ROLE OF MULTIPLE DOMAINS OF T ANTIGEN IN GENE REGULATION AND TRANSFORMATION

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SV40 large T antigen (TAg) is a dominant acting oncoprotein that elicits transformation of many cell types and induces tumors in rodents. TAg induces transformation, in part, by disabling the functions of tumor suppressors such as pRb and p53. This dissertation is aimed to determine if inactivation of Rb and p53 are the major TAg activities required for transformation or if additional activities contribute.

To determine whether Rb-family protein inactivation by the J domain of TAg is required for induction of intestinal hyperplasia, we have generated transgenic mice that express a J domain mutant (D44N) in villus enterocytes. In contrast to wild-type T antigen, the D44N mutant is unable to induce enterocyte proliferation. Unlike mice expressing wild-type TAg, mice expressing D44N do not reduce the protein levels of p130 and are also unable to dissociate p130-E2F DNA binding complexes.

To determine if Rb inactivation is sufficient for the induction of hyperplasia or if progression to dysplasia requires some activity in the C-terminus of TAg (independent of p53), I have screened several transgenic lines expressing an amino-terminal mutant of TAg (N136) in villus enterocytes. I found that these mice develop intestinal hyperplasia, although not as early as wild-type TAg does, suggesting that the inactivation of Rb family members is sufficient to induce this phenotype. Furthermore, the appearance of signs of dysplasia was significantly delayed.

I performed global analysis of gene regulation in MEFs and in mouse intestinal epithelium expressing TAg or various mutants. In mouse intestine most of the gene regulation is dependent on binding and inactivation of Rb-proteins by the LXCXE motif and J domain. Regulated genes are involved in cell cycle and proliferation. In MEFs genes belonging to cell cycle, apoptosis and growth factors are differentially regulated by TAg and its mutants. Additionally, we found upregulation of immune response genes by TAg requires the LXCXE motif and some activity mapping to the C-terminus of TAg for their regulation. Significant numbers of genes were found to be regulated independently of the LXCXE motif, J domain and p53 binding domain. This suggests activity independent of these functions.

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PREFACE

"I have not failed. I've just found 10,000 ways that won't work." - Thomas Alva Edison (1847-1931). This is what I have "in common" with Thomas Alva Edison.

I thank my mentor Dr. James Pipas who gave me shelter under his wide wings and nurtured my ambition to achieve PhD. He was also very kind to tolerate and demonstrate patience for all my 10,000 ways that would not work during the course of past 5 years. He always provided support, advice and positive feedback and gave freedom to foster new ideas. I strongly believe that it was my good fortune and luck that I had the opportunity to work with Jim.

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I have yet to come across a person as selfless as Paul Cantalupo. I acknowledge his genuine honesty, keen interest to help and valuable suggestions he has provided. He is a walking encyclopedia of Biochemistry and Microarrays and without his expertise I would not have completed my projects. One of the distinguished qualities of Paul is putting other people before himself. I truly enjoyed my interactions with Paul and his wife Paula on various social gatherings over the years.

Likewise, I thank Deepika Ahuja who gave me energy and inspiration to maintain family and professional life. Her organization and pace to work was extraordinary and quite frankly at times seemed superhuman. On number of occasions I tried to imitate her, but failed horribly. She is truly a genius and a good friend.

Over past 5 years, Jim's lab was like an extended family, very closely knit yet simple and joyous. In this extended family; Ashley Case, Shelley Nelson, Rob Culik, Danielle Oliver, Paul Eppes, Sandlin Seguin, Jeremy, Leigh Kridle, Jacqueline Moskow, Mike Miller, Chevaunne Edwards and Jennifer Markovics were always with me to laugh and cry. I had total fun with you all. I want to especially acknowledge Alison Roos, who came as undergraduate student under my supervision and became a good friend and coworker.

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Pallavi Ghosh and Anil Ojha are my friends beyond workplace. Even though they resided in Hatfull's lab, but were very much available for discussions and advise. Their post-doc

experience was very handy to carryout some difficult experiments. I always enjoyed their company both at work and outside. My best friend Poonam Agrawal was a constant source of encouragement and her friendship always enlighten my heart.

I thank my parents who instilled the enthusiasm in me for science and success since my childhood and many more things they have done to make me what I am today. My father's hardworking habits always inspire me. My brother Gopal and sister Manisha and their families were always supportive and full of love during this process. Blind faith of my nieces Aanchal and Krishnika on me always helped me to keep up the good work.

My in-laws provided a great deal of support and their wish to see me as a scientist inspired me to accomplish this PhD. Especially my mother in-law provided support from the day one when I sat for GRE. My brother in-law Nimesh and his wife Ritu were very supportive throughout these years. Nimesh participated in few of my poster presentations to encourage me, even though he was clueless about my presentations.

Most importantly and above all, my greatest inspiration is my 15 month old beautiful daughter Anusha. You can all imagine that she putout the greatest support so that I could write this thesis. At times when she needed me the most I gave her the least. You are so wonderful my dear Anusha!

Finally, my heartfelt thanks to my husband who was with me at each and every step of this thesis. Although having a very demanding career as a physician he always helped me out at home as well as at work. I want to dedicate this thesis to my husband Vikas. This thesis would not have existed if he were not on my side. Vikas, thanks for everything!

1.0 INTRODUCTION

Cancer is a complex, multi-factorial, multi-stage disease that arises due to an imbalance between growth-promoting and growth-inhibitory signals. Successive progression from one stage to the next is determined by multiple genetic events, such as mutations in tumor suppressor genes or oncogenes. Proteins encoded by these genes are involved in various cellular regulatory processes including cell proliferation, apoptosis and genomic stability. Interference with these regulatory processes give rise to phenotypic changes at the cellular level including self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, unlimited replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000).

