulator of proliferation, using the passive mechanism of

repression/de-repression through interactions with p107 and p130. This passive regulation allows for differentiation signals to transduce in the stem and progenitor cells. In the fully developed intestine, the dividing cells of the crypts are predominantly regulated by this passive mechanism of E2F4 activity, although E2F1, E2F2 and E2F3 are all expressed at low levels and regulate promoters in the more rapidly dividing progenitor cells. Their activity is controlled by pRb. As cells migrate up the crypt-villus axis, exit the cell cycle and differentiate, p130/E2F4 complexes maintain the repression of E2F-responsive gene promoters. Their repression is initiated by pRb/E2F3 or pRb/E2F2 in the progenitor cells of the crypts. In the absence of pRb activity, either due to direct mutation or interaction with T antigen, E2F3 and E2F2 are not repressed. The T antigen-directed degradation of p130 results in de-repression of E2F4-mediated activity. Thus, T antigen expression causes both the de-repression and activation of E2F-responsive genes, resulting in robust proliferation. The “activating” E2Fs are subsequently over-expressed due to the loss of their repression by p130/E2F4 complexes. This over-expression may be part of a tumor surveillance mechanism that is blocked due to the disruption of pocket protein activity. Perhaps the “activating” E2Fs can act as tumor suppressors independent of pRb, but this activity is also blocked by T antigen.

This model of Rb/E2F activity in the intestine compiled from these studies may provide a more general model of Rb/E2F activity that could be applied to normal or abnormal proliferation in different tissue types and at different stages of development. In general, the most rapid period of cellular proliferation is during development. Proliferation attenuates and becomes restricted to particular cells and tissues by the time the organism reaches its adult size. Different tissues at various developmental time points have distinct rates of proliferation or proliferative potential and are thus predicted to be regulated by specific Rb/E2F complexes. These tissues would be

affected by the loss of particular Rb and E2F family members. The “activating” E2Fs, as regulated by pRb, drive cells through the cell cycle when over-expressed, therefore causing robust proliferation. The regulation of proliferation during development may depend primarily on “activating” E2F and pRb/E2F complexes, due to the rapid proliferation. However, in homeostatic regulated proliferation in the adult, this regulation may depend primarily on “repressing” E2Fs such as E2F4 and E2F5 by interacting with pocket proteins. The “repressing” E2Fs do not drive cells through the cell cycle, but allow cells to enter through their de-repression, a more passive mechanism. E2F4 and E2F5 can also transactivate E2F-responsive promoters once de-repressed. The “activating” E2Fs are expressed downstream of the repressive E2Fs. High levels of proliferation cause the up-regulation of “activating” E2F levels. One result of their upregulation might be to signal the cell that it is undergoing high levels of proliferation. The signal is transduced through the actions of pRb, activation of ARF or other tumor suppressive functions of these E2Fs, resulting in growth arrest, senescence or apoptosis. If this signal is not transduced and there is a plethora of “activating” E2Fs, then the cell can become transformed.

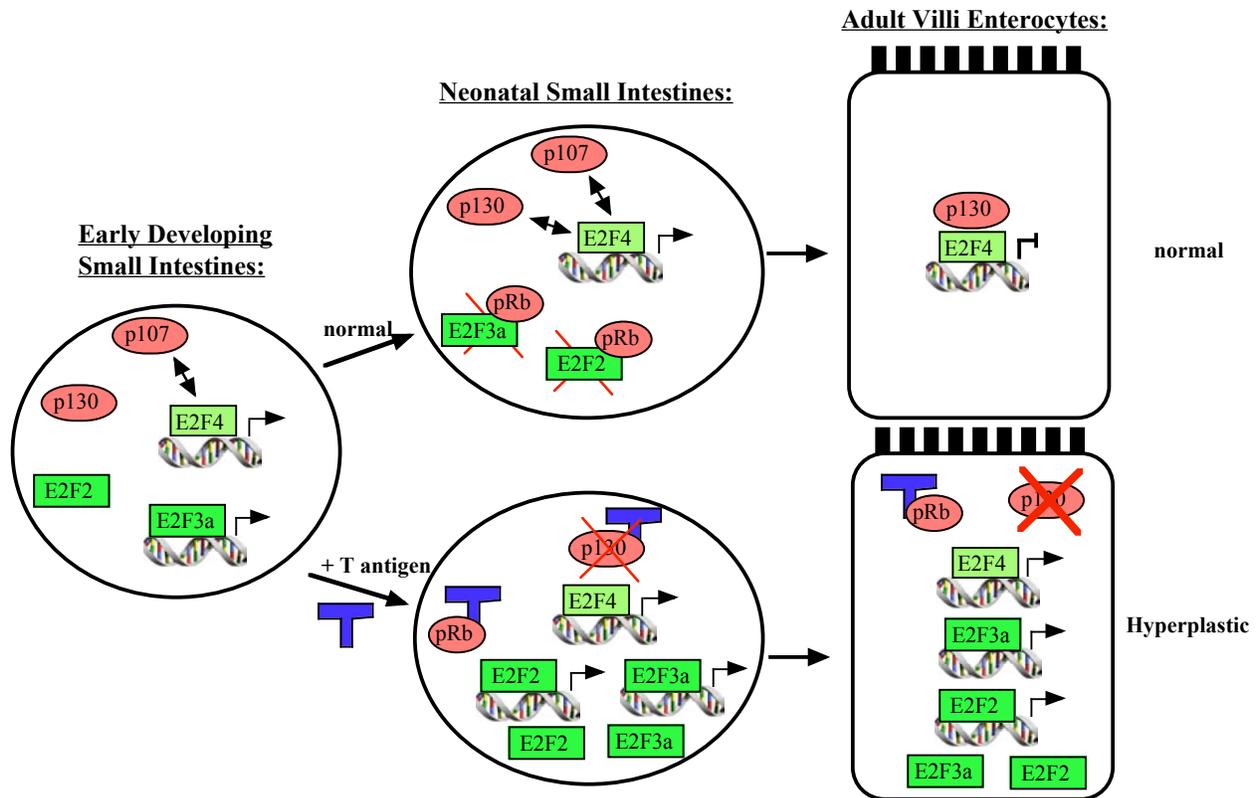


Figure 25. Timeline and mechanistic model of T antigen-induced intestinal hyperplasia. E2F3a and E2F2 are present in the early developing intestine, although the majority of DNA binding activity is E2F3a. As more of the cells differentiate and growth arrest in the normal intestine, these E2Fs are repressed and down-regulated via pRb. By adult, the main Rb/E2F complex in the growth arrested villi cells is p130/E2F4. In T antigen transgenic intestines, p130 levels are targeted by T antigen for down-regulation, thus causing de-repression of E2F4 activity. This de-repression allows for the maintained expression of E2F3a. E2F2 and E2F3a are maintained in an auto-regulatory feedback loop and prevented from repression by pRb. By adult, the main active E2F complexes in the cycling cells of the villi are E2F2, E2F3a and de-repressed E2F4. This hyperproliferation, driven by these E2Fs, causes hyperplasia.

6.2. CELL TYPE SPECIFICITY IN CANCER

A significant result from these studies is the disparate expression of T antigen and mutants along the crypt-villus axis. The T antigen mutants, *3213* and *N136*, express in the expected pattern as described for the *fabpi* promoter: highest at the tip of the villus and decreasing towards the base but none at all in the crypts. This pattern of expression is demonstrated both by immunoblot as well as preliminary immunohistochemical studies (this report and unpublished data, A. Rathi, University of Pittsburgh). *WT* T antigen expression pattern differs from the other two transgenes; highest in the mid-and-lower-villus region, lower at the tips of the villi as well as at the tops of the crypts. These results are perplexing since the same promoter construct (*fabpi*^{-1178 to +28}) drives expression of all three transgenes. It is important to delineate the cell of origin where these transgenes are expressed because if they are expressed in different cells, this adds another variable to the explanation for their different phenotypes. T antigen expression in the crypts may indicate that it is originally expressed in a progenitor cell type that is not normally growth-arrested, but is prevented from growth arrest by T antigen. If T antigen is expressed in the growth-arrested cells of the villus, then it may have to perform different tasks to induce the cell to re-enter the cell cycle after growth arrest. Thus, a difference in target cell of origin between *WT* and *N136* may explain differences there are between their respective phenotypes.

If the proliferation induced by T antigen in villus enterocytes is the same as the proliferation of progenitor cells in the crypts, then T antigen expression in the crypts should not have an effect on proliferation. However, Chandrasekaran and colleagues made the observation that there is a difference in cell cycle kinetics between normal cycling progenitor cells of the crypts and T antigen-induced cycling differentiated enterocytes (Chandrasekaran et al., 1996). TAg villi have a 40:1 ratio of BrdU labeled cells to M phase cells, whereas non-transgenic crypts

have a 12:1 ratio. This indicates that there is a difference in cell cycle kinetics between the two different cycling cell types. Specifically, more cells are in S phase at any one time in T antigen-expressing differentiated enterocytes relative to the stem and progenitor cells of the normal intestinal epithelium. The entire cell cycle might be longer in T antigen-expressing enterocytes, or S phase may be extended and the other phases condensed in these cells as compared to normal proliferating cells of the crypts. These results indicate that T antigen causes a novel type of proliferation in villi enterocytes, different from the normal proliferation of progenitor and stem cells in the crypts. These results suggest that expression of T antigen in crypt progenitor cells would have an effect on proliferation, at least in cell cycle kinetics. The outcome of that effect, however, remains unclear.

Preliminary studies of multiple lines of TAG^{N136} containing one or two copies of the chromosome carrying the transgene insertion indicate that that intestinal phenotype appears to correlate directly to expression levels of the transgene. Expression levels between zero and low detection do not result in a phenotype (data not shown). However, moderate expression in the villi, but zero to low expression in the crypts results in a hyperplastic phenotype (data not shown). In very high expressing lines or homozygous TAG^{N136} mice, high expression in both the villi and crypts results in severe hyperplasia and occasional dysplasia (data not shown). This dysplasia is not identical to the dysplasia induced by *WT* T antigen and will need further characterization by a pathologist (personal communication, M.T. Sáenz-Robles, University of Pittsburgh). Although the expression levels of these different transgenic proteins have not been stringently quantified and may not be easily comparable between one mutant and another, such as *WT* and *N136*, these qualitative observations indicate the importance of monitoring expression levels. These preliminary data led to a hypothesis of cell localization and threshold level of

expression for T antigen-induced intestinal hyperplasia and dysplasia (Fig.26a). The first 136 amino acids of T antigen are sufficient for these effects in this model, while binding and disrupting Rb activity is required. It is hypothesized that T antigen only has an effect above a threshold level of expression. Expression above this threshold in the villi causes hyperplasia, while dual expression above this threshold in the villi and crypts causes dysplasia. Future analyses of multiple lines of TAg^{wt}, TAg^{N136} and TAg³²¹³ expressing T antigen at different levels in different compartments will have to be performed for confirmation.

The cell type of origin that is targeted for cancer is becoming a widely appreciated question in the field. Cancers that are derived from different genetic disruptions or different cell types respond to distinct chemotherapies, probably due to differences in cell type-specific regulatory pathways. For instance, one particular therapy for HER-2/neu (an epidermal growth factor receptor – EGFR) over-expressing tumors, using antibodies directed towards the receptor, such as trastuzumab, is more effective than another breast-cancer therapy such as COX-2 inhibitors (Gasparini et al., 2005). COX-2 inhibitors target another type of breast cancer that is presumably derived from a different mutation in a distinct regulatory pathway. The treatment success of antibody targeting of the EGFR in breast cancer is in contrast to the relative treatment failure of antibody targeting of the EGFR in colorectal cancer (CRC). Most CRC patients do not respond to antibody drugs that target EGFR, such as cetuximab, even in the patients that show high levels of EGFR over-expression. Furthermore, there are no criteria for patient selection for these drugs in CRC like there are in breast cancer (Cohen et al., 2005). These clinical results support molecular data that suggest that tumors derived from different cells have specific molecular profiles and therefore, respond to specific therapies. Therefore, it is important to

understand the differences in tumor suppressor pathways between different cell types so that more specific cancer therapies can be developed.

Expression of T antigen in different cell types of the intestine causes distinct effects (Fig.26b). The results presented in this report demonstrate that the disruption of p53 does not play a role in the intestinal hyperplasia and dysplasia induced by T antigen expression in enterocytes. These results indicate that p53 does not function to regulate homeostasis in intestinal villi enterocytes. In contrast, transgenic expression of T antigen in goblet cells of the intestinal epithelium causes these cells to enter S phase and then die by apoptosis, thereby preventing neoplasia (Fig.26b) (Gum et al., 2001). Since T antigen is capable of binding and disrupting p53, thereby inhibiting p53-dependent apoptosis, this apoptosis in the goblet cells is probably p53-independent (Gum et al., 2001). Another possibility is that p53 does induce apoptosis in the T antigen expressing goblet cells, but that T antigen is incapable of binding, stabilizing and disrupting p53 function because there is another factor that is required for this function missing in these cells.

In another transgenic model, Paneth cell-specific promoter driven expression of T antigen in the intestinal epithelium causes the deletion of mature Paneth cells, but expands the number of “granulomucous,” intermediate cells in the crypts (Garabedian et al., 1997). These intermediate cells are the progenitor precursor cells for the Paneth and goblet cell lineage. It was noted that there is an increase in apoptosis in the crypts of the transgenic intestines. The authors suggest that these may be mature Paneth cells that commence apoptosis upon maturation due to expression of T antigen. Interestingly, T antigen-expressing cells remained intermediately differentiated in the crypts. However, as they migrate into the villus, they differentiate into mature goblet cells that retain the ability to proliferate (Fig.26b). These results indicate a non-

autonomous differentiation schedule for the intestinal epithelial cells. As opposed to expression in villus enterocytes, expression of T antigen in intermediate granulomucous cells inhibits Paneth cell formation by causing apoptosis, while it allows differentiation of goblet cells in the villi (Garabedian et al., 1997). T antigen expression in the granulomucous cells in the crypts causes proliferation, while T antigen expression in goblet cells in the villi causes entry into S phase and then apoptosis (Garabedian et al., 1997; Gum et al., 2001).

Expression of T antigen in secretin-expressing enteroendocrine cells causes regions of large intramucosal, invasive tumors in the small intestine that undergo de-differentiation, as demonstrated by loss of some enteroendocrine-specific markers (Lopez et al., 1995). These tumors are more severe than the early stages of tumorigenesis that T antigen causes when expressed in enterocytes (Fig.26b). The de-differentiation of the enteroendocrine cells in these T antigen-induced tumors is in contrast to the unaltered differentiation by T antigen expression in enterocytes. These neuroendocrine tumors in the small intestine require expression of both large T and small t antigens with intact J domains (Ratineau et al., 2000). These results suggest that small t antigen interaction with PP2A is required for formation of these neuroendocrine tumors. It remains to be determined if intestinal hyperplasia and dysplasia caused by expression of the SV40 early region in enterocytes requires expression of small t antigen and/or the J domain(s). However, transgenic mice expressing a mutant of T antigen in the J domain (*D44N*) driven by the same *fabpi* promoter have recently been generated and will soon be analyzed (personal communication, M.T. Sáenz-Robles, University of Pittsburgh).

expression of T antigen causes different phenotypes. Expression of T antigen in different cell types of the intestinal epithelium causes hyperplasia or dysplasia in the absorptive cell lineage, or S phase and or apoptosis in the paneth and goblet cell lineage, or causes aggressive tumorigenesis and de-differentiation in mature enteroendocrine cells.

6.3. SPECIFICITIES OF THE RB AND E2F FAMILIES IN CANCER

It is intriguing to speculate how it is that T antigen expression in different cell types causes distinct effects. One possibility is that specific factors in various cells present different targets for T antigen action, and these actions therefore have distinct outcomes. However, the Rb and E2F families of proteins are believed to be ubiquitous to all cell types, therefore T antigen action on these proteins are predicted to be similar for all cell types. The data presented in this report suggest that T antigen alters the levels and activities of specific Rb and E2F family members in the intestinal enterocytes, in some cases contrary to effects by T antigen in other transgenic models, such as in the choroid plexus or the lens (Pan et al., 1998; Chen et al., 2000a). These results suggest that Rb and E2F family members may play different roles in specific tissue and cell types.

6.3.1. Biological specificities of Rb and E2F family members.

The classic model of Rb/E2F functions throughout the cell cycle describes similar functions for E2F1, E2F2 and E2F3, the “activating” E2Fs, as well as similar functions for E2F4 and E2F5, the “repressing” E2Fs. All three Rb family members are able to repress E2F-dependent transcriptional activation, and each can be phosphorylated by cyclin-dependent kinases. As far as

the model describes, only one Rb should be necessary to perform its functions and only two E2Fs should be required – one “activating” E2F and one “repressing” E2F. In lower eukaryotes, this minimal-member system is the case. For instance, *Drosophila* has two Rb family members, dRBF1 and dRBF2, two E2Fs, dE2F1 and dE2F2, and one DP, dDP (Stevaux et al., 2002a). dRBF1 contains many regions of homology to all three of the Rb family members although more with p107 and p130. dRBF2 contains even more regions of homology to p130 and p107 that are not contained in dRBF1, such as the spacer region between the A and B pockets. dE2F1 is the ortholog of the “activating” E2Fs, while dE2F2 is the ortholog of the “repressing” E2Fs, (not to be confused with mammalian E2F2.) The worm, *C. elegans*, has single orthologs of Rb (*lin-35*), E2F (*efl-1*) and DP (*dpl-1*) (Ceol and Horvitz., 2001). These organisms function with only one or two Rb, E2F and DP. Therefore, a major question in the field remains: why are there so many members of the Rb and E2F families in mammals?

There are many proposed explanations for why there are so many Rb and E2F family members in mammals, each with some supporting evidence. One proposal is that the functional redundancy in these proteins is for compensation in the absence of another family member. For mammals with substantially longer generation times than flies or worms, compensation is important since dependence on one gene per cell cycle function would be more costly for us. Data that supports this comes from the phenotypes of the targeted germ-line knock-out mice that have been generated for the different genes, many of which do not result in lethality (Table 1). For instance, the targeted disruption of pRb or p130 function in the myocardium is endured and does not result in a phenotype, but the disruption of both genes results in increased proliferation and apoptosis (MacLellan et al., 2005). These results suggest that these pocket proteins are functionally redundant in the myocardium, at least in the absence of the other. Other proposals

suggest that, although the gene products have redundant functions, their expression is regulated in different ways. In this scenario, the regulatory sequences surrounding the different loci would direct the expression of the genes in specific tissue types or at specific times in development. In general, it appears that this may not be the case as the expression of the Rb and E2F family proteins is fairly ubiquitous in mouse tissues. Detailed characterization of Rb and E2F family member expression in specific cell types in these tissues has not been thoroughly investigated. In general, the expression of each Rb and E2F family member is ubiquitous in cells that grow in culture. The data that support a role for differences in tissue and cell type regulation comes from the effects of deleting one or more Rb or E2F family members, however these effects are often shared between multiple members. For instance, the deletion of both p130 and p107 affects limb development in mice, whereas the deletion of pRb alone affects erythropoiesis (Cobrinik et al., 1996; Clark et al., 2004; Spike et al., 2004).