DNA tumor viruses encode dominant acting oncoproteins and are capable of interfering with the functions of tumors suppressors and oncogenes, therefore serving as important tools to explore the nature of the molecular events leading to transformation and tumorigenesis. The studies described in this thesis are an attempt to understand the transforming activities of the DNA tumor virus SV40 in cultured cells and its tumorigenic potential in transgenic mice.

1.1 SIMIAN VIRUS 40 (SV40) AS A MODEL SYSTEM

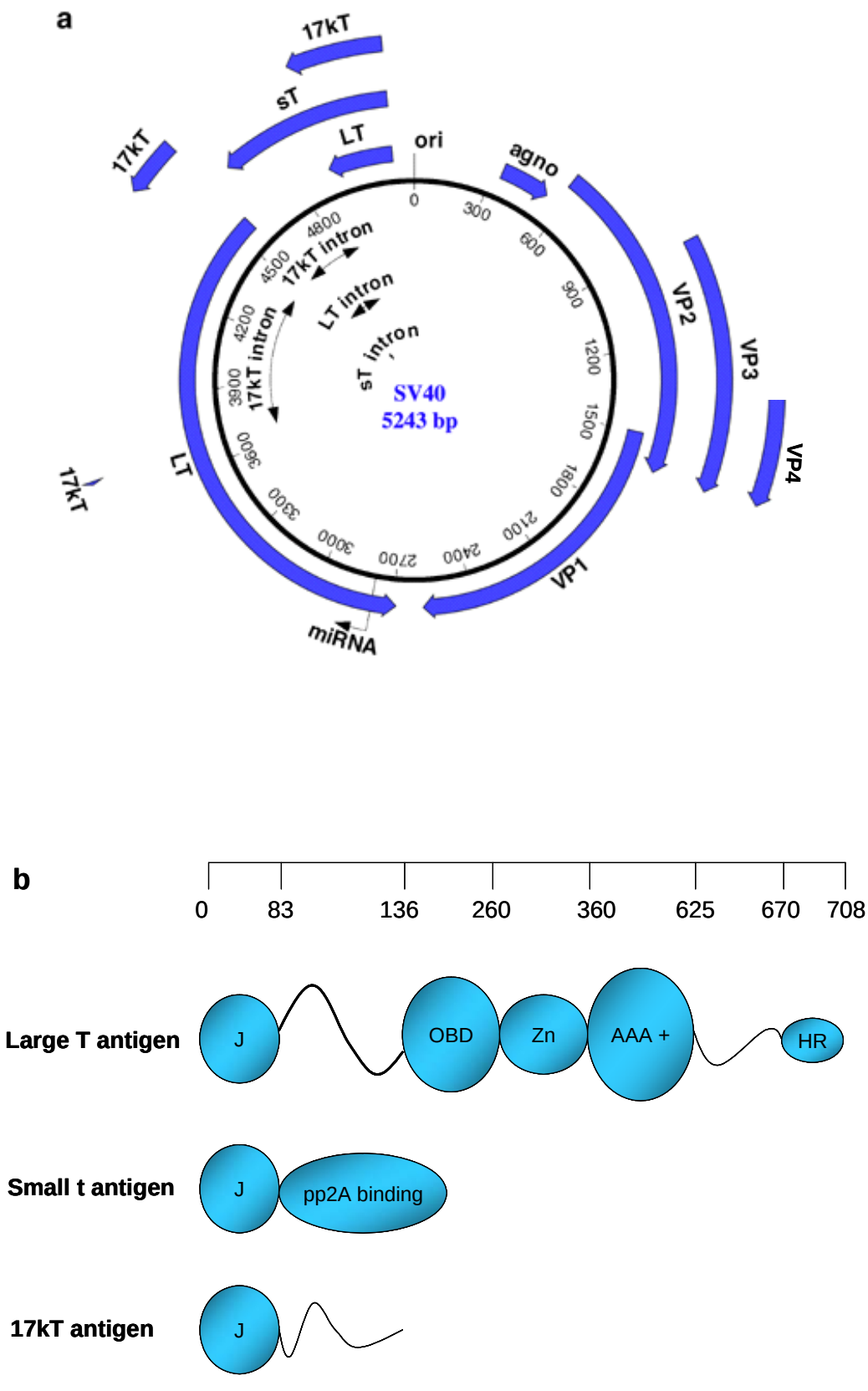
SV40 was discovered in 1960 by Sweet and Hilleman, during the screening for the presence of contaminating viruses in the poliovirus vaccine samples produced in Rhesus macaque monkey kidney cells (Sweet and Hilleman, 1960). Shortly after its discovery, SV40 was shown to induce tumors when injected into newborn hamsters or immunocompromised mice (Eddy et al., 1962; Girardi et al., 1962).The ability to induce tumors and tumor type depends on the rodent species used, route of inoculation, amount of virus, and immune status of the test animals. SV40 was shown to induce an array of different tumor types, including osteosarcoma, mesothelioma, lymphoma, and choroid plexus neoplasia (Arrington As, 2001). Similarly, SV40 induces neoplastic transformation in cell culture, as assessed by a number of different assays. Even though SV40 is extremely oncogenic in animal and cell culture model systems, no definitive link has been established between human cancers and SV40 to date (Poulin and DeCaprio, 2006).

SV40 is a member of the Polyomavirus family (Polyomaviridae); named after the ability of family members to induce multiple tumors in rodents. Polyomaviruses are a family of non-enveloped DNA viruses with icosahedral capsids containing a small, circular, double-stranded DNA genome. During SV40 infection, the virus enters the cells, uncoats, and releases its DNA. SV40 host cells tend to be terminally differentiated, quiescent cells and do not express proteins involved in DNA replication, nucleotide metabolism, chromatin assembly or proteins that are required for viral DNA replication and new virion assembly. To circumvent this problem, the early region of the SV40 genome that includes the differentially spliced tumor antigens such as large T antigen (TAg), small t antigen (sT) and 17kT antigen (17kT) transcribes immediately after infection (Fig.1). These tumor antigens interfere with cell cycle regulators such as p53 and pRb and enhance the expression of genes associated with DNA synthesis, which eventually force

cells to enter S phase. Shortly afterwards, viral DNA replication begins, resulting in the production of progeny viral DNA. Simultaneously with the initiation of viral DNA replication, the late promoter becomes active, resulting in the expression of the viral coat proteins, VP1, VP2, VP3, agno protein and recently discovered VP4 (Daniels et al., 2007) (Fig.1). This results in the assembly of progeny virions, host cell lysis and release of viral progeny, which in turn infect surrounding cells.