A third proposal explaining the multiplicity of E2F and Rb family members is that they each have subtle differences in function. These differences in “biological specificity” could be achieved by interactions with other proteins, specific to the different Rb and E2F family members, that can modulate their activity (Table 3) (Attwooll et al., 2004). This proposal could also be supported by differences in the phenotypes of the knock-out mice, since different tissue and cell types express different modulators that interact with these Rb and E2F family members, or even have differences in accessibility to E2F binding sites in specific promoters (i.e., one site could be inaccessible in heterochromatin in one cell type, but available for binding in another). It has been shown that different E2F DNA binding complexes have some differences in affinity for E2F-binding sequences, although the biological significance of these differences remains to be assessed (Tao et al., 1997).

The proto-oncogene, Myc, is a modulator of E2F activity. Myc is a transcription factor that is activated downstream of many growth factor signaling pathways, and has the ability to either induce growth or apoptosis. These two pathways appear to either work in parallel to regulate overlapping sets of genes, as many E2F-responsive gene promoters also contain Myc binding sites (E boxes), or can also behave synergistically or antagonistically, depending on their relative abundance and interactions (Santoni-Rugiu et al., 2000). In one set of experiments, it was demonstrated that Myc depends on different E2Fs for its ability to induce S phase or apoptosis (Leone et al., 2001). Specifically, E2F2 and E2F3 are required for Myc-induced S phase entry, while E2F1, and to a lesser extent E2F2, is required for Myc-induced apoptosis. These effects were also noted to be synergistic, which suggests that both Myc *and* these E2Fs could be binding the same promoters to synergistically transactivate them. On the repressive end, c-Myc interacts with pRb *in vitro* (Rustgi et al., 1991). However, only an interaction with p107 was shown *in vivo* (Beijersbergen et al., 1994). This interaction results in the repression of c-Myc transactivation and over-expression of c-Myc can release cells from a p107-mediated arrest, but not a pRb-mediated arrest in osteosarcoma cells. These data suggest a specific alternative mechanism for the growth suppressive properties of p107 through interaction with c-Myc.

Another modulator of E2F biological specificity is TFE3, a helix-loop-helix, E-box binding, transcription factor (Giangrande et al., 2003). TFE3 interacts specifically with E2F3 through the marked box domain of E2F3. However, it does *not* interact with E2F1, E2F2, or E2F4. Although TFE3 is ubiquitously expressed, it appears to confer specificity in promoter transactivation with E2F3. Both TFE3 and E2F3a are mutually dependent on each other for transactivation of the gene encoding the p68 subunit of DNA polymerase α , but not DHFR. Interestingly, co-expression of E2F3b could not transactivate the p68 promoter, and in fact the

triple expression of E2F3a, E2F3b and TFE3 resulted in repression of the promoter activity, suggesting that E2F3b can specifically antagonize E2F3a activity. Both proteins can bind TFE3.

The bridging molecular, RYBP, interacts with specific E2Fs. In this case, both E2F2 and E2F3 bind RYBP, which associates them in a complex with YY1 (Schlisio et al., 2002). The *cdc6* promoter contains both YY1 and E2F binding sites and requires both sites for its activation. The interaction between RYBP and E2F3 also depends on the marked box domain of E2F3. Together, overexpression of E2F2 or E2F3, RYBP and YY1 is required for full promoter activity. E2F1 and E2F4 do not interact with RYBP and do not behave synergistically with RYBP and YY1 to transactivate the *cdc6* promoter. These data were confirmed by chromatin immunoprecipitation experiments demonstrating that E2F2, E2F3, RYBP and YY1 are associated at the *cdc6* promoter during G₁/S phase of the cell cycle, while E2F1 is not. Interestingly, E2F6 co-immunoprecipitates with RYBP as well. In this complex, however, RYBP bridges E2F6, also through its marked box domain, with other members of the polycomb group of chromatin remodeling proteins like Ring1 and Bmi1 (Trimarchi et al., 2001). Here, different E2Fs use a shared interaction with RYBP to have antagonizing effects – activation or repression. Possibly, the absence of a transactivation domain on E2F6 allows for RYBP to recruit the other PcG proteins into this complex. Alternatively, perhaps, E2F6 behaves as a bridging molecule between these PcG proteins other PcG proteins such as EPC1 and EZH2 with which E2F6 interacts (Attwooll et al., 2005).

Two other E2F modulators bind specifically to E2F1: TopBP1, (**Topoisomerase II β -Binding Protein 1**), and GABP γ 1, an Ets-related transcription factor (Hauck et al., 2002; Liu et al., 2003). Both of these proteins bind E2F1 but *not* E2F2, E2F3 or E2F4. In the case of TopBP1, E2F1 binds it through its amino-terminus and through the sixth BRCT (**BRCA1** carboxy-

Terminal) domain of TopBP1. This interaction results in pRb-independent repression involving the recruitment of Brg/Brm, a member of the SWI/SNF chromatin remodeling complex (Liu et al., 2004). This interaction appears to be involved in a negative feedback loop since TopBP1 is a target of E2F transcriptional regulation at the G₁/S phase transition, but also plays a role in inhibiting E2F1 transactivation and apoptosis. The interaction of E2F1 with GABP γ 1 occurs through the carboxy-terminal region of E2F1 containing the Rb-interaction and transactivation domains. In contrast to the interaction with TopBP1, GABP γ 1 synergistically induces E2F1-mediated transcription to activate apoptosis (Hauck et al., 2002). The finding that E2F1 interacts with GABP γ 1 is in accord with previous data describing promoter elements of the p180 subunit of DNA polymerase α containing DNA binding elements that bind SP1, GABP and E2F1 (Izumi et al., 2000). The SP1 and GABP elements are required for basal transcription while the E2F element is required for growth-stimulated transcription. Together, these data suggest a specific interaction between E2F1 and GABP γ 1 in both functions of E2F1: growth stimulation and apoptosis.

Table 3. E2F protein interactions conferring biological specificity between family members.

Protein	E2F family member	Result of interaction	Reference
c-Myc	E2F1	apoptosis	(Leone et al., 2001)
	E2F2	S phase entry and apoptosis	
	E2F3a	S phase entry	
TFE3	E2F3a	transactivate DNA polymerase α p68 promoter	(Giangrande et al., 2003)
	E2F3b	repress DNA polymerase α p68 promoter	
RYBP	E2F2 & E2F3	complex with YY1 to synergistically transactivate cdc6 promoter	(Schlisio et al., 2002)
	E2F6	complex with PcG proteins, Ring1 and Bmi1, promoter silencing	(Trimarchi et al., 2001)
TopBP1	E2F1	repress E2F responsive promoters to inhibit apoptosis	(Liu et al., 2004)
GABPγ1	E2F1	synergistic transactivation of E2F responsive promoters to activate apoptosis	(Hauck et al., 2002)

Cell type differences in E2F function have also been described. In lymph nodes and Jurkat T cells, E2F1 positively regulates its own promoter as well as the Orc1 promoter, whereas E2F2 represses their expression (Murga et al., 2001). In this example, two “activating” E2Fs can regulate the same promoter in the same cell type, but in antagonizing modes. The mechanism by which these antagonizing effects occur remains to be elucidated.

Another cell type where E2Fs have different effects is in cardiomyocytes. Over-expression of E2Fs 1-4 are all able to stimulate cardiomyocytes into S phase, but only E2F1 and E2F3 are also able to induce apoptosis in these cells (Ebelt et al., 2005). E2F5 overexpression also showed some signs of pro-apoptotic effects as these cells had below G₁ levels of DNA content, but was unable to stimulate cells into S phase. E2F2-over-expressing cells also divided more (Ebelt et al., 2005). These data demonstrate differences between the E2Fs in their abilities to cause S phase entry, apoptosis and/or mitosis in cardiomyocytes. These authors also describe differences in gene expression in the different over-expressing cell types by quantitative RT-PCR. For instance, over-expression of E2F1-4 all significantly up-regulated cyclin E expression, all but E2F2 up-regulated cyclin D2 expression. The significance of these data remains to be determined, but it does support a model for cell type and functional specificity amongst the E2F family members.

6.3.2. Rb and E2F family members play important roles in development and differentiation

In addition to their function as cell cycle regulators, the Rb and E2F family members are also required for proper development and differentiation of particular tissues and cells (Korenjak and

Brehm, 2005). These functions further demonstrate biological specificity between the Rb and E2F family members. The pocket proteins play specific roles, both related and unrelated to their growth suppressive properties, in erythropoiesis, extra-embryonic tissue development, myogenesis, adipogenesis, chondrogenesis and osteogenesis, gametogenesis, limb development, and distinct epithelial cell differentiation (Sellers et al., 1998; Lipinski et al., 1999; Hansen et al., 2004b). The E2F family of proteins play roles, mostly related to their growth regulating properties, in neurogenesis, gametogenesis, epithelial cell differentiation, and developmental patterning (Persengiev et al., 1999; Müller et al., 2001; Cooper-Kuhn et al., 2002; Landsberg et al., 2003). A brief discussion is presented here of the roles that these proteins play in development and differentiation that are separate from their cell cycle regulatory roles.

Mice with germ line, homozygous null mutations in *RB* die early in embryogenesis, due to defects in extra-embryonic tissue development and erythropoiesis (Table 1); (de Bruin et al., 2003b). Conditional ablation of *RB* in the embryo, but not in the extra-embryonic tissue, partially rescues this phenotype, although these mice also die by birth (de Bruin et al., 2003b; MacPherson et al., 2003). One of the defects that remains is in erythropoiesis, determined to be a cell-intrinsic defect (Clark et al., 2004). The loss of Rb in erythroid cells in culture results in impaired cell cycle exit and a delay in differentiation, as measured by loss of enucleation and absence of cell type specific markers.

Another defect that remains in the embryonic-conditional *RB* null mice is in muscle growth and differentiation due to severe skeletal muscle dysplasia (de Bruin et al., 2003b). pRb regulates myoblast differentiation by binding indirectly to MyoD, a myoblast-specific transcription factor (Li et al., 2000). Together, these proteins stimulate myocyte differentiation, in part by establishing irreversible growth arrest as well as activating transcription by MEF2, a

muscle-specific transcription factor (Novitch et al., 1999). This function of pRb requires its LxCxE motif-binding domain, but seems to be independent of interaction with HDACs as addition of an HDAC inhibitor, trichostatin A, has no effect on pRb-induced myogenic differentiation (Chen and Wang, 2000b).

During osteogenesis, pRb interacts with CBFA1, a runt family transcription factor involved in osteoblast differentiation, to synergistically activate osteoblast-specific gene transcription and induce differentiation (Thomas et al., 2001). Loss of pRb inhibits late osteoblast differentiation, but not loss of p107 or p130, indicating a specific, non-redundant role for pRb in osteogenic differentiation. p107 and p130 also have overlapping roles in bone development (Cobrinik et al., 1996). p107 regulates timely expression of the osteocalcin gene in osteoblasts (van Gurp et al., 1999). During early osteoblast differentiation, osteocalcin (OC) is repressed, but upon cell cycle exit and maturation, its expression is up-regulated. p107 binds the OC promoter in a complex containing cyclin A and the homeobox protein, *CDP/cut*. This complex is down-regulated upon growth arrest, allowing for its expression. Over-expression of *CDP/cut* can repress promoter activity. These results implicate opposing roles for p107 and pRb in regulating osteoblast differentiation.

pRb also plays a role in adipocyte differentiation, specifically white adipocytes, because its inactivation by SV40 T antigen causes cells to transdifferentiate into brown adipocytes (Hansen et al., 2004b; Hansen et al., 2004a). This differentiation function is specific to pRb as p107 antagonizes pRb-induced differentiation of adipocytes (Classon et al., 2000). pRb induces this differentiation by activating the differentiation-associated transcription factor, *C/EBP α* . Inactivation of p107, however, appears to be required to induce differentiation, and this may be mediated by *PPAR γ* , as loss of p107 minimizes the requirement for *PPAR γ* to induce

differentiation. E2F4 also plays a role upstream of pRb in adipocyte differentiation, independent of its cell cycle role (Landsberg et al., 2003). Loss of E2F4 in MEFs causes cells to undergo spontaneous differentiation when maintained at confluence. Loss of E2F4 did not alter cell cycle kinetics or change E2F-responsive gene expression in these differentiated MEFs. Expression of an E2F4 protein without its Rb-binding domain had little effect on its ability to repress differentiation. These data suggest that E2F4 represses differentiation of adipocytes independently of its cell cycle regulatory functions.

E2F4 also plays a role in nerve-growth factor (NGF)-induced differentiation of PC12 cells, a pheochromocytoma cell line that undergo differentiation into sympathetic-like neurons upon growth arrest and in the presence of NGF (Persengiev et al., 1999). Loss of E2F4 diminishes differentiation of PC12 cells as induced by NGF, but does not affect the growth status. Interestingly, E2F2 also plays a role in differentiation of this cell line (Persengiev et al., 1999). In contrast to E2F4, however, E2F2 does affect the growth potential of these cells. Differentiated PC12 cells can undergo de-differentiation and re-enter the cell cycle upon withdrawal of NGF. Over-expression of E2F2 causes apoptosis upon NGF withdrawal. However, it is unclear if E2F2 is required for maintenance of a post-mitotic state in differentiated PC12 cells, or NGF is required for survival in the presence of over-expressed E2F2. Assessing the ability of PC12 cells to growth arrest and differentiate upon NGF in the absence of E2F2 would discern between these two possibilities.

In conclusion, Rb family members regulate multiple transcription factors other than E2F to activate or block differentiation. E2F family members generally block differentiation by affecting the growth status of the cell, as most cells are believed to terminally differentiate upon growth arrest. For this reason, it is hard to delineate differentiation-specific functions for the Rb

and E2F family proteins that are separate from their cell cycle regulatory functions. It is clear that their major function in the cell is to control entry and exit of the cell cycle predominantly through transcriptional regulation.

6.4. NEW PERSPECTIVES ON RB AND E2F PROTEIN FUNCTIONS

The results presented in this report demonstrate that SV40 large T antigen does not treat all Rb family members equally in the intestinal epithelium. Although T antigen binds all three pocket proteins dependent on the LxCxE motif, as expected, it causes the down-regulation of p130 levels and the up-regulation of p107 levels. The up-regulation of p107 can be explained by the fact that it is an E2F-regulated gene and many, if not all, E2F-regulated genes are up-regulated in T antigen expressing enterocytes. The down-regulation of p130 can be explained by supporting data demonstrating the T antigen can target p130 for proteasome-mediated degradation (Stubdal et al., 1997). A surprising result is that T antigen expression in villus enterocytes induces pRb/E2F2 DNA binding activity and induces pRb occupancy of several E2F-responsive gene promoters. In support, T antigen selectively disrupts p130/E2F and p107/E2F but not pRb/E2F complexes when monkey kidney cells are infected with SV40 (Sullivan et al., 2004). It appears that in the intestinal epithelium T antigen is not only inefficient at disrupting all pRb/E2F complexes, but its expression actually induces these complexes. Furthermore, if pRb is a repressor of E2F-responsive promoter transcription, how can these results be explained, especially since these promoters are associated with genes that have up-regulated transcripts?

There are several possible explanations for the specific increase of pRb activity in T antigen expressing cells. The first explanation is that the transcripts from these E2F-responsive

genes are up-regulated due to a post-transcriptional stabilization mechanism as opposed to increased transcription, therefore pRb is indeed maintaining repression of these promoters. However, if this were the case, then it might be expected that pRb would also be present on the same promoters in the non-transgenic intestinal villi, which is not the case. Another explanation is that the cycling cells in the villi of TAg^{WT}-transgenic intestines are in all stages of the cell cycle at any one point in time, therefore, sometimes the genes are repressed while other times, they are transactivated, but on a whole they are up-regulated as compared to non-transgenic. Although T antigen affects the cell cycle directly by acting downstream of the cyclin/cdk complexes that regulate pRb activity, the E2F pathway up-regulates cyclin D and cyclin E, therefore enabling these pathways to regulate pRb like in normal cycling cells. A third explanation is that pRb is not always a repressor, even in complex with E2F and bound to E2F-responsive promoters. One mechanism by which this may occur is through a balance of signals at a promoter that has two E2F binding sites. One binding site may have a pRb/E2F complex bound to it, while the other may have a pRb-free E2F complex. In such a case, the site with the pRb/E2F complex may act neutral while the site with the pRb-free E2F complex may behave dominantly to transactivate expression. However, not all of the promoters that pRb occupies in T antigen expressing villi contain two E2F binding sites. For example, the promoter for b myb contains only one E2F binding site. Accumulating data in the literature suggest still another mechanism to explain how pRb can occupy active promoters in T antigen expressing villi. This mechanism describes pRb as a transcriptional regulator not inherent to repression, but as molecule which bridges other transcription regulators together, as a “bridging molecule”.

If the only function of pRb is to repress E2F activity then the genes that are de-regulated in pRb null cells should be the same as those de-regulated in E2F-activated cells and vice versa.

However, microarray studies that performed this very type of experiment demonstrate that this is not the case. In fact, there is a limited overlap of regulated genes, indicating that this simple opposition model is incorrect (Markey et al., 2002). Cells can proliferate in the presence of active pRb, (pRb which remains hypophosphorylated due to a p16 block of cyclin D/cdk2/4) in the presence of activated c-myc or cyclin E (Alevizopoulos et al., 1997). pRb interacts with many different transcription factors to activate transcription in specific cell types to promote differentiation, like MyoD during myogenesis and C/EBP α during adipogenesis (Classon et al., 2000; Li et al., 2000). In these cases, pRb does not act as a repressor of transcription.

6.4.1. Rb family members as bridging molecules for transcription factors

The similarities between pRb's functions during cell cycle regulation and differentiation converge on pRb acting as a "bridging molecule" between the site-specific DNA binding transcription factors and either the transcriptional machinery such as TBP and RNA polymerase or histone modifying machinery such as HDACs, Suv39h1, and HP1. Therefore, pRb might behave as a bridging molecule rather than an inherent repressor. For example, pRb can bind SP1 to synergistically transactivate the hamster *dhfr* promoter (Noé et al., 1998). In another report, interaction between SP1 and pRb led to superactivation of promoter constructs that contain Rb control elements (RCEs). These "RCE"s were isolated from particular promoters that Rb expression up-regulates in *Drosophila* cells (Udvardia et al., 1995). Previous experiments have shown that an interaction between activating E2F family members and SP1 family members independent of pRb. The interaction between SP1 and pRb was not shown to be direct, and therefore could be through interaction with E2F. These two transcription factors may act synergistically to transactivate the *dhfr* promoter via recruitment of E2F and SP1. In support of

this model, another report demonstrated that there is cooperation between p130/E2F complexes and pRb/Sp1/HDAC complexes in repression of the hamster *dhfr* promoter (Chang et al., 2001). The authors suggest that in the cell cycle withdrawal, E2F1-3 complexes are first removed and then SP1 binds HDAC1 and initiates repression. pRb then binds to this complex and is then replaced or supported by the more stable p130/E2F/DP complex. This confers a TSA-(HDAC inhibitor) insensitive repression of the promoter.

6.4.2. “Activating” E2Fs are not required for proliferation

There are several lines of evidence that suggest that E2Fs1-3 may not be required to drive cells through the cell cycle and that these E2Fs may, in fact, play a role in tumor suppression or at least tumor surveillance. In this scenario, these E2Fs rise in level as the cell cycles, in a type of auto-regulatory feedback mechanism. Therefore, these E2Fs are associated with cycling cells, however they are not driving the cells through the cycle, but merely being regulated by the cell cycle. Alternatively, they drive the cell cycle in some cell types while behaving as stress surveillance molecules in other cell types, reflecting their dual functions as both oncogenes and tumor suppressors.

In studies of triple E2F1, E2F2 and E2F3 knock-out (TKO) MEFs, the authors concluded that the “activating” E2Fs, E2F1-3, are “essential” for S phase and cell cycle progression, these same experiments demonstrated that these E2Fs are not required for cell cycle progression (Wu et al., 2001). These TKO MEFs display a clear defect in growth, this could be explained by the concomitant up-regulation of p21 in these cells (Wu et al., 2001). Ectopic expression of Cyclin E and cdk2 were able to induce BrdU incorporation in these E2F1-3 TKO MEFs (Wu et al., 2001). These data demonstrate that S phase can proceed without E2F1, E2F2 or E2F3, a result that is in

clear contrast to the classic model of the Rb/E2F pathway. Two questions arise from these data: 1) What drives these cells through S phase without “activating” E2Fs? 2) What, then, are the functions of the activating E2Fs?

If E2Fs are required to drive cells through S phase, then perhaps E2F4 and E2F5 are performing the required E2F activities, at least in the absence of “activating” E2Fs. Although these E2Fs have been classically referred to as “repressive” E2Fs, they both have transactivation domains, albeit smaller than their homologous domains in the “activating” E2Fs, and they both have the ability to drive promoter activity, particularly if DP1 or DP2 is co-expressed (DeGregori et al., 1997; Vaishnav et al., 1998). E2F5 can even act as an oncogene in cooperation with ras to transform primary rat kidney cells (BRK cells), and is amplified in some breast tumors (Polanowska et al., 2000). These data indicate that E2F5 can drive proliferation to induce tumors, similar to “activating” E2Fs.

Evidence from genetic studies suggests that E2F4 can drive cells through the cell cycle. E2F4 knock-out mice die perinatally, suggesting that it is required for normal adult homeostasis (Gaubatz et al., 2000). It was suggested that this reflects a defect in differentiation because E2F4/5 double knock-out MEFs display a defect in growth arrest (Gaubatz et al., 2000). Detailed analysis of particular homeostatic tissues like the small intestine have shown that E2F4 knock-out mice display defects in crypt formation, where the dividing and undifferentiated stem and progenitor cells are located, while the differentiated cells of the villus were not affected in these mice (Rempel et al., 2000). These data demonstrate that the loss of E2F4 affects homeostatic proliferation, rather than merely growth arrest and differentiation. In support of a role for E2F4 in regulation of proliferation in the intestinal epithelium, transgenic mice that express SV40 large T antigen in the intestinal villi have a delayed onset of a hyperplastic phenotype and live longer

in an E2F4^{+/-} background (unpublished data, M.T. Sáenz-Robles, University of Pittsburgh). In another genetic study, loss of E2F4 suppressed tumor formation by the loss of pRb (Lee et al., 2002). Results of this nature would usually be interpreted such that E2F4 activity is partially responsible for tumor formation in the absence of pRb. However, the authors proceeded to provide data to explain their results such that in the absence of E2F4, “activating” E2Fs form repressive complexes that suppress tumor formation in pRb^{+/-} mice (Lee et al., 2002). These results do not contradict a model that says E2F4 can drive proliferation in the absence of pRb. Mouse embryo fibroblasts were prepared from these E2F4^{-/-}, Rb^{+/-} mice and subjected to BrdU incorporation and measurement. The results demonstrate again that loss of E2F4 completely suppresses the induction of BrdU incorporation from loss of pRb in MEFs (Lee et al., 2002). These results support a role for E2F4 in driving S phase. These results should be interpreted similarly to the interpretation of studies where pRb knock-out mice were crossed into individual “activating” E2F knock-out backgrounds to ascertain any specificities in mediating phenotypes between these three E2Fs (Saavedra et al., 2002). Loss of pRb leads to ectopic proliferation in the retina, as measured by BrdU labeling. Combined loss of any of the three E2Fs, E2F1, E2F2 and E2F3, reduced the number of cells incorporating BrdU (Saavedra et al., 2002). The authors interpret this to mean that each of these E2Fs contributes to the ectopic proliferation caused by loss of pRb. In conclusion, the loss of E2F1, E2F2, E2F3, or E2F4 can suppress the effects of the loss of pRb, indicating that all four E2Fs drive proliferation.

There is conflicting evidence in the literature that E2Fs, both “activators” and “repressors”, are required for cell cycle progression at all. In one study, ectopic expression of a dominant negative form of E2F-2 or DP1 in normal breast epithelial cells resulted in a block of apoptosis induced by serum starvation, but did not alter the rate of cell proliferation (Bargou et

al., 1996). In fact, these cells were capable of inducing tumor growth in SCID mice. These results suggest that proliferation can be driven in an E2F-independent manner and that the normal, overall function of the E2Fs is tumor suppressive. However, another study reported that the expression of dominant-negative forms of E2F or DP retained E2F DNA binding activity, indicating that endogenous activity remained in these cells despite reduction in E2F-responsive promoter activity (Maehara et al., 2005). For this study, endogenous E2F/DP activity was inhibited by transfection of a short-hairpin RNA (shRNA) containing complementary sequences to the DP1 transcript, thus effectively eliminating DP1 in particular cell lines that predominantly express DP1 and not DP2. Transfection of the DP1-shRNA in cancer cell lines that do not express functional pRB or p53 resulted in senescence, indicating that these cancer cells require E2F/DP activity for growth. This study did not address the function of the endogenous E2F/DP activity in normal cells, however. Therefore, these results only apply to the abnormal growth of cancer cells. Another study transfected cells with plasmids containing multiple E2F sites to sequester E2F complexes from their endogenous site to block E2F/DP activity in various human cell lines (He et al., 2000a). Upon sequestration, both the *dhfr* and *b-myb* promoter activities were significantly reduced in all cell types tested, consistent with evidence that suggests that E2F sites function primarily as repressive elements. The authors hypothesized that these sites are necessary for growth-arrest. They tested this hypothesis by inducing growth arrest by gamma-irradiation and then measuring BrdU incorporation. The amount of cells in S phase increased in a concentration-dependent manner for irradiated cells transfected with the sequestration plasmid (He et al., 2000a). These results indicate that the endogenous function of the E2F/DP complexes is to block S phase entry upon growth arrest signals such as from gamma-irradiation. Altogether,

these studies indicate that the sum of all E2F/DP activity is to function normally to regulate growth-arrest and abnormally to promote growth of cancer cells.

6.4.3. “Activating” E2Fs as tumor surveillance factors.

If the normal function of all net E2F/DP activity is to assist in growth arrest and E2F4 and E2F5 are the primary E2Fs that perform this function, then the question remains, what is the normal function of the “activating” E2Fs, E2F1, E2F2, and E2F3? One possibility is that these E2Fs normally function as stress or tumor surveillance molecules. In humans, high levels of E2F1 expression and low levels of pRb staining, correlate with disease-free survival in SCC of the anterior tongue (Kwong et al., 2003). In breast cancer patients, however, E2F1 expression is correlated with poor survival after chemotherapy (Han et al., 2003). The most compelling evidence is the phenotype of E2F1 or E2F2 knock-out mice. They develop multiple types of tumors, suggesting that E2F1 and E2F2 play roles as tumor suppressors (Table 1). However, E2F1 knock-out mice also display testicular atrophy and E2F2 knock-out mice have defects in pancreatic islet beta cell growth, suggesting that these E2Fs also play a important roles in growth promotion in particular cells.

Many reports demonstrate that E2F1 induces apoptosis, and that this is important in tumor suppression. There is further evidence that sets the “activating” E2Fs in a stress response pathway. DNA damaging agents and hypoxia cause the up-regulation of E2F1 expression in p53 and pRb null cells without a concomitant increase in E2F1 transactivation (O'Connor and Lu, 2000). In fact, some cell lines show reduced E2F reporter expression upon UV-irradiation, although E2F1 was induced. Possibly, E2F1 is induced, but its transactivation activity is blocked. p14^{ARF} binds to E2F-1 in its amino terminal region (Eymin et al., 2001). Co-expression of ARF

and E2F-1 in p53 knock-out MEFs caused a reduction in E2F-responsive promoter activity. Interestingly, *mdm2* was required for the reduction in promoter activity, but not for the interaction between E2F1 and ARF. Together, these results indicate that stress can activate E2F1 expression, and that ARF can bind E2F1 and prevent E2F1-mediated promoter transactivation. A logical question that follows is why is E2F1 up-regulated if its transactivational activity is blocked?

One study directly implicates E2F1 in DNA damage repair, an alternative function of E2F1 from transactivation (Liu et al., 2003). E2F1 was shown to mediate the repression of Topoisomerase II β in MEFs and melanoma cell lines (Jiao et al., 2005). Upon expression of the TopoII inhibitor and chemotherapeutic, genotoxic agent, doxorubicin, E2F1 is released from the TopoII β promoter, leading to the de-repression of TopoII β . Then TopoII β co-localizes with apoptosis-associated heterochromatin (AAHF). The authors suggest that heterochromatin formation precedes E2F1-dependent drug-induced apoptosis (Jiao et al., 2005).

E2F1 induction upon DNA damage does not always result in apoptosis. Sometimes a checkpoint DNA repair pathway is induced through interactions with TopBP1. After DNA damage, E2F1 becomes phosphorylated by the DNA damage-induced ATM, allowing for its association with TopBP1 (TopoII binding protein 1) (Liu 2003). This interaction leads to repression of E2F1-regulated promoter activity, dependent on the Brg1/Brm chromatin remodelers (Liu et al., 2004). TopBP1 then relocalizes E2F1 to discrete nuclear foci containing BRCA1, which associates to regions of stalled replication forks (Liu et al., 2003). These results suggest a mechanism by which E2F1 mediates apoptosis after genotoxic stress. Although E2F2 and E2F3a have been shown to induce apoptosis various studies, mechanisms for how they perform their pro-apoptotic functions remain to be elucidated.

The up-regulation of ‘activating’ E2Fs during the cell cycle may also provide a mechanism for the cell to monitor its own level of proliferation through signaling to pRb. pRb is required to sense this abnormally high level of proliferation and activate the cellular defense mechanism of senescence. In support for a specific role of pRb-mediated repression, Rb/E2F repressor complexes differ in their stability at promoters, suggesting that one repressor complex may behave more transiently while another is more permanent. Over-expression of E2F1 cannot displace stable pRb/E2F repressor complexes at promoters, which recruit HP1, but can displace p130/E2F or p107/E2F repressor complexes (Young et al., 2004). These results support a more important role for pRb in mediating senescence than in regulating normal, quiescent growth arrest. In support of this role, pRb is required for HPV E2-induced senescence (Wells et al., 2000b; Psyrrri et al., 2004). p130/E2F complexes probably do not play a role in senescence since double knock-out E2F4 and E2F5 MEFs retain the ability to senesce, suggesting that E2F4 and E2F5 are not required for senescence (Gaubatz et al., 2000).

6.4.4. A revised model of Rb/E2F function.

From these new perspectives of Rb and E2F family protein functions, a revised model of Rb/E2F function throughout the normal cell cycle is presented here (Fig.27). Most cells in the adult organism undergo a very tightly regulated cell cycle and are regulated by E2F4/pocket protein/DP complexes. In growth arrest, chromatin modifiers such as HDAC are tethered to E2F-responsive promoters by p130/E2F4/DP complexes, actively repressing their transcription. As cells receive external signals to divide, cyclin D levels rise and it complexes with cdk4 resulting in phosphorylation of p130. This causes the release of HDACs or other repressive chromatin modifiers from p130/E2F4/DP complexes, which in turn results in the de-repression of certain

genes such as cyclin E and p107. Cyclin E binds cdk2 resulting in phosphorylation of p130, causing its release from the E2F4/DP complex. On some promoters the entire complex is released, but on others p130-free E2F4/DP can transactivate gene transcription. At the same time, around the G₁/S boundary, E2F4 is made as well as p107. p107 binds E2F4 and translocates it into the nucleus. According to the model, Rb-free E2F4/DP complexes transactivate low levels of E2F1, E2F2 and E2F3a, and these proteins bind to pRb. Some Rb-free E2F1-3a contributes to the progression of S phase, by more robust transactivation of E2F-responsive genes. This model suggests that E2F3b and pRb terminate S phase. The activating E2Fs bind cyclin A, are phosphorylated and targeted for degradation. Rb-free E2F4 is also unstable and targeted for degradation. However, as the cells divide and more E2F4 is synthesized, p130 binds it in the cytoplasm and translocates it into the nucleus where it binds E2F-responsive promoters and represses their transcription.

Cell type differences also contribute to this model. In cells that are terminally differentiating, these E2F-responsive promoters are maintained as repressed by the p130/E2F4 or p130/E2F5 complexes. These complexes may be replaced with E2F6/PcG complexes that form heterochromatin-like structures in some cells, silencing the expression of those genes permanently. Possibly this is accomplished by the accumulation of E2F6 through cell cycles, transactivated by E2F. The accumulation of E2F6 through sequential cell cycles could be a mechanism by which the cells counts the number of cell cycles until permanently terminating the cell cycle. Some cell types that need to be poised to proliferate quickly by external signals may depend more heavily on the activating E2Fs 1-3a, and their interaction with pRb. In these cells, the p19ARF and p53 pathways are predicted to be important to balance the effect of proliferation with apoptosis. For instance, lymphocytes might be predominantly regulated by E2F1, E2F2, and

E2F3a for transactivation of E2F-responsive genes. As these cells progress through multiple rounds of division, their levels of E2F1, E2F2 and E2F3a rise until above a particular threshold, resulting in the induction of the p53 pathway, and subsequent growth-arrest or apoptosis.

In cases of abnormal induction of growth, either by constitutive signaling of growth factors or presence of a viral oncogene, p130 dissociates from E2F4/DP complexes on promoters, de-repressing their transcription. The cell then enters the cell cycle and produces activating E2Fs. As the activating E2Fs rise, pRb senses this and attempts to shut down promoter activity by binding it through the activating E2Fs. This may result in the formation of heterochromatin-like structures that permanently silence their expression and cause senescence. However, constitutive activation of cyclin-dependent kinase activity or expression of a viral oncogene that binds Rb and disrupts Rb/E2F complexes would inactivate Rb, preventing it from sensing the activating E2Fs. Alternatively, the activating E2Fs could signal to upregulate ARF. ARF could both disrupt E2F/DP DNA binding complexes, abrogating their transactivating abilities, as well as block the degradation of p53, stabilizing it for activation of apoptosis. Cancer cells then need to block this pathway for their survival.