Figure 1. SV40 genomic organization and early gene products.

(a) SV40 genomic DNA is composed of three elements: the early and late coding units and the regulatory region. The early unit encodes large T antigen (TAg), small t antigen (sT), 17kT antigen (17kT). The late unit encodes the four structural proteins (VP1, VP2, VP3 and VP4) and the agnoprotein (agno) and a pre-microRNA (miRNA). The regulatory region (*ori*) contains sequences for the early and late promoter and the origin of replication. Figure from (Ahuja et al., 2005). (**b**) SV40 large T antigen contains the following domains and motifs: J domain (J), Rb-protein-binding motif (LXCXE), nuclear localization signal (NLS), origin-binding domain (OBD), Zn domain (Zn), ATPase domain (AAA+), variable region (VR), and host-range domain (HR). Small t antigen is comprised of the J domain and pp2A binding domain. 17kT is comprised of the first 131 amino acids of large T antigen including the J domain and LXCXE motif, plus an additional four unique amino acids. Residue numbers are indicated above the domain structure. Figure modified from Ahuja *et al*. 2005.


a
17kT
sT
LT
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agno
17kT
17kT intron
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SV40
5243 bp
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VP3
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3300
3600
3900
4200
4500
4800
b
0
83
136
260
360
625
670
708
Large T antigen
J
OBD
Zn
AAA +
HR
Small t antigen
J
pp2A binding
17kT antigen
J

Because of the potent transforming activities of SV40 proteins in multiple cellular environments, this virus has become a model system for human cancer research. The SV40 genome may be the most heavily mutagenized DNA molecule per base pair and has been intensively studied to provide a model system for the study of basic eukaryotic cell processes, including DNA replication, transcription, malignant transformation, and signal transduction. The advantage of using this viral system for studying replication is that only one viral protein (T antigen) is required to initiate DNA replication. In combination with a wealth of mutants and *in vitro* assays, this has led to an understanding of how eukaryotic DNA replication is initiated (Bullock, 1997). Additionally, SV40 encodes dominant acting transforming proteins (TAg, sT, and 17kT). In particular, the expression of TAg alone is sufficient to induce transformation in multiple cell types (Bryan and Reddel, 1994; Chen et al., 1992; Hahn et al., 1999; Ibaraki et al., 1998), while small t antigen (sT) assists in transforming some cell types, and in some situations expression of sT by itself is sufficient to induce a transformed phenotype (Mungre et al., 1994; Porras et al., 1996; Porras et al., 1999; Rundell et al., 1998). The 17kT is expressed during infection in smaller amounts relative to other T antigens. There is limited information available about the functions of 17kT. One of the studies suggests that 17kT may function in fine-tuning SV40 mediated cell cycle control (Zerrahn et al., 1993).

SV40 is a model for understanding cellular functions as each of its transforming proteins target one or more key regulatory proteins within a cell and thus alter the cell's growth/survival properties. Thus T antigen serves as a molecular probe to find essential factors required for cellular processes and navigate researchers through the complicated interactions in the cellular environment.

1.2 FUNCTIONAL DOMAINS OF SV40 LARGE TUMOR ANTIGEN

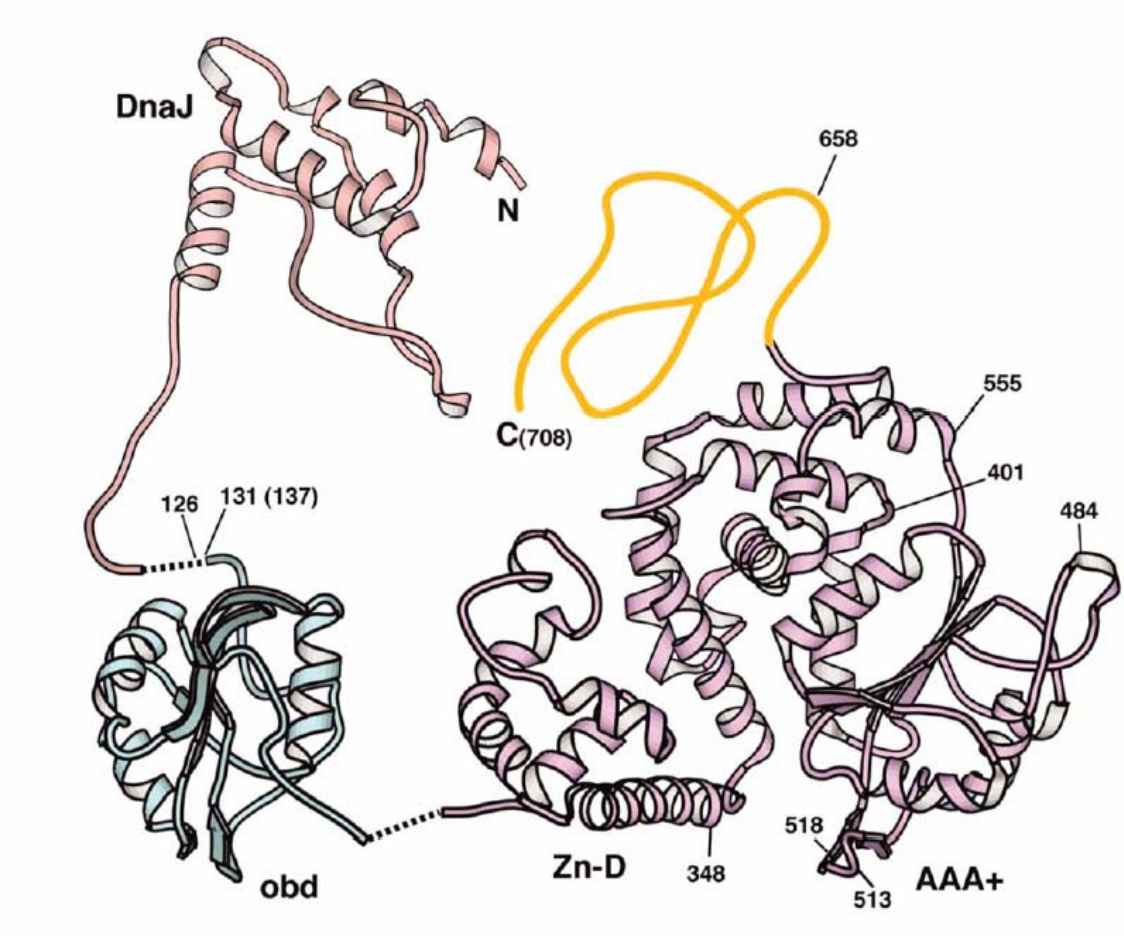
SV40 large T antigen (TAg) is a 708 amino acid protein that contains many independently folding domains (Fig.2). A brief structural description of the various domains, motifs and linker regions of TAg follows.