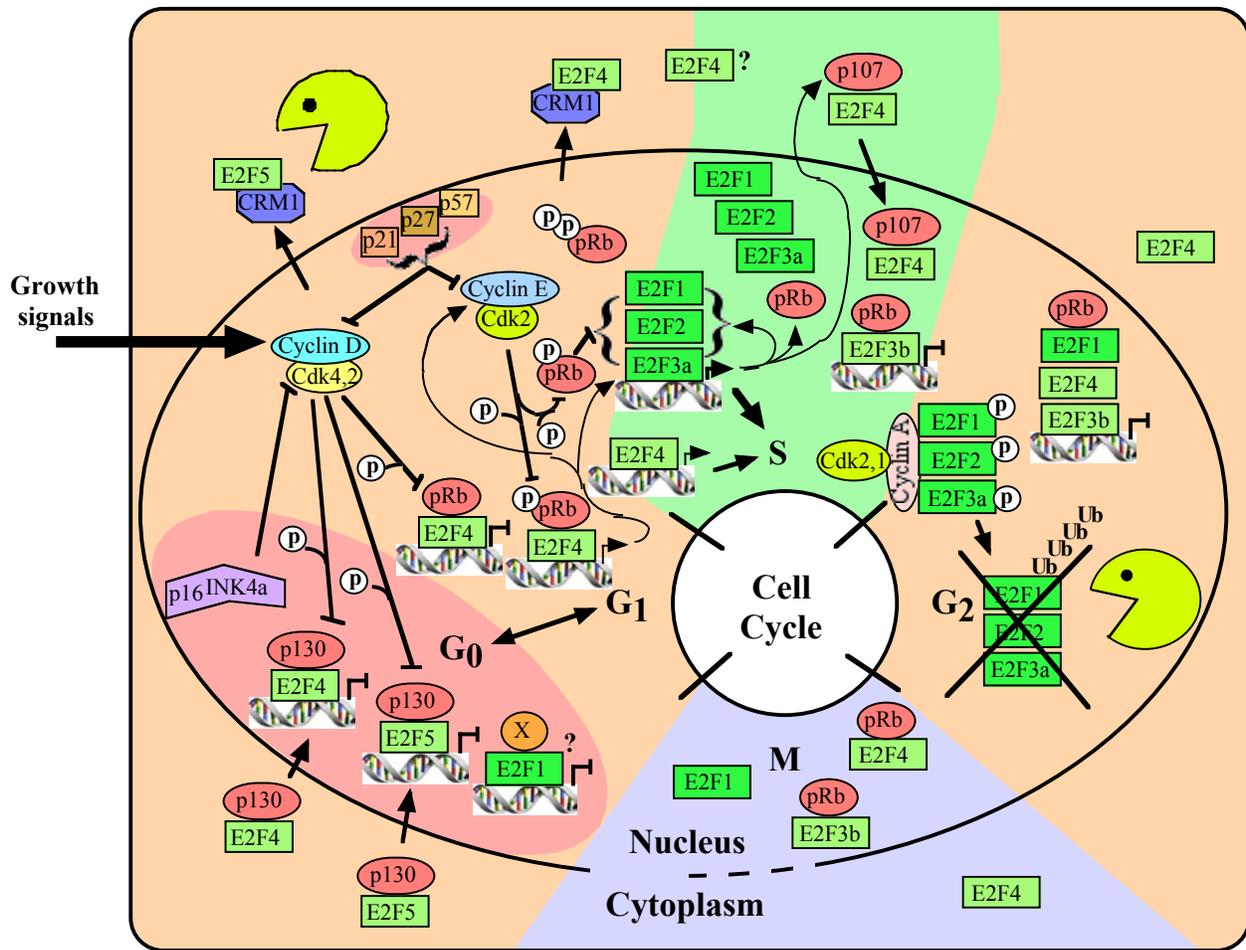


Figure 27. Revised model of Rb/E2F function throughout the cell cycle. In growth arrest, G₀, p130/E2F4 and p130/E2F5 complexes are bound to and repressing E2F-responsive gene promoters. E2F1 is also bound to specific promoters in an Rb-independent repressive complex. Growth signals transduced to the nucleus activate cyclin D/cdk complexes, which, in turn, phosphorylate p130/E2F complexes, activating transition into G₁. p16^{INK4a} expression can specifically inhibit cyclin D/cdk complexes, sending cells into growth arrest. Hypophosphorylated pRb/E2F4 activates the transcription of some genes such as cyclin E and the “activating” E2Fs (1-3a). Then, hypo-phosphorylated pRb/E2F1, pRb/E2F2, and pRb/pE2F3a complexes bind E2F-responsive promoters. Hyper-phosphorylation of pRb by cyclin E/cdk complexes cause the dissociation of pRb from these E2F complexes, allowing for their transactivation of S phase gene synthesis and transition into S phase. E2F4 and E2F5 are exported from the nucleus by CRM1-dependent mechanisms where they are degraded by the proteasome (represented by pacman cartoon). During S phase, p107 is expressed, binds remaining E2F4, targeting it for import into the nucleus. E2F3b is bound to and repressing specific E2F-responsive promoters such as for p19ARF. After replication, E2Fs1-

3a are bound and phosphorylated by cyclin A/cdk complexes and targeted for ubiquitination and proteasome-mediated degradation, causing the transition into G₂. Throughout G₂ and M phase, E2F3b and E2F4 are present in the nucleus and cytoplasm, respectively, with or without pRb. After division, p130 binds to E2F4 and E2F5, importing them into the nucleus to bind E2F-responsive promoters for repression upon cell cycle exit. Upon permanent growth potential withdrawal, pRb/E2F complexes or E2F6/PcG complexes replace these less stable p130/E2F complexes and forms heterochromatin at the promoters, permanently silencing their activity.

6.5. SIGNIFICANCE

Of the three Rb family members, pRb is the only one that is found mutated in human cancers, suggesting that pRb is the only true tumor suppressor of the family. It is not understood why pRb is singled-out in this way. However, the revised model of Rb/E2F function put forth in this thesis suggests a possible explanation and allows for new ideas to emerge for cancer treatments. If activating E2Fs can act both as oncogenes and as signaling molecules for stress or also as tumor suppressors, then pRb plays a crucial role in both relaying those signals and repressing the oncogenic functions of E2F. Since most adult cells do not express high levels of ‘activating’ E2Fs, even in proliferating cells, these E2Fs may serve as a way to specifically target abnormally dividing cells. In tumors that are defective in Rb function or over-express oncogenic E2Fs, targeting cells with over-active ‘activating’ E2Fs could provide a beneficial new way to specifically target the tumor cells. However, it is important not to target all E2Fs as it is clear that some E2Fs, such as E2F4 and E2F5 and occasionally E2F1, are required to maintain growth-arrest.

To target cells with high activity of activating E2Fs, plasmids with E2F sites that have been empirically determined to preferentially bind pRb-free ‘activating’ E2F complexes could be

delivered to the cells through viral-coated delivery methods such as adenoviral vectors (Fig.28). These E2F sites could drive the expression of a modular protein that contains the E2F interaction domain from a specific E2F-binding protein hybridized to the GAL4 DNA binding domain. This would thus allow the interaction of the heterologous protein with a specific, targeted E2F. For example, E2F3 could be targeted by using the E2F3-binding domain from TFE3 hybridized to the GAL4 binding domain. Several E2Fs, such as E2F2 and E2F3, could be targeted through their region of interaction on RYBP. The hybrid protein could then bind GAL4 DNA binding sequences also contained on the vector. These sequences would then drive the expression of another gene encoding a cellular toxin such as diphtheria toxin, thus killing the tumor cell. This hypothetical design allows for cells that express high levels of “activating” E2Fs to transactivate the expression of a hybrid protein. This hybrid then specifically targets one or two E2Fs to transactivate the expression of a cellular toxin, thus killing the cell. This design allows for at least two levels of specificity before killing the cell, so that non-specific effects would hopefully be minimized. Such an application would have to be researched at each level before general use however.

The underlying significance of this study is that different cell types at different points in development use different mechanisms to regulate normal and abnormal growth and growth arrest. It is clear that a study in one cell type cannot be generalized to all cell types, or even as the definitive mechanism that occurs in that cell type at all points in development or disease. Cancer treatment must evolve to consider each cell type that the tumor is derived from as well as the regulation of growth in the normal cells throughout the rest of the body. It would be a fantastic discovery if a single treatment could destroy all tumor types and not any normally dividing cells in the body. However, this is unlikely, and even if it were to be discovered, as a

microevolutionary process, cancer cells would evolve to bypass that treatment as well. Therefore, the goal of cancer treatment should remain to destroy the entire cancer and to improve the quality of life of cancer patients - but not at the expense of other cells.

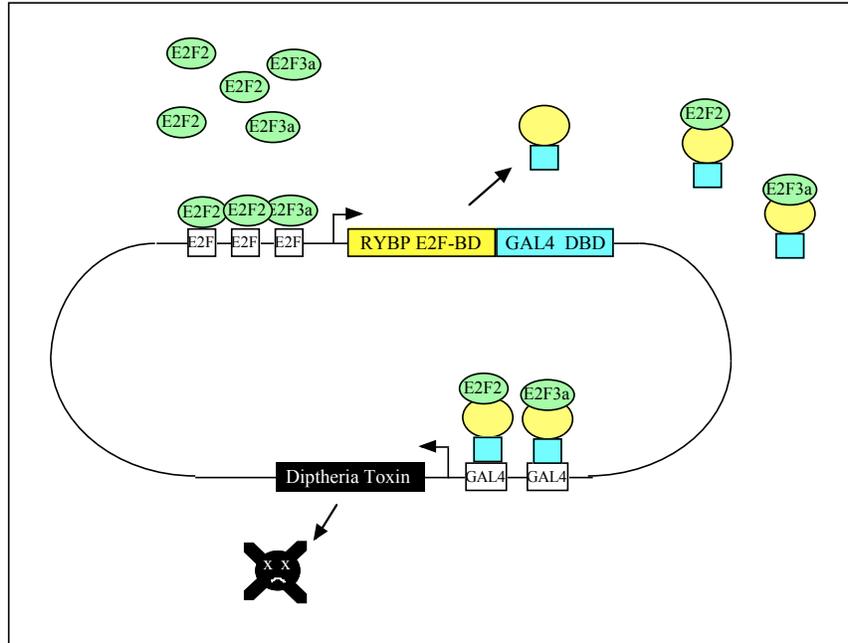


Figure 28. Hypothetical viral vector for ‘activator’ E2F-based cancer therapy. This hypothetical viral vector targets cells with over-expressing, “activating” E2Fs. The E2F binding sites on the vector should be empirically designed to preferentially bind “activating” E2Fs such as E2F2 and E2F3a. These E2Fs then activate the transcription of a heterologous protein containing the E2F interaction domain of a specific E2F interacting partner such as RYBP fused to the GAL4 DNA binding domain. This protein would then complex with specific E2Fs such as E2F2 or E2F3a, then bind to the GAL4 binding sites in the vector, activating the transcription of a cellular toxin such as diphtheria toxin. Translation of this toxin subsequently causes the cell to die. Theoretically, this therapy specifically targets abnormally dividing cells that have signaled their abnormal proliferation through up-regulation of “activating” E2Fs. The virus that encodes this vector might also be designed to bind and invade specific cell types, dependent on their membrane receptors.

BIBLIOGRAPHY

- Adams, M.R., Sears, R., Nuckolls, F., Leone, G., and J.R. Nevins. 2000. Complex transcriptional regulatory mechanisms control expression of the E2F3 locus. *Molecular and Cellular Biology* **20**(10): 3633-3639.
- Ahuja, D., Sáenz-Robles, M.T., and J.M. Pipas. *in press*. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. *Oncogene*.
- Akiyama, T., Ohuchi, T., Sumida, S., Matsumoto, K., and K. Toyoshima. 1992. Phosphorylation of the retinoblastoma protein by cdk2. *Proceedings of the National Academy of Sciences USA* **89**: 7900-7904.
- Alevizopoulos, K., Vlach, J., Hennecke, S., and B. Amati. 1997. Cyclin E and c-Myc promote cell proliferation in the presence of p16^{INK4a} and of hypophosphorylated retinoblastoma family proteins. *The EMBO Journal* **16**(17): 5322-5333.
- Ali, S.H., and J.A. DeCaprio. 2001. Cellular transformation by SV40 large T antigen: interaction with host proteins. *Cancer Biology* **11**: 15-22.
- Ali, S.H., Kasper, J.S., Arai, T., and J.A. DeCaprio. 2004. Cul7/p185/p193 binding to Simian Virus 40 large T antigen has a role in cellular transformation. *Journal of Virology* **78**(6): 2749-2757.
- Angus, S.P., Mayhew, C.N., Solomon, D.A., Braden, W.A., Markey, M.P., Okuno, Y., Cardoso, M.C., Gilbert, D.M., and E.S. Knudsen. 2004. RB reversibly inhibits DNA replication via two temporally distinct mechanisms. *Molecular and Cellular Biology* **24**(12): 5404-5420.
- Apostolova, M.D., Ivanova, I.A., Dagnino, C., D'Souza, S.J.A., and L. Dagnino. 2002. Active nuclear import and export pathways regulate E2F-5 subcellular localization. *The Journal of Biological Chemistry* **277**(37): 34471-34479.
- Araki, K., Nakajima, Y., Eto, K., and M. Ikeda. 2003. Distinct recruitment of E2F family members to specific E2F-binding sites mediates activation and repression of the *E2F1* promoter. *Oncogene* **22**: 7632-7641.
- Armitage, P., and R. Doll. 1954. The age distribution of cancer and a multi-stage theory of carcinogenesis. *British Journal of Cancer* **8**: 1-12.

- Aslanian, A., Phillip J. Iaquina, Raluca Verona, and Jacqueline A. Lees. 2004. Repression of the Arf tumor suppressor by E2F3 is required for normal cell cycle kinetics. *Genes and Development* **18**: 1413-1422.
- Attwooll, C., Lazzerini Denchi, E., and Helin, K. 2004. The E2F family: specific functions and overlapping. *The EMBO Journal* **23**: 4709-4716.
- Attwooll, C., Oddi, S., Cartwright, P., Prosperini, E., Agger, K., Steensgaard, P., Wagener, C., Sardet, C., Moroni, M.C., and Helin, K. 2005. A novel repressive E2F6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. *Journal of Biological Chemistry* **280**(2): 1199-1208.
- Bagchi, S., Raychaudhuri, B., and J.R. Nevins. 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. *Cell* **62**(4): 659-669.
- Balsitis, S., Dick, F., Lee, D., Farrell, L., Hyde, R.K., Griep, A.E., Dyson, N., and P.F. Lambert. 2005. Examination of the pRb-dependent and pRb-independent functions of E7 in vivo. *Journal of Virology* **79**(17): 11392-11402.
- Bargou, R.C., Wagener, C., Bommert, K., Arnold, W., Daniel, P.T., Mapara, M.Y., Grinstein, E., Royer, H.D., and B. Dörken. 1996. Blocking of transcription factor E2F/DP by dominant-negative mutants in a normal breast epithelial cell line efficiently inhibits apoptosis and induces tumor growth in SCID mice. *Journal of Experimental Medicine* **183**: 1205-1213.
- Bates, S., Phillips, A.C., Clark, P.A., Stott, F., Peters, G., Ludwig, R.L., and K.H. Vousden. 1998. p14ARF links the tumour suppressors RB p53. *Nature* **395**: 124-125.
- Battle, E., Henderson, J.T., Beghtel, H., van den Born, M.M., Sancho, E., Huls, G., Meeldijk, J., Roberston, J., van de Wetering, M., Pawson, T., and H. Clevers. 2002. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrin B. *Cell* **111**(2): 251-263.
- Beachy, T.M., Cole, S.L., Cavender, J.F., and M.J. Tevethia. 2002. Regions and activities of simian virus 40 T antigen that cooperate with an activated *ras* oncogene in transforming primary rat embryo fibroblasts. *Journal of Virology* **76**: 3145-3157.
- Beaulieu, J., Nichols, B., and A. Quaroni. 1989. Posttranslational regulation of sucrase-isomaltase expression in intestinal crypt and villus cells. *The Journal of Biological Chemistry* **264**(33): 20000-20011.
- Beijersbergen, R.L., Hijmans, E.M., Zhu, L., and R. Bernards. 1994. Interaction of c-Myc with the pRb-related protein p107 results in inhibition of c-Myc-mediated transactivation. *The EMBO Journal* **13**(17): 4080-4086.

- Ben-Porath, I., and R.A. Weinberg. 2004. When cells get stressed: an integrative view of cellular senescence. *Journal of Clinical Investigation* **113**(1): 8-13.
- Bhattacharya, S., Garriga, J., Calbó, J., Yong, T., Haines, D.S., and X. Graña. 2003. SKP2 associates with p130 and accelerates p130 ubiquitylation and degradation in human cells. *Oncogene* **22**(16): 2443-2451.
- Black, E.P., Huang, E., Dressman, H., Rempel, R., Laakso, N., Asa, S.L., Ishida, S., West, M., and J.R. Nevins. 2003. Distinct gene expression phenotypes of cells lacking Rb and Rb family members. *Cancer Research* **63**: 3715-3723.
- Blake, M.C., and J.C. Azizkhan. 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. *Molecular and Cellular Biology* **9**(11): 4994-5002.
- Bookchin, D., and J. Schumacher. 2004. *The virus and the vaccine: the true story of a cancer-causing monkey virus, contaminated polio vaccine, and the millions of Americans exposed*. St. Martin's Press, New York.
- Botz, J., Zerfass-Thome, K., Spitkovsky, D., Delius, H., Vogt, B., Eilers, M., Hatzigeorgiou, A., and P. Jansen-Dürr. 1996. Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Molecular and Cellular Biology* **16**(7): 3401-3409.
- Boucher, M., Jean, D., Vézina, A., and N. Rivard. 2004. Dual role of MEK/ERK signaling in senescence and transformation of intestinal epithelial cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **286**(5): 736-746.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry* **72**: 248-254.
- Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A.H.F.M., Schlegelberger, B., Stein, H., Dörken, B., Jenuwein, T., and C.A. Schmitt. 2005. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* **436**: 660-665.
- Buchkovich, K., Duffy, L.A., and E. Harlow. 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* **58**(6): 1097-1105.
- Buda, A., Sands, C., and M.A. Jepson. 2005. Use of fluorescence imaging to investigate the structure and function of intestinal M cells. *Advanced Drug Delivery Reviews* **57**: 123-134.
- Budelier, K., and J. Schorr. 2002. Manipulation of DNA: Purification and concentration of DNA from aqueous solutions. in *Current Protocols in Molecular Biology* (ed. R.B. F.M. Ausubel, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl), pp. 2.1.1-2.1.9. John Wiley and Sons, Inc., New York.

- Cairns, J. 1975. Mutation selection and the natural history of cancer. *Nature* **225**: 197-200.
- Campanero, M.R., and E.K. Flemington. 1997. Regulation of E2F through ubiquitin-proteasome-dependent degradation: Stabilization by the pRb tumor suppressor protein. *Proceedings of the National Academy of Sciences USA* **94**: 2221-2226.
- Campanero, M.R., Armstrong, M.I., and E.K. Flemington. 2000. CpG methylation as a mechanism for the regulation of E2F activity. *Proceedings of the National Academy of Sciences USA* **97**(12): 6481-6486.
- Carreira, S., Goodall, J., Aksan, I., La Rocca, S.A., Galibert, M.D., Denat, L., Larue, L., and C.R. Goding. 2005. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. *Nature* **433**: 764-769.
- Ceol, C.J., and H.R. Horvitz. 2001. dpl-1 DP and elf-1 E2F act with lin-35 Rb to antagonize Ras signaling in *C. elegans* vulval development. *Molecular Cell* **7**(3): 461-473.
- Chandrasekaran, C., Coopersmith, C.M., and J.I. Gordon. 1996. Use of normal and transgenic mice to examine the relationship between terminal differentiation of intestinal epithelial cells and accumulation of their cell cycle regulators. *Journal of Biological Chemistry* **271**: 28414-28421.
- Chang, Y.C., Illenye, S., and N.H. Heintz. 2001. Cooperation of E2F-p130 and Sp1-pRb complexes in repression of the Chinese hamster dhfr gene. *Molecular and Cellular Biology* **21**(4): 1121-1131.
- Chellappan, S., Kraus, V.B., Kroger, B., Munger, K., Howley, P.M., Phelps, W.C., and J.R. Nevins. 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. *Proceedings of the National Academy of Sciences USA* **89**: 4549-4553.
- Chen, C., Kang, Y., Siegel, P.M., and J. Massagué. 2002. E2F4/5 and p107 as Smad cofactors linking the TGF β receptor to *c-myc* repression. *Cell* **110**: 19-32.
- Chen, J., and T. Van Dyke. 1991. Uniform cell-autonomous tumorigenesis of the choroid plexus by papovavirus large T antigens. *Molecular and Cellular Biology* **11**: 5968-5976.
- Chen, Q., Hung, F., Fromm, L., and P.A. Overbeek. 2000a. Induction of cell cycle entry and cell death in postmitotic lens fiber cells by overexpression of E2F1 or E2F2. *Investigative Ophthalmology & Visual Science* **41**(13): 4223-4231.
- Chen, Q., Liang, D., Fromm, L.D., and P.A. Overbeek. 2004a. Inhibition of lens fiber cell morphogenesis by expression of a mutant SV40 large T antigen that binds CREB-binding protein/p300 but not pRb. *The Journal of Biological Chemistry* **279**(17): 17667-17679.

- Chen, T., and J.Y.J. Wang. 2000b. Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function. *Molecular and Cellular Biology* **20**(15): 5571-5580.
- Chen, W., Arroyo, J.D., Timmons, J.C., Possemato, R., and W.C. Hahn. 2005a. Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. *Cancer Research* **65**(18): 8183-8192.
- Chen, W., Possemato, R., Campbell, K.T., Plattner, C.A., Pallas, D.C., and W.C. Hahn. 2004b. Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell* **5**(2): 127-136.
- Chen, Y., and X. Zhao. 1998. Shaping limbs by apoptosis. *Journal of Experimental Zoology* **282**(6): 691-702.
- Chen, Z., Trotman, L.C., Shaffer, D., Lin, H., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., Cordon-Cardo, C., and P.P. Pandolfi. 2005b. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436**: 725-730.
- Chittenden, T., Livingston, D.M., and J.A. DeCaprio. 1993. Cell cycle analysis of E2F in primary human T cells reveals novel E2F complexes and biochemically distinct forms of free E2F. *Molecular and Cellular Biology* **13**(7): 3975-3983.
- Chittenden, T., Livingston, D.M., and W.G. Kaelin Jr. 1991. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. *Cell* **65**(6): 1073-1082.
- Chondrogianni, N., Stratford, F.L.L., Trougakos, I.P., Friguet, B., Rivett, A.J., and E.S. Gonos. 2003. Central role of the proteasome in senescence and survival of human fibroblasts. *The Journal of Biological Chemistry* **278**(30): 28026-28037.
- Clark, A.J., Doyle, K.M., and P.O. Humbert. 2004. Cell-intrinsic requirement for pRb in erythropoiesis. *Blood* **104**(5): 1324-1326.
- Clarke, A.R., Maandag, E.R., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A., and H. te Riele. 1992. Requirement for a functional Rb-1 gene in murine development. *Nature* **359**: 328-330.
- Classon, M., and N. Dyson. 2001. p107 and p130: Versatile proteins with interesting pockets. *Experimental Cell Research* **264**: 135-147.
- Classon, M., Kennedy, B.K., Mulloy, R., and E. Harlow. 2000. Opposing roles of pRB and p107 in adipocyte differentiation. *Proceedings of the National Academy of Sciences USA* **97**(20): 10826-10831.

- Cobrinik, D. 2005. Pocket proteins and cell cycle control. *Oncogene* **24**: 2796-2809.
- Cobrinik, D., Lee, M., Hannon, G., Mulligan, G., Bronson, R.T., Dyson, N., Harlow, E., Beach, D., Weinberg, R.A., and T. Jacks. 1996. Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes and Development* **10**: 1633-1644.
- Cohen, S.J., Cohen, R.B., and N.J. Meropol. 2005. Targeting signal transduction pathways in colorectal cancer - more than skin deep. *Journal of Clinical Oncology* **23**(23): 5374-5385.
- Cohn, S.M., Simon, T.C., Roth, K.A., Birkenmeier, E.H., and J.I. Gordon. 1992. Use of transgenic mice to map *cis*-acting elements in the intestinal fatty acid binding protein gene (*Fabpi*) that control its cell lineage-specific and regional patterns of expression along the duodenal-colonic and crypt-villus axes of the gut epithelium. *The Journal of Cell Biology* **119**(1): 21-44.
- Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benjuria, A., Zaballos, A., Flores, J.M., Barbacid, M., Beach, D., and M. Serrano. 2005. Senescence in premalignant tumours. *Nature* **436**: 642.
- Cooper-Kuhn, C.M., Vroemen, M., Brown, J., Ye, Hong, Thompson, M.A., Winkler, J., and H.G. Kuhn. 2002. Impaired adult neurogenesis in mice lacking the transcription factor E2F1. *Molecular and Cellular Neuroscience* **21**(2): 312-323.
- Coopersmith, C.M., and J.I. Gordon. 1997a. Gamma-Ray-induced apoptosis in transgenic mice with proliferative abnormalities in their intestinal epithelium: re-entry of villus enterocytes into the cell cycle does not affect their radioresistance but enhances the radiosensitivity of the crypt by inducing p53. *Oncogene* **15**: 131-141.
- Coopersmith, C.M., Chandrasekaran, C., McNevin, M.S., and J.I. Gordon. 1997b. Bi-transgenic mice reveal that K-ras^{Val12} augments a p53-independent apoptosis when small intestinal villus enterocytes reenter the cell cycle. *Journal of Cell Biology* **138**: 167-179.
- Cress, W.D., and J.R. Nevins. 1996. A role for a bent DNA structure in E2F-mediated transcription activation. *Molecular and Cellular Biology* **16**(5): 2119-2127.
- Dagnino, L., Fry, C.J., Bartley, S.M., Farnham, P., Gallie, B.L., and R.A. Phillips. 1997. Expression patterns of the E2F family of transcription factors during murine epithelial development. *Cell Growth and Differentiation* **8**: 553-563.
- Dannenbergh, J., van Rossum, A., Schuijff, L., and H. te Riele. 2000. Ablation of the retinoblastoma gene family deregulates G₁ control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes and Development* **14**: 3051-3064.

- Datta, A., Sen, J., Hagen, J., Korgaonkar, C.K., Caffrey, M., Quelle, D.E., Hughes, D.E., Ackerson, T.J., Costa, R.H., and P. Raychaudhuri. 2005. ARF directly binds DP1: interaction with DP1 coincides with the G₁ arrest function of ARF. *Molecular and Cellular Biology* **25**(18): 8024-8036.
- de Boer, J., and J.H.J. Hoeijmaker. 2000. Nucleotide excision repair and human syndromes. *Carcinogenesis* **21**(3): 453-450.
- de Bruin, A., Maiti, B., Jakoi, L., Timmers, C., Buerki, R., and G. Leone. 2003a. Identification and characterization of *E2F7*, a novel mammalian *E2F* family member capable of blocking cellular proliferation. *Journal of Biological Chemistry* **278**(43): 42041-42049.
- de Bruin, A., Wu, L., Saavedra, H.I., Wilson, P., Yang, Y., Rosol, T.J., Weinstein, M., Robinson, M.L., and G. Leone. 2003b. Rb function in extraembryonic lineages suppresses apoptosis in the CNS of Rb-deficient mice. *Proceedings of the National Academy of Sciences USA* **100**(11): 6546-6551.
- DeCaprio, J.A., Ludlow, J.W., Lynch, D., Furukawa, Y., Griffin, J., Piwnicka-Worms, H., Huang, C.M., and D.M. Livingston. 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* **58**(6): 1085-1095.
- DeCaprio, J.A., Ludlow, J.W., Figge, J., Shew, J.Y., Huang, C.M., Lee, W.H., Marsilio, E., Paucha, E., and D.M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**(2): 275-283.
- DeGregori, J. 2002. The genetics of the E2F family of transcription factors: shared functions and unique roles. *Biochimica et Biophysica Acta* **1602**: 131-150.
- DeGregori, J., Leone, G., Miron, A., Jakoi, L., and J.R. Nevins. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. *Proceedings of the National Academy of Sciences USA* **94**: 7245-7250.
- Deschênes, C., Alvarez, L., Lizotte, M., Vézina, A., and N. Rivard. 2004. The nucleocytoplasmic shuttling of E2F4 is involved in the regulation of human intestinal epithelial cell proliferation and differentiation. *Journal of Cellular Physiology* **199**: 262-273.
- Di Stefano, L., Jensen, M.R., and K. Helin. 2003. E2F7, a novel E2F featuring DP-independent repression of a subset of E2F-regulated genes. *The EMBO Journal* **22**(23): 6289-6298.
- Dick, F.A., and N. Dyson. 2003. pRB contains an E2F1-specific binding domain that allows E2F1-induced apoptosis to be regulated separately from other E2F activities. *Molecular Cell* **12**(3): 639-649.
- Dickmanns, A., Zeltvogel, A., Simmersbach, F., Weber, R., Arthur, A.K., Dehde, S., Wildeman, A.G., and E. Fanning. 1994. The kinetics of simian virus 40-induced progression of

- quiescent cells into S phase depend on four independent functions of large T antigen. *Journal of Virology* **68**: 5496-5508.
- Dimova, D.K., and N.J. Dyson. 2005. The E2F transcriptional network: old acquaintances with new faces. *Oncogene* **24**: 2810-2826.
- Ding, Q., Wang, Q., Dong, Z., and B.M. Evers. 2000. Characterization and regulation of E2F activity during Caco-2 cell differentiation. *American Journal of Physiology - Cell Physiology* **278**: C110-C117.
- Djelloul, S., Forgue-Lagitte, M., Hermelin, B., Mareel, M., Bruyneel, E., Baldi, A., Giordano, A., Chastre, E., and C. Gespach. 1997. Enterocyte differentiation is compatible with SV40 large T expression and loss of p53 function in human colonic Caco-2 cells. *FEBS Letters* **406**: 234-242.
- Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J.S., and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**: 215-221.
- Dyson, N. 1998. The regulation of E2F by pRB-family proteins. *Genes and Development* **12**: 2245-2262.
- Dyson, N., Howley, P.M., Munger, K., Harlow, E. 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**(4893): 934-937.
- Ebelt, H., Hufnagel, N., Neuhaus, P., Gajawada, P., Simm, A., Müller-Werdan, U., Werdan, K., and T. Braun. 2005. Divergent Siblings: E2F2 and E2F4 but not E2F1 and E2F3 induce DNA synthesis in cardiomyocytes without activation of apoptosis. *Circulation Research*.
- Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D., and T. Hunt. 1983. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**(2): 389-396.
- Eymin, B., Karayan, L., Séité, P., Brambilla, C., Brambilla, E., Larsen, C., and S. Gazzéri. 2001. Human ARF binds E2F1 and inhibits its transcriptional activity. *Oncogene* **20**: 1033-1041.
- Field, S.J., Tsai, F.Y., Kuo, F., Zubiaga, A.M., Kaelin, W.G., Jr., Livingston, D.M., Orkin, S.H., and M.E. Greenberg. 1996. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* **85**(4): 549-561.
- Fields, B.N., editor-in-chief; Chanock, M.D., Knipe, D.M., Melnick, J.L., Roizman, B., Shope, R.E., ed. 1985. *Virology*. Raven, New York.

- Flemington, E.K., Speck, S.H., and J.W.G. Kaelin. 1993. E2F-1 mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proceedings of the National Academy of Sciences USA* **90**(6914-6918).
- Frolov, M.V., and N.J. Dyson. 2004. Molecular mechanisms of E2F-dependent activation and pRb-mediated repression. *Journal of Cell Science* **117**: 2173-2181.
- Garabedian, E.M., Roberts, L.J.J., McNevin, M.S., and J.I. Gordon. 1997. Examining the role of paneth cells in the small intestine by lineage ablation in transgenic mice. *The Journal of Biological Chemistry* **272**(38): 23729-23740.
- Gartel, A.L., Goufman, E., Tevosian, S.G., Shih, H., Yee, A.S., and A.L. Tyner. 1998. Activation and repression of p21(WAF1/CIP1) transcription by RB binding proteins. *Oncogene* **17**(26): 3463-3469.
- Gasparini, G., Longo, R., Torino, F., and A. Morabito. 2005. Therapy of breast cancer with molecular targeting agents. *Annals of Oncology* **16**(Supplement 4): iv28-iv36.
- Gatenby, R.A., and T.L. Vincent. 2003. An evolutionary model of carcinogenesis. *Cancer Research* **63**(19): 6212-6220.
- Gaubatz, S., Lindeman, G.J., Ishida, S., Jakoi, L., Nevins, J.R., Livingston, D.M., and R.E. Rempel. 2000. E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. *Molecular Cell* **6**: 729-735.
- Gebauer, F., and M.W. Hentze. 2004. Molecular mechanisms of translational control. *Nature Reviews: Molecular Cell Biology* **5**(10): 827-835.
- Giangrande, P.H., Hallstrom, T.C., Tunyaplin, C., Calame, K., and J.R. Nevins. 2003. Identification of E-box factor TFE3 as a functional partner for the E2F3 transcription factor. *Molecular and Cellular Biology* **23**(11): 3707-3720.
- Ginsberg, D., Vairo, G., Chittendon, T., Xiao, Z.X., Xu, G., Wydner, K.L., DeCaprio, J.A., Lawrence, J.B., and D.M. Livingston. 1994. E2F-4, a new member of the E2F transcription factor family, interacts with p107. *Genes and Development* **8**(22): 2665-2679.
- Girling, R., Partridge, J.F., Bandara, L.R., Burden, N., Totty, N.F., Hsuan, J.J., and N.B. La Thangue. 1993. A new component of the transcription factor DRTF1/E2F. *Nature* **362**(6415): 83-87.
- Graña, X., Garriga, J., and X. Mayol. 1998. Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene* **17**: 3365-3383.
- Gum, J., J.R., Hicks, J.W., Gillespie, A.M., Rius, J.L., Treseler, P.A., Kogan, S.C., Carlson, E.J., Epstein, C.J., and Y.S. Kim. 2001. Mouse intestinal goblet cells expressing SV40 T

- antigen directed by the *MUC2* mucin gene promoter undergo apoptosis upon migration to the villi. *Cancer Research* **61**: 3472-3479.
- Hagemeyer, C., Cook, A., and T. Kouzarides. 1993. The retinoblastoma protein binds E2F residues required for activation *in vivo* and TBP binding *in vitro*. *Nucleic Acids Research* **21**(22): 4998-5004.
- Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., and R.A. Weinberg. 2002. Enumeration of the simian virus 40 early region elements necessary for human cell transformation. *Molecular and Cellular Biology* **22**(7): 2111-2123.
- Halaban, R. 2005. Rb/E2F: a two edged sword in the melanocytic system. *Cancer Metastasis Review* **23**(2): 3.
- Han, S., Park, K., Bae, B.N., Kim, K.H., Kim, H.J., Kim, Y.D., and H.Y. Kim. 2003. E2F1 expression is related with the poor survival of lymph node-positive breast cancer patients treated with fluorouracil, doxorubicin and cyclophosphamide. *Breast Cancer Research and Treatment* **82**: 11-16.
- Hansen, J.B., Jorgensen, C., Petersen, R.K., Hallenborg, P., De Matteis, R., Petrovic, N., Enerback, S., Nedergaard, J., Cinti, S., te Riele, H., and K. Kristiansen. 2004a. Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. *Proceedings of the National Academy of Sciences USA* **101**(12): 4112-4117.
- Hansen, J.B., te Riele, H., and K. Kristiansen. 2004b. Novel function of the retinoblastoma protein in fat: regulation of white versus brown adipocyte differentiation. *Cell Cycle* **3**(6): 774-778.
- Haramis, A.G., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G.J.A., and H. Clevers. 2004. De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* **303**: 684-686.
- Harbour, J.W., and D.C. Dean. 2000. Chromatin remodeling and Rb activity. *Current Opinion in Cell Biology* **12**: 685-689.
- Harlow, E., Crawford, L.V., Pim, D.C., and N.M. Williamson. 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. *Journal of Virology* **39**: 861-869.
- Harper, J.W., and S.J. Elledge. 1999. Skipping into the E2F1-destruction pathway. *Nature Cell Biology* **1**: E5-E7.
- Hauck, A.L., Swanson, K.S., Kenis, P.J.A., Leckband, D.E., Gaskins, H.R., and L.B. Schook. 2005. Twists and turns in the development and maintenance of the mammalian small intestine epithelium. *Birth Defects Research (Part C)* **75**: 58-71.

- Hauck, L., Kaba, R.G., Lipp, M., Dietz, R., and R. von Harsdorf. 2002. Regulation of E2F1-dependent gene transcription and apoptosis by the ETS-related transcription factor GABPgamma1. *Molecular and Cellular Biology* **22**(7): 2147-2158.
- Hauff, S.M., Kim, S.H., Schmidt, G.H., Pease, S., Rees, S., Harris, S., Roth, K.A., Hansbrough, J.R., Cohn, S.M., Ahnen, D.J., Wright, N.A., Goodland, R.A., and J.I. Gordon. 1992. Expression of SV-40 T antigen in the small intestinal epithelium of transgenic mice results in proliferative changes in the crypt and reentry of villus-associated enterocytes into the cell cycle but has no apparent effect on cellular differentiation programs and does not cause neoplastic transformation. *Journal of Cell Biology* **117**: 825-839.
- He, S., Cook, B.L., Deverman, B.E., Weihe, U., Zhang, F., Prachand, V., Zheng, J., and S.J. Weintraub. 2000a. E2F is required to prevent inappropriate S-phase entry of mammalian cells. *Molecular and Cellular Biology* **20**(1): 363-371.
- He, X.C., Zhang, J., Tong, W., Tawfik, O., Ross, J., Scoville, D.H., Tian, Q., Zeng, X., He, X., Wiedemann, L.M., Mishina, Y., and L. Lheng. 2004. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt- β -catenin signaling. *Nature Genetics* **36**(10): 1117-1121.
- He, Y., Armanious, M.K., Thomas, M.J., and W.D. Cress. 2000b. Identification of E2F-3B, an alternative form of E2F-3 lacking a conserved N-terminal region. *Oncogene* **19**: 3422-3433.
- Helin, K., Harlow, E., and A. Fattaey. 1993a. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Molecular and Cellular Biology* **13**(10): 6501-6508.
- Helin, K., Lees, J.A., Vidal, M., Dyson, N., Harlow, E., and A. Fattaey. 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. *Cell* **70**(2): 337-350.
- Helin, K., Wu, C.L., Fattaey, A.R., Lees, J.A., Dynlacht, B.D., Ngwu, C., and E. Harlow. 1993b. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. *Genes and Development* **7**(10): 1850-1861.
- Hiebert, S.W., Blake, M., Azizkhan, J., and J.R. Nevins. 1991. Role of E2F transcription factor in E1A-mediated *trans* activation of cellular genes. *Journal of Virology* **65**(7): 3547-3552.
- Higashitsuji, H., Itoh, K., Nagao, T., Dawson, S., Nonoguchi, K., Kido, T., Mayer, R.J., Shigeki, A., and T. Fujita. 2000. Reduced stability of retinoblastoma protein by gankyrin, an oncogenic ankyrin-repeat protein overexpressed in hepatomas. *Nature Medicine* **6**(1): 96-99.