1.2.1 The J domain

The first 70 amino acids of TAg have sequence identity with the J domain of the DnaJ class of molecular chaperones (Kelley and Landry,1994). The J domain of TAg binds to hsc70, the major DnaK homologue present in mammalian cells, and stimulates its ATPase activity (Srinivasan et al., 1997; Sullivan et al., 2001). This domain is also essential for DNA replication, virion assembly, and tumorigenesis (Peden and Pipas, 1992; Pipas et al., 1983; Spence and Pipas, 1994b; Srinivasan et al., 1989). The residues histidine-proline-aspartate (HPD) are absolutely conserved within the J domain and mutations in the HPD motif disrupt the above functions of the J domain (Srinivasan et al., 1997). The E3 ubiquitin ligase, Cul7, from the cullin family of proteins (Ali et al., 2004) binds TAg through residues 69-83 (Kasper et al., 2005). Cullins serve as a scaffold for the SCF multi-component complex that functions as an E3 ubiquitin ligase with Skp1 and F-box proteins.

Figure 2: Structural representation of the TAg monomer.

The J domain (DnaJ), origin binding domain (obd), zinc binding domain (Zn-D), and ATPase domain (AAA+) are assembled together based on previous NMR and crystal structures. The linkers between domains are represented by dashed lines. No structural information is available for the Host Range Domain (residues 628-708, in yellow). Figure from Xiaojiang S.Chen, USC.



1.2.2 The N-terminal flexible linker

The N-terminal linker of TAg consists of residues 83-134. This small region contains binding sites for several important cellular proteins. Residues 103-107 constitute an LXCXE motif, which is essential for the interaction of TAg with Rb family members (DeCaprio et al., 1988). Another TAg target is Bub1, a mitotic spindle checkpoint protein, which interacts with TAg residues 89-97. This interaction is probably through a conserved WEXWW sequence that is found in almost all large TAgs (Cotsiki et al., 2004). Finally, the nuclear localization signal (NLS), P(126) KKKRKV(132), is found in this region.

1.2.3 Origin binding domain

The origin binding domain extends from amino acids 135-249 and is required for TAg to bind to the SV40 DNA origin of replication. The SV40 genome contains a 64 base pair origin of replication and consists of a central palindrome of four GAGGC base pairs. This region is flanked downstream by a 17 base pair AT rich region and upstream by an imperfect inverted repeat known as the early palindrome (Deb et al., 1986; Deb et al., 1987). TAg double hexamers prefer to bind to pentanucleotides 1 and 3 (Joo et al., 1998; Joo et al., 1997). By cooperative mechanism the first hexamer facilitates the assembly of a second hexamer to form a dodecamer (Parsons et al., 1991). The two halves of the dodecamer are then arranged in a “head to head” manner. This is important for viral DNA replication but plays no known role in transformation.

1.2.4 Zinc binding domain

The zinc binding domain stretches from amino acids 250-370 and contains a zinc finger motif ($CX_2CX_{15}HX_3H$) which includes residues C302, C305, H317, and H320 (Pipas, 1992). Mutation of these residues renders the virus non-viable and efficiency of transformation is reduced by 50%. (Loeber et al., 1989). Recent crystallographic studies revealed that zinc domain has a globular fold instead of typical zinc finger structure (Li et al., 2003a). Additionally, this fold is stabilized by the coordination of a zinc atom through the zinc finger motif. The zinc binding domain is required for hexamerization, since a deletion mutant containing residues 303-627 abolished hexamerization but deletion mutant containing residues 251-627 (with intact zinc binding motif) did not (Li et al., 2003a).

1.2.5 ATPase domain and p53 interaction site

The ATPase domain extends from residues 371-624 and is essential for both viral replication and transformation. The ATPase domain is a canonical AAA+ ATPase domain, which binds and hydrolyzes ATP (Bradley et al., 1987; Erzberger and Berger, 2006). Hexamerization of TAg requires ATP binding, but not hydrolysis (Mastrangelo et al., 1989). Based on structural studies, the TAg hexamer exist in an "all or none binding mode". In this mode all subunits exist in the same state: ATP bound, ADP bound, or nucleotide free (Gai et al., 2004), therefore suggesting a concerted mechanism for ATP hydrolysis and ADP release. In contrast to hexamerization, ATP hydrolysis is required for TAg helicase activity (Mastrangelo et al., 1989). The older mapping studies suggested bipartite interaction of p53 with TAg requiring residues 350-450 and 550-650 (Kierstead and Tevethia, 1993). However, recently Li and coworkers demonstrated that this

assignment does not exist, and the residues required for p53 binding cluster together on the surface of TAg (Li et al., 2003a). The p53 residues essential for TAg interaction clusters on the same side of the structure as the p53 DNA binding domain. This suggests that TAg interacts with the DNA-binding surface of p53 therefore blocking its ability to bind promoters (Li et al., 2003a). However this may not be entirely true as evident by several studies suggesting that amino terminal sequences are also involved in blocking p53 function (Quartin et al., 1994; Rushton et al., 1997).