- Higgins, C., Chatterjee, S., and V. Cherington. 1996. The block of adipocyte differentiation by a C-terminally truncated, but not by full-length, simian virus 40 large tumor antigen is dependent on an intact retinoblastoma susceptibility protein family binding domain. *Journal of Virology* **70**(2): 745-752.
- Hijmans, E.M., Voorhoeve, P.M., Beijersbergen, R.L., van't Veer, L.J., and R. Bernards. 1995. E2F-5, a new E2F family member that interacts with p130 in vivo. *Molecular and Cellular Biology* **15**: 3082-3089.
- Howes, K.A., Ransom, H., Papermaster, D.S., Lasudry, J.G., Albert, D.M., and J.J. Windle. 1994. Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. *Genes and Development* **8**(11): 1300-1310.
- Hu, Q.J., Bautista, C., Edwards, G.M., Defeo-Jones, D., Jones, R.E., and E. Harlow. 1991. Antibodies specific for the human retinoblastoma protein identify a family of related polypeptides. *Molecular and Cellular Biology* **11**(11): 5792-5799.
- Hu, Q.J., Dyson, J., and E. Harlow. 1990. The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. *EMBO Journal* **9**(4): 1147-1155.
- Huang, H.J., Yee, J.K., Shew, J.Y., Chen, P.L., Bookstein, R., Friedmann, T., Lee, E.Y., and W.H. Lee. 1988. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science* **242**(4885): 1563-1566.
- Humbert, P.O., Rogers, C., Landsberg, R.L., Trimarchi, J.M., Dandapani, S., Brugnara, C., Erdman, S., Schrenzel, M., Bronson, R.T., and J.A. Lees. 2000a. E2F4 is essential for normal erythrocyte maturation and neonatal viability. *Molecular Cell* **6**: 281-291.
- Humbert, P.O., Verona, R., Trimarchi, J.M., Rogers, C., Dandapani, S., and J.A. Lees. 2000b. E2F3 is critical for normal cellular proliferation. *Genes and Development* **14**(6): 690-703.
- Hwang, M.S., Kim, Y.S., Choi, N.H., Park, J.H., Oh, E.J., Kwon, E.J., Yamaguchi, M., and M.A. Yoo. 2002. The caudal homeodomain protein activates Drosophila E2F gene expression. *Nucleic Acids Research* **30**(23): 5029-5035.
- Imperiale, M.J. 2001. Oncogenic transformation by the human polyomaviruses. *Oncogene* **20**: 7917-7923.
- Ishida, S., Huang, E., Zuzan, H., Spang, R., Leone, G., West, M., and J.R. Nevins. 2001. Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. *Molecular and Cellular Biology* **21**(14): 4684-4699.

- Ivey-Hoyle, M., Conroy, R., Huber, H.E., Goodhart, P.J., Oliff, A., and D.C. Heimbroom. 1993. Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. *Molecular and Cellular Biology* **13**(12): 7802-7812.
- Iwai, A., Marusawa, H., Kiuchi, T., Higashitsuji, H., Tanaka, K., Fujita, J., and T. Chiba. 2003. Role of a novel oncogenic protein, gankyrin, in hepatocyte proliferation. *Journal of Gastroenterology* **38**(8): 751-758.
- Izumi, M., Yokoi, M., Nishikawa, N.S., Miyazawa, H., Sugino, A., Yamagishi, M., Yamaguchi, M., Matsukage, A., Yatagai, F., and F. Hanaoka. 2000. Transcription of the catalytic 180-kDa subunit gene of mouse DNA polymerase alpha is controlled by E2F, an Ets-related transcription factor, and Sp1. *Biochimica et Biophysica Acta* **1492**(2-3): 341-352.
- Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., and R.A. Weinberg. 1992. Effects of an Rb mutation in the mouse. *Nature* **359**: 295-300.
- Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., halachmi, S., Bronson, R.T., and R.A. Weinberg. 1994. Tumor spectrum analysis in p53-mutant mice. *Current Biology* **4**: 1-7.
- Jensen, D.E., Black, A.R., Swick, A.G., and J.C. Azizkhan. 1997. Distinct roles for SP1 and E2F sites in the growth/cell cycle regulation of the DHFR promoter. *Journal of Cellular Biochemistry* **67**: 24-31.
- Jiang, Z., Zacksenhaus, E., Gallie, B.L., and R.A. Phillips. 1997. The retinoblastoma gene family is differentially expressed during embryogenesis. *Oncogene* **14**: 1789-1797.
- Jiao, W., Lin, H., Timmons, J., Nagaich, A.K., Ng, S., Misteli, T., and S.G. Rane. 2005. E2F-dependent repression of topoisomerase II regulates heterochromatin formation and apoptosis in cells in melanoma-prone mutation. *Cancer Research* **65**(10): 4067-4077.
- Johnson, D.G., Ohtani, K., and J.R. Nevins. 1994. Autoregulatory control of *E2F1* expression in response to positive and negative regulators of cell cycle progression. *Genes and Development* **8**: 1514-1525.
- Kaelin, W.G., Jr., Krek, W., Sellers, W.R., DeCaprio, J.A., Ajchenbaum, F., Fuchs, C.S., Chittendon, T., Li, Y., Farnham, P.J., and M.A. Blanas. 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. *Cell* **70**(2): 351-364.
- Kamarova, E.A., Christov, K., Faerman, A.I., and A.V. Gudkov. 2000. Different impact of p53 and p21 on the radiation response of mouse tissues. *Oncogene* **19**: 3791-3798.
- Karlseder, J., Rotheneder, H., and E. Wintersberger. 1996. Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. *Molecular and Cellular Biology* **16**(4): 1659-1667.

- Kasper, J.S., Kuwabara, H., Arai, T., Ali, S.H., and J.A. DeCaprio. 2005. Simian virus 40 large T antigen-s association with the CUL7 SCF complex contributes to cellular transformation. *Journal of Virology* **79**(18): 11685-11692.
- Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., and C.J. Sherr. 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes and Development* **7**(3): 331-342.
- Kierstead, T.D., and M.J. Tevethia. 1993. Association of p53 binding and immortalization of primary C57BL/6 mouse embryo fibroblasts by using simian virus 40 T-antigen mutants bearing internal overlapping deletion mutations. *Journal of Virology* **64**: 1817-1829.
- Kim, K.K., Soonpaa, M.H., Wang, H., and L.J. Field. 1995. Developmental expression of p107 mRNA and evidence for alternative splicing of the p107 (RBL1) gene product. *Genomics* **28**: 520-529.
- Kim, S.H., Roth, K.A., Coopersmith, C.M., Pipas, J.M., and J.I. Gordon. 1994. Expression of wild-type and mutant simian virus 40 large tumor antigens in villus-associated enterocytes of transgenic mice. *Cell Biology* **91**: 6914-6918.
- Kim, S.H., Roth, K.A., Moser, A.R., and J.I. Gordon. 1993. Transgenic mouse models that explore the multistep hypothesis of intestinal neoplasia. *The Journal of Cell Biology* **123**(4): 877-893.
- Kohn, M.J., Bronson, R.T., Harlow, E., Dyson, N.J., and L. Yamasaki. 2003. *Dp1* is required for extra-embryonic development. *Development* **130**: 1295-1305.
- Kondo, Y., Rose, I., Young, G.P., and R.H. Whitehead. 1984. Growth and differentiation of fetal rat small intestinal epithelium in tissue culture. Relationship to fetal age. *Experimental Cell Research* **153**(1): 121-134.
- Korenjak, M., A. Brehm. 2005. E2F-Rb complexes regulating transcription of genes important for differentiation and development. *Current Opinion in Genetics and Development* **15**(5): 520-527.
- Kovesdi, I., Reichel, R., and J.R. Nevins. 1986. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. *Proceedings of the National Academy of Sciences USA* **84**(8): 2180-2184.
- Kovesdi, I., Reichel, R., and J.R. Nevins. 1987. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. *Proceedings of the National Academy of Sciences USA* **84**(8): 2180-2184.
- Krek, W., Ewen, M.E., Shirodkar, S., Arany, Z., Kaelin, Jr., W.G., and D.M. Livingston. 1994. Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. *Cell* **78**: 161.

- Kwong, R.A., Nguyen, T.V., Bova, R.J., Kench, J.G., Cole, I.E., Musgrove, E.A., Henshall, S.M., and R.L. Sutherland. 2003. Overexpression of E2F-1 is associated with increased disease-free survival in squamous cell carcinoma of the anterior tongue. *Clinical Cancer Research* **9**: 3705-3711.
- Lande-Diner, L., and H. Cedar. 2005. Silence of the genes--mechanisms of long-term repression. *Nature Reviews: Genetics* **6**(8): 648-654.
- Landsberg, R.L., Sero, J.E., Danielian, P.S., Yuan, T.L., Lee, E.Y., and J.A. Lees. 2003. The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. *Proceedings of the National Academy of Sciences USA* **100**(5): 2456-2461.
- Lang, G.A., Iwakuma, T., Suh, Y., Liu, G., Rao, A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C., El-Naggar, A.K., and G. Lozano. 2004. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell* **119**: 861-872.
- Lasorella, A., Iavarone, A., and M.A. Israel. 1996. Id2 specifically alters regulation of the cell cycle by tumor suppress proteins. *Molecular and Cellular Biology* **16**(6): 2570-2578.
- Lasorella, A., Uo, T., and A. Iavarone. 2001. Id proteins at the cross-road of development and cancer. *Oncogene* **20**(58): 8326-8333.
- LeCouter, J.E., Kablar, B., Hardy, W.R., Ying, C., Megeney, L.A., May, L.L., and M.A. Rudnicki. 1998a. Strain-dependent myeloid hyperplasia, growth deficiency, and accelerated cell cycle in mice lacking the Rb-related *p107* gene. *Molecular and Cellular Biology* **18**(12): 7455-7465.
- LeCouter, J.E., Kablar, B., Whyte, P.F.M., Ying, C., and M.A. Rudnicki. 1998b. Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. *Development* **125**: 4669-4679.
- Ledl, A., Schmidt, D., and S. Muller. 2005. Viral oncoproteins E1A and E7 and cellular LxCxE proteins repress SUMO modification of the retinoblastoma tumor suppressor. *Oncogene* **24**(23): 3810-3818.
- Lee, E.Y., Cam, H., Ziebold, U., Rayman, J.B., Lees, J.A., and B.D. Dynlacht. 2002. E2F4 loss suppresses tumorigenesis in *Rb* mutant mice. *Cancer Cell* **2**(463-472).
- Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H., and A. Bradley. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359**: 288-294.

- Lee, M., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., and T. Jacks. 1996. Targeted disruption of *p107*: functional overlap between *p107* and *Rb*. *Genes and Development* **10**: 1621-1632.
- Lee, W.H., Bookstein, R., Hong, F., Young, L.J., Shew, J.Y., and E.Y. Lee. 1987a. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* **235**(4794): 1394-1399.
- Lee, W.H., Shew, J.Y., Hong, F.D., Sery, T.W., Donoso, L.A., Young, L.J., Bookstein, R., and E.Y. Lee. 1987b. The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. *Nature* **329**(6140): 642-645.
- Lees, E., Faha, B., Dulic, V., Reed, S.I., and E. Harlow. 1992. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. *Genes and Development* **6**: 1874-1885.
- Lees, J.A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and K. Helin. 1993. The retinoblastoma protein binds to a family of E2F transcription factors. *Molecular and Cellular Biology* **13**(12): 7813-7825.
- Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R.S., and J.R. Nevins. 1998. E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes and Development* **12**(14): 2120-2130.
- Leone, G., Sears, R., Huang, E., Rempel, R., Nuckolls, F., Park, C., Giangrande, P., Wu, L., Saavedra, H.I., Field, S.J., Thompson, M.A., Yang, H., Fujiwara, Y., Greenberg, M.A., Orkin, S., Smith, C., and J.R. Nevins. 2001. Myc requires distinct E2F activities to induce S phase and apoptosis. *Molecular Cell* **8**: 105-113.
- Levine, A.J. 1997. p53, the cellular gatekeeper for growth and division. *Cell* **88**(3): 323-331.
- Li, D., Zhao, R., Lilyestrom, W., Gai, D., Zhang, R., DeCaprio, J.A., Fanning, E., Jochimiak, A., Szakonyi, G., and X.S. Chen. 2003a. Structure of the replicative helicase of the oncoprotein SV40 large tumour antigen. *Nature* **423**: 512-518.
- Li, F., Coonrod, A., and M. Horwitz. 2000. Selection of a dominant negative retinoblastoma protein (RB) inhibiting satellite myoblast differentiation implies an indirect interaction between MyoD and RB. *Molecular and Cellular Biology* **20**(14): 5129-5139.
- Li, F.X., Shu, J.W., Hogan, C.J., and J. DeGregori. 2003b. Defective gene expression, S phase progression, and maturation during hematopoiesis in E2F1/E2F2 mutant mice. *Molecular and Cellular Biology* **23**(10): 3607-3622.
- Li, Y., Graham, C., Lacy, S., Duncan, A.M., and P. Whyte. 1993. The adenovirus E1A-associated 130-kDa protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. *Genes and Development* **7**: 2366-2377.

- Li, Y., Slansky, J.E., Myers, D.J., Drinkwater, N.R., Kaelin, W.G., and P.J. Farnham. 1994. Cloning, chromosomal location, and characterization of mouse *E2F1*. *Molecular and Cellular Biology* **14**(3): 1861-1869.
- Lickert, H., Domon, C., Huls, G., Wehrle, C., Duluc, I., Clevers, H., Meyer, B.I., Freund, J.N., and R. Kemler. 2000. Wnt/ β -catenin signaling regulates the expression of the homeobox gene *Cdx1* in embryonic intestine. *Development* **127**(17): 3805-3813.
- Lin, S.Y., Black, A.R., Kostic, D., Pajovic, S., Hoover, C.N., and J.C. Azizkhan. 1996. Cell cycle-regulated association of E2F1 and Sp1 is related to their functional interaction. *Molecular and Cellular Biology* **16**(4): 1668-1675.
- Lindeman, G.J., Dagnino, L., Gaubatz, S., Xu, Y., Bronson, R.T., Warren, H.B., and D.M. Livingston. 1998. A specific; nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. *Genes and Development* **12**: 1092-1098.
- Lindeman, G.J., Gaubatz, S., Livingston, D.M., and D. Ginsberg. 1997. The subcellular localization of E2F-4 is cell-cycle dependent. *Proceedings of the National Academy of Sciences USA* **94**: 5095-5100.
- Linzer, D.I., and A.J. Levine. 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17**: 43-52.
- Lipinski, M.M., and T. Jacks. 1999. The retinoblastoma gene family in differentiation and development. *Oncogene* **18**(55): 7873-7882.
- Liu, K., Lin, F.T., Ruppert, J.M., and W.C. Lin. 2003. Regulation of E2F1 by BRCT domain-containing protein TopBP1. *Molecular and Cellular Biology* **23**(9): 3287-3304.
- Liu, K., Luo, Y., Lin, F.T., and W.C. Lin. 2004. TopBP1 recruits Brg1/Brm to repress E2F1-induced apoptosis, a novel pRb-independent and E2F1-specific control for cell survival. *Genes and Development* **18**(6): 673-686.
- Lopez, M.J., Upchurch, B.H., Rindi, G., and A.B. Leiter. 1995. Studies in transgenic mice reveal potential relationships between secretin-producing cells and other endocrine cell types. *The Journal of Biological Chemistry* **270**(2): 885-891.
- Louvard, D., Kedinger, M., and H.P. Hauri. 1992. The differentiating intestinal epithelial cell: establishment and maintenance of functions through interactions between cellular structures. *Annual Review of Cell Biology* **8**: 157-195.
- Lowe, S.W., and H.E. Ruley. 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes and Development* **7**(4): 535-545.

- Ludlow, J.W., Shon, J., Pipas, J.M., Livingston, D.M., and J.A. DeCaprio. 1990. The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. *Cell* **60**(3): 387-396.
- Lukas, J., Parry, D., Aagaard, L., Mann, D.J., Bartkova, J., Strauss, M., Peters, G., and J. Bartek. 1995. Rb-dependent cell cycle inhibition by p16^{CDKN2} tumour suppressor. *Nature* **375**: 503-506.
- Lukas, J., Peterson, B.O., Holm, K., Bartek, J., and K. Helin. 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes p16^{INK4a}-mediated growth suppression. *Molecular and Cellular Biology* **16**: 1047-1057.
- Lund, A.H., and M. van Lohuizen. 2004. Epigenetics and cancer. *Genes and Development* **18**: 2315-2335.
- MacLellan, W.R., Garcia, A., Oh, H., Frenkel, P., Jordan, M.C., Roos, K.P., and M.D. Schneider. 2005. Overlapping roles of pocket proteins in the myocardium are unmasked by germ line deletion of p130 plus heart-specific deletion of Rb. *Molecular and Cellular Biology* **25**(6): 2486-2497.
- Macleod, K.F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and T. Jacks. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes and Development* **9**: 935-944.
- MacPherson, D., Sage, J., Crowley, D., Trumpp, A., Bronson, R.T., and T. Jacks. 2003. Conditional mutation of *Rb* causes cell cycle defects without apoptosis in the central nervous system. *Molecular and Cellular Biology* **23**(3): 1044-1053.
- Maehara, K., Yamakoshi, K., Ohtani, N., Kubo, Y., Takahashi, A., Arase, S., Jones, N., and E. Hara. 2005. Reduction of total E2F/DP activity induces senescence-like cell cycle arrest in cancer cells lacking functional pRB and p53. *The Journal of Cell Biology* **168**(4): 553-560.
- Magae, J., Wu, C.L., Illenye, S., Harlow, E., and N.H. Heintz. 1996. Nuclear localization of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. *Journal of Cell Science* **109**: 1717-1726.
- Magro, P.G., Russo, A.J., Li, W., Banerjee, D. and J.R. Bertino. 2004. p14^{ARF} expression increases dihydrofolate reductase degradation and paradoxically results in resistance to folate antagonists in cells with non-functional p53. *Cancer Research* **64**: 4338-4345.
- Maiti, B., Li, J., de Bruin, A., Gordon, F., Timmers, C., Opavsky, R., Patil, K., Tuttle, J., Cleghorn, W., and G. Leone. 2005. Cloning and characterization of mouse *E2F8*, a novel mammalian *E2F* family member capable of blocking cellular proliferation. *Journal of Biological Chemistry* **280**(18): 18211-18220.