1.2.6 Variable region and host range domain

The C-terminal variable region (625-669) is found in only a few polyomavirus TAgs. The host range lies at the extreme C terminus (670-708). Wild-type SV40 can replicate in BSC40 and CV-1 monkey kidney cell lines, however mutations in the host range domain prevent viral replication in CV-1 cells but have no effect on SV40 growth in BSC40 cells (Pipas, 1985). Host range mutants are cold-sensitive, can not grow in BSC40 at 32°C and are defective for growth in CV-1 cells at all temperatures (Pipas, 1985). This region also allows human adenovirus to grow normally in nonpermissive monkey cells and therefore is also referred to as the adenovirus helper function (Cole et al., 1979). It has been suggested that this region also contains an activity required for viral assembly and therefore host range mutants are defective in viral growth (Spence and Pipas, 1994a).

1.3 T ANTIGEN BINDS CELLULAR TARGETS TO ELICIT TRANSFORMATION

Neoplastic transformation is defined by the expanded proliferation and/or extended survival of a cell or tissue. Several different assays are used to assess transformation by SV40 TAg. Each of these assays imposes growth restrictive conditions on cells in culture. Normal cells are unable to proliferate and/or survive in these conditions, however transformed cells not only survive but are also able to proliferate. Five main assays have been employed to identify transformed cells in culture. These are:

(1) Immortalization: Primary cells can not grow in culture indefinitely. After a certain number of passages they undergo growth arrest and irreversible senescence. SV40-transformed primary cells are immortal and can proliferate indefinitely.

(2) Focus formation: Untransformed cells grow on an appropriate surface until they reach to confluence and form a monolayer. In contrast, SV40-transformed cells continue proliferating on the surface of a growth arrested monolayer of untransformed cells, resulting in formation of dense regions of multilayered cells, referred to as foci.

(3) Anchorage independence: Normal cells require a surface to adhere and proliferate. SV40-transformed cells proliferate in the absence of such contact with a surface and grow as multicellular balls when suspended in semi-solid media such as agarose.

(4) Growth in low serum: Normal cells can not grow in media containing low concentrations of serum or growth factors. However, SV40-transformed cells continue to grow in medium with little or no serum.

(5) Saturation density: Saturation density is defined as the maximum number of cells per unit area of culture surface. Because SV40-transformed cells do not show contact inhibition, they

continue to grow beyond a monolayer and thus reach higher or indefinite saturation densities than untransformed cells.

All these transformation assays are performed using either primary cells or established cell lines. Each of these systems has inherent advantages and disadvantages. Primary cells such as mouse embryo fibroblasts (MEFs) are useful because, although they have a limited passage in cell culture, they carry the same genetic composition as the animal from which they were derived. Additionally, primary cells can be cultured from specific strains of mice, such as gene knockout or transgenic lines, which allow the study of the related biological effect. One of the disadvantages of using primary cell cultures is that they contain many different cell types that may respond differently to oncogenic signals. On the other hand, cell line establishment results from genetic changes that select for cell populations that are immortal. The specific genetic changes carried by a cell line in response to oncogenic and death stimuli affect their biological characteristics and are often unknown. One of the major advantages of using an established cell line is that they are clonal (genetically identical cells) and therefore respond similarly to oncogenic stimuli.

Cell culture systems are very good for studying the molecular aspects of regulating signaling pathways. However they have limited value in predicting how perturbing specific pathways alters cellular behavior in the context of tissue. Furthermore, cultured cells are not necessarily in a native state since they are cultured on a plastic surface in medium supplemented with fetal bovine serum or the equivalent. These abnormal conditions may alter specific cellular responses. Therefore, animal models are essential to examine the influence of oncogenic signals in the native environment.

Most of our understanding about the mechanism of SV40-induced transformation stems from cell culture studies. In addition, series of transgenic mice expressing TAg in various tissues have been generated by different research groups to test the relevance of these targets, identified originally in cell culture studies, in the context of a natural tissue setting. In this chapter, I provide an overview about the targets of TAg and putative mechanisms by which TAg induces transformation. Two of the extensively studied targets of TAg are the major tumor suppressors, pRb and p53. T antigen can bind and inactivate pRb and p53 and therefore serves as an important tool to elucidate the function of these tumor suppressors.

1.3.1 RB

The retinoblastoma susceptibility gene (*RB*) was the first tumor suppressor gene to be cloned. In familial cases of retinoblastoma, a germ line mutation in the *RB* gene is inherited, and affected individuals develop retinal tumors in which the loss of the second *RB* allele is the rate limiting step (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Later, *RB* was found to be mutated in other human cancers, including osteosarcoma, soft tissue sarcoma, and carcinomas of breast, lung, bladder and prostate origin. Reintroduction of wild-type *RB* into *RB*-deficient tumor cell lines suppresses their neoplastic phenotype, consistent with a role for *RB* as a tumor suppressor (Riley et al., 1994). The *RB* gene product, pRb, is a nuclear phosphoprotein and has been shown to interact with the E1A oncoprotein encoded by adenovirus (Whyte et al., 1988). Soon after, the viral oncogenes E7 from HPV and TAg from SV40 were also shown to form a stable complex with pRb (DeCaprio et al., 1988; Dyson et al., 1989; Munger et al., 1989). Adenovirus E1A, HPV E7 and T antigen from all primate polyomaviruses contain an LXCXE motif (X= any amino acid residue) (Chellappan et al., 1992) that is essential for their interaction with pRb. The

importance of this sequence in mediating the interactions with Rb-proteins and in transformation was first demonstrated for the adenovirus E1A protein (Whyte et al., 1988) and later on for HPV E7 and SV40 TAg (DeCaprio et al., 1988; Dyson et al., 1989; Munger et al., 1989). The inactivation of Rb-proteins by the TAg appear to be a common feature of DNA tumor viruses as co-immunoprecipitaion assays performed by Dyson and coworkers show a stable protein complex between the TAgs of mouse, monkey, baboon, or human polyomaviruses and Rb-proteins (Dyson et al., 1990). The region of pRb which interacts with the LXCXE motif overlaps with positions of naturally occurring mutations in the *RB* gene, suggesting that all the above mentioned viral oncoproteins target a site of pRb that is important for its tumor suppressive function (Hu et al., 1990).

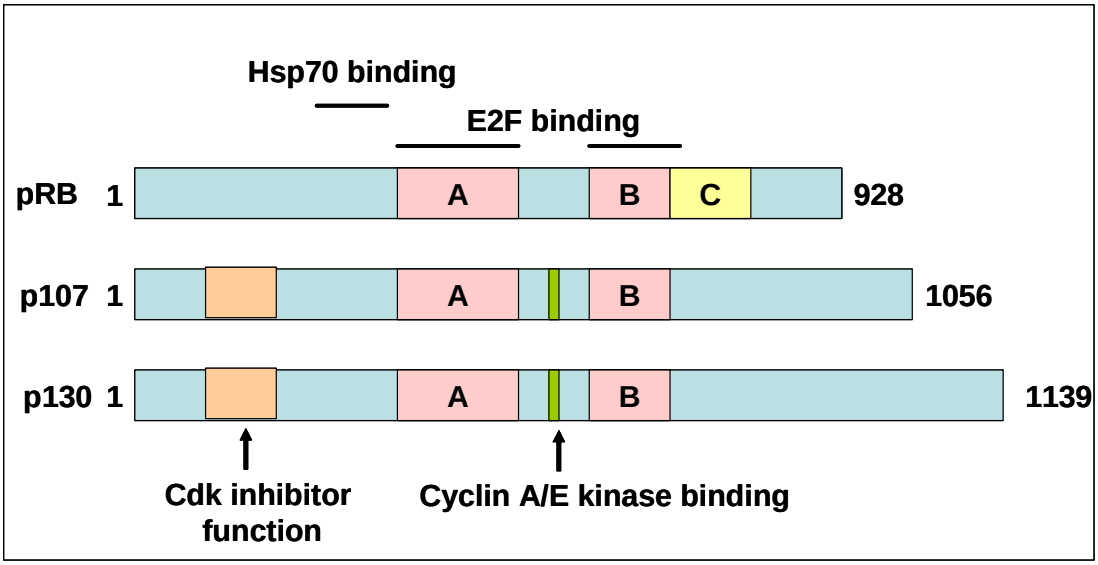
Many elegant biochemical studies have demonstrated that pRb functions as a central cell cycle regulator. Cell cycle progression in mammalian cells is driven by the sequential activation of cyclin –dependent kinases (cdks), which mediate phosporylation of critical cellular substrates and allow cells to pass through certain cell cycle transition points (Sherr and Roberts, 1995). pRb has been shown to be a key substrate of G1 cyclin/cdk complexes, including cyclin D/cdk4 or cdk6 and cyclin E/cdk2, both *in vitro* and *in vivo* (Riley et al., 1994; Weinberg, 1995). In quiescent cells or cells in early G1, pRb is present in a hypophosphorylated form. As G1 cyclin / cdk complexes are activated, cells progress towards the G1/ S boundary, and the overall levels of pRb phosphorylation increase. pRb is maintained in this hyperphosphorylated form throughout S, G2 and M phases of the cell cycle. As cells emerge from S phase, pRb loses multiple phosphate groups thereby becoming active and capable of binding to E2F transcription factors, thus blocking transcription of S-phase genes (Riley et al., 1994; Weinberg, 1995). The cyclin / cdk complexes that negatively regulate pRb function are, in turn, themselves negatively

regulated by cell cycle inhibitors (CDKIs), such as p16 (Sherr, 1996). Genetic mutations in many parts of the Rb pathway, including Rb itself, CDKIs, cyclins and E2Fs have been linked to cancer (Sherr, 2000).

pRb is a member of a family of transcriptional repressors that includes p107 and p130. These three proteins are closely related and all contain bipartite domains, termed the A and B domains, which are essential for repression of E2Fs (Fig.3). The A and B domains mediate physical interaction with certain viral oncoproteins, such as adenovirus E1A and SV40 large T antigen, and cellular factors such as members of the E2F family of transcription factors (Kim et al., 2001; Ross et al., 1999; Sellers et al., 1995). pRb consists of multiple domains, some of which bind directly to specific transcription factors and others that recruit histone deacetylases (Dyson, 1998; Harbour and Dean, 2000b; Lipinski and Jacks, 1999; Nevins, 1998). pRb represses transcription directly through its repression domains, and indirectly by recruiting histone deacetylase and chromatin modifying complexes (Harbour and Dean, 2000b). Additionally, p107 and p130 share a conserved spacer region between their A and B domains, through which they interact with cyclin A/Cdk2 and cyclin E/Cdk2 (Lees et al., 1992; Li et al., 1993). p107 and p130 also share an amino-terminal region that can inhibit Cdk activity (Woo et al., 1997).

Figure 3: Domain map of RB-family members.

The three Rb family members share sequence homology in domains A and B. E2Fs bind to a separate surface formed at the intersection of the A and B domain. pRb contains domain C at its carboxy terminus which can facilitate a specific interaction with E2F1 (Dick and Dyson, 2003). p107 and p130 contain sequences in the spacer region which serves as a cyclin A/E kinase binding site. The amino terminus of p107 and p130 contain sequences which are involved in Cdk inhibitor function. The Hsp70 binding site of pRb is located in the region (residues 301-372) ouside the A pocket (Inoue et al., 1995).