- Markey, M.P., Angus, S.P., Strobeck, M.W., Williams, S.L., Gunawardena, R.W., Aronow, B.J., and E.S. Knudsen. 2002. Unbiased analysis of RB-mediated transcriptional repression identifies novel targets and distinctions from E2F action. *Cancer Research* **62**(6587-6598): November 15.
- Markovics, J.A., Carroll, P.A., Sáenz-Robles, M.T., Pope, H., Coopersmith, C.M., and J.M. Pipas. 2005. Intestinal dysplasia induced by simian virus 40 T antigen is independent of p53. *Journal of Virology* **79**(12): 7492-7502.
- Martelli, F., Hamilton, T., Silver, D.P., Sharpless, N.E., Bardeesy, N., Rokas, M., DePinho, R.A., Livingston, D.M., and S.R. Grossman. 2001. p19ARF targets certain E2F species for degradation. *Proceedings of the National Academy of Sciences USA* **98**(8): 4455-4460.
- Marti, A., Wirbelauer, C., Scheffner, M., and W. Krek. 1999. Interaction between ubiquitin-protein ligase SCF^{SKP2} and E2F-1 underlies the regulation of E2F-1 degradation. *Nature Cell Biology* **1**: 14-19.
- Martinez-Balbás, M.A., Bauer, U., Nielsen, S.J., Brehm, A., and T. Kouzarides. 2000. Regulation of E2F1 activity by acetylation. *The EMBO Journal* **19**(4): 662-671.
- Marzio, G., Wagener, C., Gutierrez, M.I., Cartwright, P., Helin, K., and M. Giacca. 2000. E2F family members are differentially regulated by reversible acetylation. *The Journal of Biological Chemistry* **275**(15): 10887-10892.
- Mayol, S., Grana, X., Baldi, A., Sang, N., Hu, Q., and A. Giordano. 1993. Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. *Oncogene* **8**(9): 2561-2566.
- Merritt, A.J., Potten, C.S., Kemp, C.J., Hickmann, J.A., Balmain, A., Lane, D.P., and P.A. Hall. 1994. The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. *Cancer Research* **54**: 614-617.
- Michaloglou, C., Vredeveld, L.C.W., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M.A.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and D.S. Peeper. 2005. BRAF^{E600}-associated senescence-like cell cycle arrest of human naevi. *Nature* **436**: 720-724.
- Mittnacht, S. 1998. Control of pRB phosphorylation. *Current Opinion in Genetics and Development* **8**(1): 21-27.
- Moberg, K., Starz, M.A., and J.A. Lees. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Molecular and Cellular Biology* **16**(4): 1436-1449.
- Moley, K.H., and M.M. Mueckler. 2000. Glucose transport and apoptosis. *Apoptosis* **5**(2): 99-105.

- Moser, A.R., Pitot, H.C., and W.F. Dove. 1990. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **247**: 322-324.
- Mudryj, M., Devoto, S.H., Hiebert, S.W., Hunter, T., Pines, J., and J.R. Nevins. 1991. Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. *Cell* **65**(7): 1243-1253.
- Mudryj, M., Hiebert, S.W., and J.R. Nevins. 1990. A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. *The EMBO Journal* **9**(7): 2179-2184.
- Munger, K., Werness, B.A., Dyson, N., Phelps, W.C., Harlow, E., and P.M. Howley. 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO Journal* **8**(13): 4099-4105.
- Mungre, S., Enderle, K., Turk, B., Porrás, A., Wu, Y., Mumby, M.C., and K. Rundell. 1994. Mutations which affect the inhibition of protein phosphatase 2A by simian virus 40 small-t antigen in vitro decrease viral transformation. *Journal of Virology* **68**(3): 1675-1681.
- Murga, M., Fernández-Capetillo, O., Field, S.J., Moreno, B., Borlado, L.R., Fujiwara, Y., Balomenos, D., Vicario, A., Carrera, A.C., Orkin, S.H., Greenberg, M.E., and A.M. Zubiaga. 2001. Mutation of E2F2 in mice causes enhanced T lymphocyte proliferation, leading to the development of autoimmunity. *Immunity* **15**: 959-970.
- Mysorekar, I.U., Lorenz, R.G., and J.I. Gordon. 2002. A gnotobiotic transgenic mouse model for studying interactions between small intestinal enterocytes and intraepithelial lymphocytes. *The Journal of Biological Chemistry* **277**(40): 37811-37819.
- Müller, H., Bracken, A.P., Vernell, R., Moroni, M.C., Christians, F., Grasselli, E., Prosperini, E., Vigo, E., Oliner, J.D., and K. Helin. 2001. E2Fs regulate the expression of genes involved in differentiation, development, proliferation and apoptosis. *Genes and Development* **15**(3): 267-285.
- Narita, M., Nuñez, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and S.W. Lowe. 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113**: 703-716.
- Nevins, J.R. 1998. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. *Cell Growth and Differentiation* **9**: 585-593.
- Nguyen, D.X., Baglia, L.A., Huang, S.M., Baker, C.M., and D.J. McCance. 2004. Acetylation regulates the differentiation-specific functions of the retinoblastoma protein. *The EMBO Journal* **23**(7): 1609-1018.

- Novitch, B.G., Spicer, D.B., Kim, P.S., Cheung, W.L., and A.B. Lassar. 1999. pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. *Current Biology* **9**(9): 449-459.
- Noé, V., Alemany, C., Chasin, L.A., and C.J. Ciudad. 1998. Retinoblastoma protein associates with SP1 and activates the hamster *dihydrofolate reductase* promoter. *Oncogene* **16**: 1931-1938.
- O'Connor, D.J., and X. Lu. 2000. Stress signals induce transcriptionally inactive E2F-1 independently of p53 and pRb. *Oncogene* **19**: 2369-2376.
- O'Shea, C.C. 2005. DNA tumor viruses - the spies who lyse us. *Current Opinion in Genetics and Development* **15**: 18-26.
- Ohta, T., and Y. Xiong. 2001. Phosphorylation- and SKP1-independent *in vitro* ubiquitination of E2F1 by multiple ROC-Cullin ligases. *Cancer Research* **61**: 1347-1353.
- Olgiate, J., Ehmann, G.L., Vidyarthi, S., Hilton, M.J., and S.L. Bachenheimer. 1999. Herpes simplex virus induces intracellular redistribution of E2F4 and accumulation of E2F pocket protein complexes. *Virology* **258**(2): 257-270.
- Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and T. Jacks. 119. Mutant *p53* gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* **119**: 847-860.
- Oren, M. 1994. Relationship of p53 to the control of apoptotic cell death. *Seminars in Cancer Biology* **5**(3): 221-227.
- Oruetxebarria, I., Venturini, F., Kekarainen, T., Houweling, A., Zuijderduijn, L.M.P., Mohd-Sarip, A., Vries, R.G.J., Hoeben, R.C., and C.P. Verrijzer. 2004. p16^{INK4a} is required for hSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumor cells. *The Journal of Biological Chemistry* **279**(5): 3807-3816.
- Osifchin, N.E., Jiang, D., Ohtani-Fujita, N., Fujita, T., Carroza, M., Kim, S., Sakai, T., and P.D. Robbins. 1994. Identification of a p53 binding site in the human retinoblastoma susceptibility gene promoter. *The Journal of Biological Chemistry* **269**(9): 6383-6389.
- Ouellett, M.M., Chen, J., Wright, W.E., and J.W. Shaw. 1992. Complexes containing the retinoblastoma gene product recognize different DNA motifs related to the E2F binding site. *Oncogene* **7**(6): 1075-1081.
- Padmanabhan, B., Adachi, N., Kataoka, K., and M. Hoikoshi. 2004. Crystal structure of the homolog of the oncoprotein Gankyrin, an interactor of Rb and CDK4/6. *The Journal of Biological Chemistry* **279**(2): 1546-1552.

- Pallas, D.C., Shahrik, L.K., Martin, B.L., Jaspers, S., Miller, T.B., Brautigam, D.L., and T.M. Roberts. 1990. Polyoma small and middle T antigens and SV40 small t antigen for stable complexes with protein phosphatase 2A. *Cell* **60**(1): 167-176.
- Pan, H., Yin, C., Dyson, N.J., Harlow, E., Yamasaki, L., and T. Van Dyke. 1998. Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. *Molecular Cell* **2**(3): 283-292.
- Parisi, T., Pollice, A., Di Cristofano, A., Calabro, V., and G. La Mantia. 2002. Transcriptional regulation of the human tumor suppressor p14(ARF) by E2F1. *Biochemistry and Biophysics Research Communication* **291**: 1138-1145.
- Park, K., Rue, S., Lee, I., Kim, H., Lee, I., Ahn, J.D., Kim, H., Yu, T., Kwak, J., Heintz, N.H., Magae, J., and Y. Chang. 2003. Modulation of SP1-dependent transcription by a *cis*-acting E2F element in *dhfr* promoter. *Biochemical and Biophysical Research Communications* **306**: 239-243.
- Peden, K.W., Srinivasan, A., Vartikar, J.V., and J.M. Pipas. 1998. Effects of mutations within the SV40 large T antigen ATPase domain on viral replication and transformation. *Virus Genes* **16**(2): 153-165.
- Persengiev, S.P., I.I. Kondova, and D.L. Kilpatrick. 1999. E2F4 actively promotes the initiation and maintenance of nerve growth factor-induced cell differentiation. *Molecular and Cellular Biology* **19**(9): 6048-6056.
- Peto, R. 1977. Cancer epidemiology, multistage models, and short-term mutagenicity tests. in *Origins of Human Cancer* (ed. H.H. Hiatt, Watson, J.D., and J.A. Winsten), pp. 1403-1428. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Pietenpol, J.A., and Z.A. Stewart. 2002. Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. *Toxicology* **181-182**: 475-481.
- Pipas, J.M., and A.J. Levine. 2001. Role of T antigen interactions with p53 in tumorigenesis. *Seminars in Cancer Biology* **11**(1): 23-30.
- Pipas, J.M., Peden, K.W.C., and D. Nathans. 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene. *Molecular and Cellular Biology* **3**(2): 203-213.
- Pohlers, M., Truss, M., Frede, U., Scholz, A., Strehle, M., Kuban, R.J., Hoffmann, B., Morkel, M., Birchmeier, C., and C. Hagemeier. 2005. A role for E2F6 in the restriction of male-germ-cell-specific gene expression. *Current Biology* **15**(11): 1051-1057.
- Polager, S., and D. Ginsberg. 2003. E2F mediates sustained G₂ arrest and down-regulation of stathmin and AIM-1 expression in response to genotoxic stress. *The Journal of Biological Chemistry* **278**(3): 1443-1449.

- Polanowska, J., Le Cam, L., Orsetti, B., Vallés, H., Fabbriozio, E., Fajas, L., Taviaux, S., Theillet, C., and C. Sardet. 2000. Human *E2F5* is oncogenic in primary rodent cells and is amplified in human breast tumors. *Genes, Chromosomes and Cancer* **28**: 126-130.
- Potten, C.S., Wilson, J.W., and C. Booth. 1997. Regulation and significance of apoptosis in the stem cells of the gastrointestinal epithelium. *Stem Cells* **15**: 82-93.
- Powell, P.C. 1976. The clonal evolution of tumor cell populations. *Science* **194**: 23-28.
- Prives, C., and P.A. Hall. 1999. The p53 pathway. *Journal of Pathology* **187**: 112-126.
- Psyrrri, A., DeFilippis, R.A., Edwards, A.P.B., Yates, K.E., Manuelidis, L., and D. DiMaio. 2004. Role of the retinoblastoma pathway in senescence triggered by repression of the human papillomavirus E7 protein in cervical carcinoma cells. *Cancer Research* **64**(9): 3079-3086.
- Pucilowska, J.B., McNaughton, K.K., Mohapatra, N.K., Hoyt, E.C., Zimmermann, E.M., Sartor, R.B., and P.K. Lund. 2000. IGF-I and procollagen alpha1(I) are coexpressed in a subset of mesenchymal cells in active Crohn's disease. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **279**: G1307-G1322.
- Purdle, C.A., Harrison, D.J., Peter, A., Dobble, L., White, S., Howie, S.E., Salter, D.M., Bird, C.C., Wyllie, A.H., Hooper, M.L., and A.R. Clarke. 1994. Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. *Oncogene* **9**: 603-609.
- Qi, Y., Gregory, M.A., Li, Z., Brousal, J.P., West, K., and S.R. Hann. 2004. p19ARF directly and differentially controls the functions of c-Myc independently of p53. *Nature* **431**: 712-717.
- Quelle, D.E., Zindy, F., Ashmun, R.A., and C.J. Sherr. 1995. Alternative reading frames of the *INK4a* tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83**: 993-1000.
- Radtke, F., and H. Clevers. 2005. Self-renewal and cancer of the gut: Two sides of the same coin. *Science* **307**: 1904-1909.
- Ramond, M.J., Martinot-Peignoux, M., and S. Erlinger. 1985. Dome formation in the human colon carcinoma cell line Caco-2 in culture. Influence of ouabain and permeable supports. *Biological Cell* **54**(1): 89-92.
- Ratineau, C., Ronco, A., and A.B. Leiter. 2000. Role of the amino-terminal domain of simian virus 40 early region in inducing tumors in secretin-expressing cells in transgenic mice. *Gastroenterology* **119**(5): 1305-1311.

- Reichel, R., Kovessi, I., and J.R. Nevins. 1988. Activation of a preexisting cellular factor as a basis for adenovirus E1A-mediated transcription control. *Proceedings of the National Academy of Sciences USA* **85**(2): 387-390.
- Rempel, R.E., Saenz-Robles, M.T., Storms, R., Morham, S., Ishida, S., Engel, A., Jakoi, L., Melhem, M.F., Pipas, J.M., Smith, C., and J.R. Nevins. 2000. Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Molecular Cell* **6**: 293-306.
- Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R.A., and B.D. Dynlacht. 2002. E2F integrates cell cycle progression with DNA repair, replication, and G₂/M checkpoints. *Genes and Development* **16**(2): 245-256.
- Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L., and A.P. Wolffe. 2000. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nature Genetics* **25**(3): 338-342.
- Rotheneder, H., Geymayer, S., and E. Haidweger. 1999. Transcription factor of the Sp1 family: interaction with E2F and regulation of the murine thymidine kinase promoter. *Journal of Molecular Biology* **293**(5): 1005-1015.
- Ruiz, S., Segrelles, C., Bravo, A., Santos, M., Perez, P., Leis, H., Jorcano, J.L., and J.M. Paramio. 2004. Abnormal epidermal differentiation and impaired epithelial-mesenchymal tissue interactions in mice lacking the retinoblastoma relative p107 and p130. *Development* **130**: 2341-2353.
- Russell, J.L., Weeks, R.L., Berton, T.R., and D.G. Johnson. 2005. E2F1 suppresses skin carcinogenesis via the ARF-p53 pathway. In *Oncogene*.
- Russell, R.G., Lasorella, A., Dettin, L.E., and A Iavarone. 2004. Id2 drives differentiation and suppresses tumor formation in the intestinal epithelium. *Cancer Research* **64**: 7220-7225.
- Rustgi, A.K., Dyson, N., and R. Bernards. 1991. Amino-terminal domains of c-myc and N-myc proteins mediate binding to the retinoblastoma gene product. *Nature* **352**: 541-544.
- Saavedra, H.I., Maiti, B., Timmers, C., Altura, R., Tokuyama, Y., Fukasawa, K., and G. Leone. 2003. Inactivation of E2F3 results in centrosome amplification. *Cancer Research* **3**(4): 333-346.
- Saavedra, H.I., Wu, L., de Bruin, A., Timmers, C., Rosol, T.J., Weinstein, M., Robinson, M.L., and G. Leone. 2002. Specificity of E2F1, E2F2, and E2F3 in mediating phenotypes induced by loss of Rb. *Cell Growth and Differentiation* **13**: 215-225.
- Sachsenmeier, K.F., James M. Pipas. 2001. Inhibition of Rb and p53 is insufficient for SV40 T-antigen transformation. *Virology* **283**(1): 40-48.

- Sage, J., Mulligan, G.J., Attardi, L.D., Miller, A., Chen, S., Williams, B., Theodorou, E., and T. Jacks. 2000. Targeted disruption of the three Rb-related genes leads to loss of G₁ control and immortalization. *Genes and Development* **14**: 3037-3050.
- Sancho, E., Batlle, E., and H. Clevers. 2004. Signaling pathways in intestinal development and cancer. *Annual Review of Cell and Developmental Biology* **20**: 695-723.
- Sansom, O.J., Reed, K.R., van de Wetering, M., Muncan, V., Winton, D.J., Clevers, H., and A.R. Clarke. 2004. Cyclin D1 is not an immediate target of β -catenin following Apc loss in the intestine. *The Journal of Biological Chemistry* **280**(31): 28463-28467.
- Santoni-Rugiu, E., Falck, J., Mailand, N., Bartek, J., and J. Lukas. 2000. Involvement of Myc activity in a G₁/S-promoting mechanism parallel to the pRb/E2F pathway. *Molecular and Cellular Biology* **20**(10): 3497-3509.
- Sardet, C., Vidal, M., Cobrinik, D., Geng, Y., Onufryk, C., Chen, A., and R.A. Weinberg. 1995. E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. *Proceedings of the National Academy of Sciences USA* **92**: 2403-2407.
- Schlisio, S., Halperin, T., Vidal, M., and J.R. Nevins. 2002. Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function. *The EMBO Journal* **21**(21): 5775-5786.
- Schonhoff, S.E., Giel-Moloney, M., and A.B. Leiter. 2004. Minireview: Development and differentiation of gut endocrine cells. *Endocrinology* **145**(6): 2639-2644.
- Schwarz, J.K., Devoto, S.H., Smith, E.J., Chellappan, S.P., Jakoi, L., and J.R. Nevins. 1993. Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. *The EMBO Journal* **12**(3): 1013-1020.
- Sears, R., Ohtani, K., and J.R. Nevins. 1997. Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. *Molecular and Cellular Biology* **17**(9): 5227-5235.
- Sell, S. 2004. Stem cell origin of cancer and differentiation therapy. *Critical Reviews in Oncology/Hematology* **51**: 1-28.
- Sellers, W.R., Novitch, B.G., Miyake, S., Heith, A., Otterson, G.A., Kaye, F.J., Lassar, A.B., and W.G. Kaelin, Jr. 1998. Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. *Genes and Development* **12**: 95-106.
- Serrano, M., Hannon, G.J., and D. Beach. 1993. A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/cdk4. *Nature* **366**(704-707).