Rb-family members have different patterns of expression throughout the cell cycle. Whereas pRb is expressed steadily through all stages of the cell cycle, p130 expression is high in growth arrested cells and p107 expression peaks during the S phase of the cell cycle (Classon and Harlow, 2002). The Rb family proteins have unique and overlapping functions in cell cycle control, differentiation and inhibition of oncogenic transformation. The RB gene is mutated in a variety of human tumors, but no specific mutations of p107 or p130 in cancers have been reported to date.

Many of the insights into the role of the Rb family in proliferation and differentiation have been obtained from mouse knockout models. Inactivation of RB in mice results in unscheduled cell proliferation, apoptosis and widespread developmental defects, leading to embryonic death by day 13.5 (Jacks and Weinberg 1992; Lee and Bradley 1992). In certain genetic backgrounds, mice with null mutations in either *p107* or *p130* are viable and normal (Cobrinik et al., 1996; LeCouter et al., 1998a; LeCouter et al., 1998b; Lee et al., 1996), while mice lacking both *p107* and *p130* die at birth with defects in endochondral bone development, epidermal differentiation and epithelial /mesenchymal interactions (Cobrinik et al., 1996). This suggests that p107 and p130 perform overlapping functions. Similarly, pRb may have overlapping functions with p130 that are not shared with p107 and vice versa (Ruiz et al., 2004).

Rb family members interact with many transcription factors such as E2F, MyoD, myogenin, C/EBPα,β,γ NF-IL6, Pax-3, Mhox, Chx10, AP-2 (Lipinski and Jacks, 1999), c-Abl (Welch and Wang, 1993), and CDP/cut (van Wijnen et al., 1996; Webster et al., 1998). Among all these transcription factors, interaction of Rb family members with E2F is the most studied and probably most important for its growth suppressive functions.

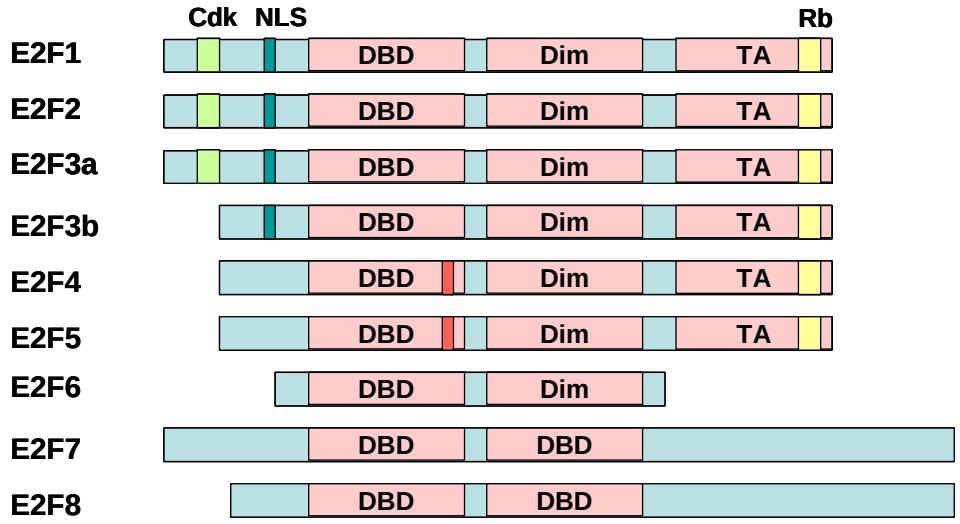
E2F is a family of transcription factors that consists of at least eight known members (E2F1-8). E2F1, E2F2 and E2F3a are transcriptional activators and interact with pRb, while E2F3b, E2F4 and E2F5 are transcriptional repressors that preferentially bind p107 and p130, although E2F4 can also interact with pRb (Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995). These associations also occur at distinct times during the cell cycle, in part due to the differential expression patterns of Rb-family members at different stages of the cell cycle. Most of the information about *in vivo* promoter occupancy of individual Rb and E2F family members throughout different stages of the cell cycle is provided by two different research groups using chromatin immnoprecipation (ChIP) assay (Takahashi et al., 2000; Wells et al., 2000). pRb interacts with E2Fs in both quiescent and cycling cells, p130 interacts with E2Fs primarily in quiescent cells and p107 is predominantly associated with E2Fs in the S phase of the cell cycle. On the other hand, E2F6, E2F7, and E2F8 lack the domain required for interacting with Rb proteins, and function as repressors (Fig.4). While E2F1 through E2F6 bind DNA through heterodimerization with the DNA binding partner DP-1 or DP-2, E2F7 and E2F8 can bind DNA directly. Dimerization with DP proteins is required for high affinity DNA-binding, as well as for interacting with the Rb-family.

Much information about the potential functions and activities of individual E2F family members derives from cell culture studies and animal knock out models. Overexpression of any activator E2F protein is sufficient to induce quiescent cells to re-enter the cell cycle (Johnson et al., 1993; Lukas et al., 1996; Qin et al., 1994). Moreover, the combined loss of E2F1, E2F2 and E2F3 completely blocks the proliferation of MEFs (Wu et al., 2001). On the other hand, E2F1 deficient mice are viable and fertile but tumor-prone due to a defect in the ability to induce apoptosis (Field et al., 1996; Yamasaki et al., 1996). E2F2 null mice exhibit increased

lymphocyte proliferation and develop autoimmunity (Murga et al., 2001). E2F1 (-/-); E2F2 (-/-) mice are also viable and show defects in proliferation and maturation of hematopoietic cells, diabetes, tumorigenesis and lack of spermatogenesis (Li et al., 2003b; Zhu et al., 2001). E2F3 (-/-) mice exhibit partially penetrant embryonic lethality and growth defects, and E2F3 (-/-) MEFs show reduced cell cycle entry (Humbert et al., 2000b). E2F4 is abundantly expressed in many cell types, and displays the most abundant DNA-binding activity of the E2Fs (Moberg et al., 1996). Depending on the genetic background, only a small percentage of E2F4 (-/-) mice are viable and show defects in hematopoiesis, crypt formation in the intestinal epithelium and craniofacial defects (Humbert et al., 2000a; Rempel et al., 2000). E2F5 null mice die prematurely due to hydrocephaly (Lindeman et al., 1998). E2F4 and E2F5 double knock out mice are embryonic lethal and this suggests a redundancy between functions of E2F4 and E2F5 (Gaubatz et al., 2000). Finally, E2F6 (-/-) mice are viable and show defects in homeotic patterning and in restriction of male germ cell gene expression (Pohlers et al., 2005; Storre et al., 2002). All these studies with E2F gene knockouts revealed unique as well as overlapping roles for E2F family members in cell proliferation and cell fate in mouse development.