- Shah, K.V. 1985. Chapter 19: Papovaviruses. in *Virology* (ed. B.N. Fields, editor-in-chief; Chanock, R.M., Knipe, D.M., Melnick, J.L., Roizman, B., Shope, R.E.), pp. 371-391. Raven Press, New York.
- Shan, B., and W. Lee. 1994a. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Molecular and Cellular Biology* **14**(12): 8166-8173.
- Shan, B., Chang, C., Jones, D., and W. Lee. 1994b. The transcription factor E2F-1 mediates the autoregulation of RB gene expression. *Molecular and Cellular Biology* **14**(1): 299-309.
- Sharpless, N.E., and R.A. DePinho. 2005. Crime and punishment. *Nature* **436**: 636-637.
- Shenolikar, S. 1996. Analysis of protein phosphorylation: Detection of phosphorylation by enzymatic techniques. in *Current Protocols in Molecular Biology* (ed. R.B. F.M. Ausubel, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl), pp. 18.15.11-18.15.14. John Wiley and Sons, Inc., New York.
- Sherr, C.J. 2001. The INK4a/ARF network in tumour suppression. *Nature Reviews: Molecular Cell Biology* **2**(10): 731-737.
- Sherr, C.J., and F. McCormick. 2002. The RB and p53 pathways in cancer. *Cancer Cell* **2**: 103-112.
- Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'amico, M., Pestell, R., and A. Ben-Ze'ev. 1999. The cyclin D1 gene is a target of the β -catenin/LEF-1 pathways. *Proceedings of the National Academy of Sciences USA* **96**(10): 5522-5527.
- Singh, P., Wong, S.W., and W. Hong. 1994. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. *The EMBO Journal* **13**(14): 3329-3338.
- SivaRaman, L., Subramanian, S., and B. Thimmappaya. 1986. Identification of a factor in HeLa cells specific for an upstream transcriptional control sequence of an E1A-inducible adenovirus promoter and its relative abundance in infected and uninfected cells. *Proceedings of the National Academy of Sciences USA* **83**: 5914-5918.
- Smith, E.J., Leone, G., and J.R. Nevins. 1998. Distinct mechanisms control the accumulation of the Rb-related p107 and p130 proteins during cell growth. *Cell Growth and Differentiation* **9**(4): 297-303.
- Solway, J., Seltzer, J., Samaha, F.F., Kim, S., Alger, L.E., Niu, Q., Morrissey, E.E., Ip, H.S., and M.S. Parmacek. 1995. Structure and expression of a smooth muscle cell-specific gene, SM22alpha. *Journal of Biological Chemistry* **270**: 13460-13469.
- Spike, B.T., Dirlam, A., Dibling, B.C., Marvin, J., Williams, B.O., Jacks, T., and K.F. Macleod. 2004. The Rb tumor suppressor is required for stress erythropoiesis. *The EMBO Journal* **23**(21): 4319-4329.

- Srinivasan, A., McClellan, A.J., Vartikar, J., Marks, I., Cantalupo, P., Li, Y., Whyte, P., Rundell, K., Brodsky, J., and J.M. Pipas. 1997. The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain. *Molecular and Cellular Biology* **17**: 4761-4773.
- Srinivasan, A., Peden, K.W.C., and J.M. Pipas. 1989. The large tumor antigen of simian virus 40 encodes at least two distinct transforming functions. *Journal of Virology* **63**(12): 5459-5463.
- Stainier, D.Y.R. 2005. No organ left behind: Tales of gut development and evolution. *Science* **307**: 1902-1904.
- Stevaux, O., and N.J. Dyson. 2002a. A revised picture of the E2F transcriptional network and RB function. *Current Opinion in Cell Biology* **14**(6): 684-691.
- Stevaux, O., Dimova, D., Frolov, M.V., Taylor-Harding, B., Morris, E., and N. Dyson. 2002b. Distinct mechanisms of E2F regulation by *Drosophila* RBF1 and RBF2. *The EMBO Journal* **21**(18): 4927-4937.
- Storre, J., Elsässer, H., Fuchs, M., Ullmann, D., Livingston, D.M., and S. Gaubatz. 2002. Homeotic transformations of the axial skeleton that accompany a targeted deletion of *E2F6*. *EMBO reports* **3**(7): 695-700.
- Stott, F.J., Bates, S., James, M.C., McConnell, B.B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K.H., and G. Peters. 1998. The alternative product from the human *CDKN2A* locus, p14^{ARF}, participates in a regulatory feedback loop with p53 and MDM2. *The EMBO Journal* **17**(17): 5001-5014.
- Stubdal, H., Zalvide, J., Campbell, K.S., Schweitzer, C., Roberts, T.M., and J.A. DeCaprio. 1997. Inactivation of pRB-related proteins p130 and p107 mediated by the J domain of Simian Virus 40 large T antigen. *Molecular and Cellular Biology* **17**(9): 4979-4990.
- Sullivan, C.S., and J.M. Pipas. 2002. T antigens of simian virus 40: Molecular chaperones for viral replication and tumorigenesis. *Microbiology and Molecular Biology Reviews* **66**(2): 179-202.
- Sullivan, C.S., Baker, A.E., and J.M. Pipas. 2004. Simian virus 40 infection disrupts p130-E2F and p107-E2F complexes but does not perturb pRb-E2F complexes. *Virology* **320**(2): 218-228.
- Sullivan, C.S., Cantalupo, P., and J.M. Pipas. 2000. The molecular chaperone activity of simian virus 40 large T antigen is required to disrupt Rb-E2F family complexes by an ATP-dependent mechanism. *Molecular and Cellular Biology* **20**(17): 6233-6243.

- Sullivan, C.S., Grundhoff, A.T., Tevethia, S., Pipas, J.M., and D. Ganem. 2005. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature* **435**: 682-686.
- Symonds, H., Krall, L., Remington, L., Sáenz-Robles, M.T., Lowe, S., Jacks, T., and T. Van Dyke. 1994. p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell* **78**: 703-711.
- Sáenz-Robles, M.T., Sullivan, C.S., and J.M. Pipas. 2001. Transforming functions of Simian virus 40. *Oncogene* **20**: 7899-7907.
- Sáenz-Robles, M.T., Symonds, H., Chen, J., and T. Van Dyke. 1994. Induction versus progression of brain tumor development: differential functions for the pRB- and p53-targeting domains of simian virus 40 T antigen. *Molecular and Cellular Biology* **14**: 2686-2698.
- Takahashi, Y., Rayman, J.B., and B.D. Dynlacht. 2000. Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes and Development* **14**: 804-816.
- Tao, Y., Kassatly, R.F., Cress, W.D., and J.M. Horowitz. 1997. Subunit composition determines E2F DNA-binding site specificity. *Molecular and Cellular Biology* **17**(12): 6994-7007.
- Tedesco, D., Caruso, M., Fischer-Fantuzzi, L., and C. Vesco. 1995. The inhibition of cultured myoblast differentiation by the simian virus 40 large T antigen occurs after myogenin expression and Rb up-regulation and is not exerted by transformation-competent cytoplasmic mutants. *Journal of Virology* **69**(11): 6947-6957.
- Tevethia, M.J., Bonneau, R.H., Griffith, J.W., and L. Mylin. 1997. A simian virus 40 large T-antigen segment containing amino acids 1-127 and expressed under the control of the rat elastase-1 promoter produces pancreatic acinar carcinomas in transgenic mice. *Journal of Virology* **71**: 8157-8166.
- Thomas, D.M., Carty, S.A., Piscopo, D.M., Lee, J., Wang, W., Forrester, W.C., and P.W. Hinds. 2001. The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Molecular Cell* **8**: 303-316.
- Thomas, R.C., Edwards, M.J., and R. Marks. 1996. Translocation of the retinoblastoma gene product during mitosis. *Experimental Cell Research* **223**: 227-232.
- Thompson, D.L., Kalderon, D., Smith, A.E., and M.J. Tevethia. 1990. Dissociation of pRB-binding and anchorage-independent growth from immortalization and tumorigenicity using SV40 mutants producing N-terminally truncated large T antigens. *Virology* **178**: 15-34.

- Tommasi, S., and G.P. Pfeifer. 1997. Constitutive protection of E2F recognition sequences in the human thymidine kinase promoter during cell cycle progression. *The Journal of Biological Chemistry* **272**(48): 30483-30490.
- Traber, P.G., and G.D. Wu. 1995. Intestinal Development and Differentiation. in *Gastrointestinal Cancers: Biology; Diagnosis and Therapy* (ed. A.K. Rustgi), pp. 21-43. Lippincott-Raven, Philadelphia.
- Trimarchi, J.M., and J.A. Lees. 2002. Sibling rivalry in the E2F family. *Nature Reviews: Molecular Cell Biology* **3**: 11-20.
- Trimarchi, J.M., Fairchild, B., Verona, R., Moberg, K., Andon, N., and J.A. Lees. 1998. E2F-6, a member of the E2F family that can behave as a transcriptional repressor. *Proceedings of the National Academy of Sciences USA* **95**: 2850-2855.
- Trimarchi, J.M., Fairchild, B., Wen, J., and J.A. Lees. 2001. The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. *Proceedings of the National Academy of Sciences USA* **98**(4): 1519-1524.
- Tsunokawa, Y., Takebe, N., Kasamatsu, T., Terada, M., and T. Sugimura. 1986. Transforming activity of human papillomavirus type 16 DNA sequences in a cervical cancer. *Proceedings of the National Academy of Sciences USA* **83**: 2200-2203.
- Udvardi, A.J., Templeton, D.J., and J.M. Horowitz. 1995. Functional interactions between the retinoblastoma (Rb) protein and Sp family members: Superactivation by Rb requires amino acids necessary for growth suppression. *Proceedings of the National Academy of Sciences USA* **92**(9): 3953-3957.
- Uemura, K., Saka, M., Nakagawa, T., Kawasaki, N., Thiel, S., Jensenius, J.C., and T. Kawasaki. 2002. L-MBP is expressed in epithelial cells of mouse small intestine. *Journal of Immunology* **169**: 6945-6950.
- Vairo, G., Livingston, D.M., and D. Ginsberg. 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes and Development* **9**(7): 869-891.
- Vaishnav, Y.N., Vaishnav, M.Y., and V. Pant. 1998. The molecular and functional characterization of E2F-5 transcription factor. *Biochemical and Biophysical Research Communications* **242**: 586-592.
- van der Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Battle, E., Coudreuse, D., Haramis, A., Tjon-Pon-Fon, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., and H. Clevers. 2002. The β -catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**: 241-250.

- van Gurp, M.F., Pratap, J., Luong, M., Javed, A., Hoffman, H., Giordano, A., Stein, J.L., Neufeld, E.J., Lian, J.B., Stein, G.S., and A.J. van Wijnen. 1999. The CCAAT displacement protein/*cut* homeodomain protein represses osteocalcin gene transcription and forms complexes with the retinoblastoma protein-related protein p107 and cyclin A. *Cancer Research* **59**: 5980-5988.
- Vogelstein, B., and K.W. Kinzler. 1993. The multistep nature of cancer. *Trends in Genetics* **9**: 138-141.
- Walters, J.R.F. 2005. Recent findings in the cell and molecular biology of the small intestine. *Current Opinion in Gastroenterology* **21**: 135-140.
- Watanabe, G., Albanese, C., Lee, R.J., Reutens, A., Vairo, G., Henglein, B., and R.G. Pestell. 1998. Inhibition of cyclin D1 kinase activity is associated with E2F-mediated inhibition of cyclin D1 promoter activity through E2F and Sp1. *Molecular and Cellular Biology* **18**(6): 3213-3222.
- Watson, A.J.M., and D.M. Pritchard. 2000. Lessons from genetically engineered animal models VII. Apoptosis in intestinal epithelium: lessons from transgenic and knockout mice. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **278**(G1-G5).
- Weber, J.D., Jeffers, J.R., Rehg, J.E., Randle, D.H., Lozano, G., Roussel, M.F., Sherr, C.J., and G.P. Zambetti. 200. p53-independent functions of the p19^{ARF} tumor suppressor. *Genes and Development* **14**(18): 2358-2365.
- Weintraub, S.J., Prater, C.A., and D.C. Dean. 1992. Retinoblastoma protein switches the E2F site from positive to negative element. *Nature* **358**: July 16.
- Wells, J., Boyd, K.E., Fry, C.J., Bartley, S.M., and P.J. Farnham. 2000a. Target gene specificity of E2F and pocket protein family members in living cells. *Molecular and Cellular Biology* **20**(16): 5797-5807.
- Wells, J., Graveel, C.R., Bartley, S.M., Madore, S.J., and P.J. Farnham. 2002. The identification of E2F1-specific target genes. *Proceedings of the National Academy of Sciences USA* **99**(6): 3890-3895.
- Wells, J.M., Illenye, S., Magae, J., Wu, C., and N.H. Heintz. 1997. Accumulation of E2F-4•DP-1 DNA binding complexes correlates with induction of *dhfr* gene expression during the G1 to S phase transition. *The Journal of Biological Chemistry* **272**(7): 4483-4492.
- Wells, S.I., Francis, D.A., Karpova, A.Y., Dowhanick, J.J., Benson, J.D., and P.M. Howley. 2000b. Papillomavirus E2 induces senescence in HPV-positive cells via pRB- and p21^{CIP}- dependent pathways. *The EMBO Journal* **19**(21): 5762-5771.

- Whyte, P., Buchkovich, K.H., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A., and E. Harlow. 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334**: 124-129.
- Wilson, J.W., Pritchard, D.M., Hickman, J.A., and C.S. Potten. 1998. Radiation-induced p53 and p21^{WAF-1/CIP-1} expression in the murine intestinal epithelium. *The American Journal of Pathology* **153**(3): 899-909.
- Woo, M.S., Sánchez, I., and B.D. Dynlacht. 1997. p130 and p107 use a conserved domain to inhibit cellular cyclin-dependent kinase activity. *Molecular and Cellular Biology* **17**(7): 3566-3579.
- Wu, L., Timmers, C., Maiti, B., Saavedra, H.I., Sang, L., Chong, G.T., Nuckolls, F., Giangrande, P., Wright, F.A., Field, S.J., Greenberg, M.E., Orkin, S., Nevins, J.R., Robinson, M.L., and G. Leone. 2001. The E2F1-3 transcription factors are essential for cellular proliferation. *Nature* **414**: 457-462.
- Xu, G., D.M. Livingston, and W. Krek. 1995. Multiple members of the E2F transcription factor family are the products of oncogenes. *Proceedings of the National Academy of Sciences USA* **92**: 1357-1361.
- Xu, M., Sheppard, K., Peng, C., Yee, A.S., and H. Piwnicka-Worms. 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. *Molecular and Cellular Biology* **14**(12): 8420-8431.
- Yamasaki, L. 2003. Role of the RB tumor suppressor in cancer. *Cancer Treatment Research* **115**: 209-239.
- Yamasaki, L., Bronson, R., Williams, B.O., Dyson, N.J., Harlow, E., and T. Jacks. 1998. Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-)mice. *Nature Genetics* **18**(4): 360-364.
- Yamasaki, L., Jacks, T., Bronson, R., Goillet, E., Harlow, E., and N.J. Dyson. 1996. Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* **85**: 537-548.
- Yan, Y., Frisen, J., Lee, M.H., Massague, J., and M. Barbacid. 1997. Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes and Development* **11**(8): 973-983.
- Yang, S., Lickteig, R.L., Estes, R., Rundell, K., Walter, G., and M.C. Mumbly. 1991. Control of protein phosphatase 2A by simian virus 40 small-t antigen. *Molecular and Cellular Biology* **11**(4): 1988-1995.
- Yang, X.H., and T.L. Sladek. 1995. Overexpression of the E2F-1 transcription factor gene mediates cell transformation. *Gene Expression* **4**(4-5): 195-204.

- Yee, A.S., Raychaudhuri, P., Jakoi, L., and J.R. Nevins. 1989. The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. *Molecular and Cellular Biology* **9**(2): 578-585.
- Yee, A.S., Reichel, R., Kovesdi, I., and J.R. Nevins. 1987. Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex. *EMBO Journal* **6**(7): 2061-2068.
- Yokota, Y. 2001. Id and development. *Oncogene* **20**(58): 8390-8398.
- Young, A.P., and G.D. Longmore. 2004. Differences in stability of repressor complexes at promoters underlie distinct roles for Rb family members. *Oncogene* **23**: 814-823.
- Young, A.P., Nagarajan, R., and G.D. Longmore. 2003. Mechanisms of transcriptional regulation by Rb-E2F segregate by biological pathway. *Oncogene* **22**: 7209-7217.
- Zhang, H.S., and D.C. Dean. 2001. Rb-mediated chromatin structure regulation and transcriptional repression. *Oncogene* **20**(3134-3138).
- Zhang, H.S., Postigo, A.A., and D.C. Dean. 1999. Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGF β , and contact inhibition. *Cell* **97**: 53-61.
- Zhang, Y., and S.P. Chellappan. 1995. Cloning and characterization of human DP2, a novel dimerization partner of E2F. *Oncogene* **10**(11): 2085-2093.
- Zhang, Y., Venkatraj, V.S., Fischer, S.G., Warburton, D., and S.P. Chellappan. 1997. Genomic cloning and chromosomal assignment of the E2F dimerization partner TFDP gene family. *Genomics* **39**: 95-98.
- Zhu, J.W., Field, S.J., Gore, L., Thompson, M., Yang, H., Fujiwara, Y., Cardiff, R.D., Greenberg, M., Orkin, S.H., and J. DeGregori. 2001. E2F1 and E2F2 determine thresholds for antigen-induced T-cell proliferation and suppress tumorigenesis. *Molecular and Cellular Biology* **21**(24): 8547-8564.
- Zhu, L., Zhu, L., Xie, E., and L. Chang. 1995. Differential roles of two tandem E2F sites in repression of the human promoter by retinoblastoma and p107 proteins. *Molecular and Cellular Biology* **15**(7): 3552-3562.
- Ziebold, U., Lee, E.Y., Bronson, R.T., and J.A. Lees. 2003. E2F3 loss has opposing effects on different pRB-deficient tumors, resulting in suppression of pituitary tumors but metastasis of medullary thyroid carcinomas. *Molecular and Cellular Biology* **23**(18): 6542-6552.
- zur Hausen, H. 2000. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *Journal of the National Cancer Institute* **92**(9): 690-698.
- zur Hausen, H. 2001. Oncogenic DNA viruses. *Oncogene* **20**: 7820-7823.

Zwicker, J., Liu, N., Engeland, K., Lucibello, F.C., and R. Müller. 1996. Cell cycle regulation of E2F site occupation *in vivo*. *Science* **271**(5255): 1595-1597.