Figure 4: Domain map of E2F family members.

E2F1-8 shares sequence homology in their DNA binding domain (DBD). The 'activating' E2Fs, E2F1-3 shares sequence homology in their nuclear localization signal (NLS), dimerization domain (Dim), transactivation domain (TA) and Rb-binding motifs. E2F1-3a, but not E2F3b, shares a homologous amino-terminal cyclin A/cdk2 interaction domain. The 'repressing' E2Fs, E2F4 and E2F5, share sequence homology in their nuclear export signal (NES, showed in red), DBD, Dim, TA and Rb-binding motif. E2F6 shares sequence homology only in its DBD and Dim with the other E2Fs, otherwise it is very different from other family members. E2F7 and E2F8 share sequence homology only in their DBD with the other E2Fs, however they contain two DBDs instead of one.



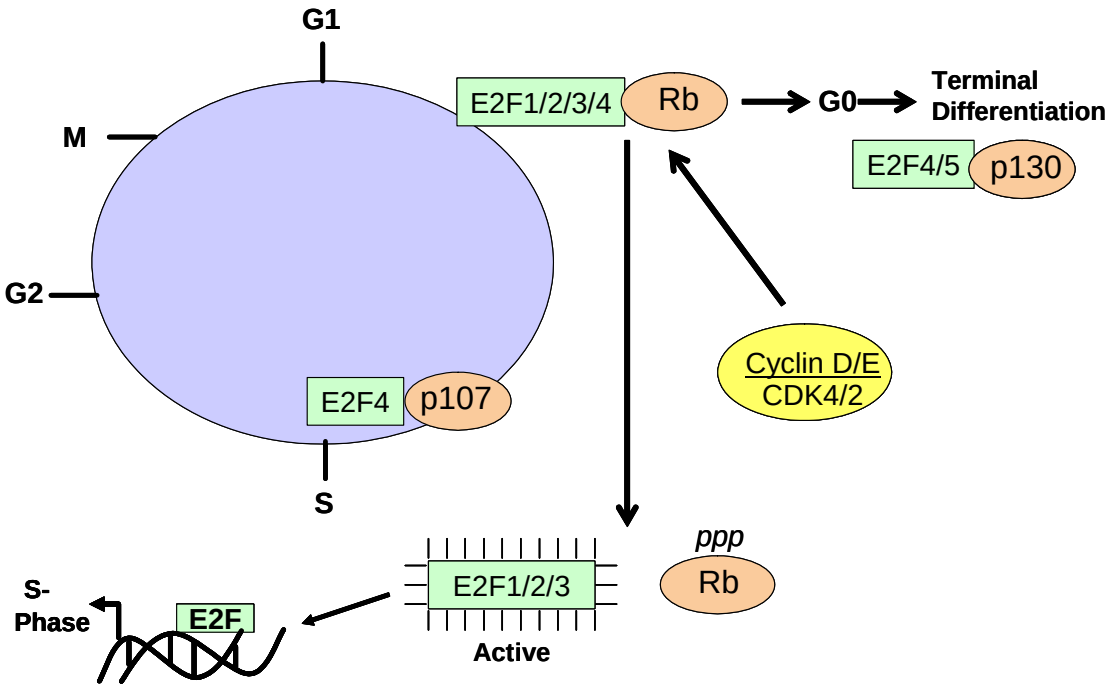
1.3.1.1 RB/E2F regulation throughout the cell cycle

Hypophosphorylated pRb family members block transcription by recruiting chromatin remodeling complexes, (such as SWI/SNF, histone deacetylases (HDACs), or the polycomb group (PcG) proteins) as well as methylases to E2F-binding sites (Harbour and Dean, 2000a; Harbour and Dean, 2001). Furthermore, pRb can repress transcription by physically blocking the transcriptional activation domain of E2F and therefore prevent the assembly of the preinitiation complex. Normal S-phase progression requires the relief of transcriptional repression by removal of RB proteins from promoters, followed by the subsequent activation of these promoters by E2F1-3. In a normal cell cycle, phophorylation of RB-proteins by Cdks leads to their dissociation from E2Fs. Subsequently, free E2Fs then stimulate the transcription of genes that encode proteins controlling cell cycle progression and DNA replication (such as cyclins A, D1 and E, dihydrofolate reductase, thymidine kinase, Cdk2, cdc2, cdc6, DNA polymerase a, c-Myc, c-Myb), and also genes whose products are involved in DNA repair, differentiation and apoptosis. E2F-regulated genes are not expressed in quiescent cells because their promoters are occupied primarily with p130/E2F4 complexes, which repress transcription. Inactivation of pRb by phosphorylation results in the replacement of these complexes by activating E2F1–3 and results in the transactivation of E2F-target genes (Fig.5). The retinoblastoma proteins keep cells in the G1 phase primarily by repression of E2F-mediated transcription, however other mechanisms may apply. In fact, p107 and p130 can also prevent cell cycle progression by acting as Cdk2 inhibitors (Dynlacht et al., 1997; Zhu et al., 1995).

The role of the Rb and E2F pathways in cellular growth control has been the focal point of research from many years and substantial progress has been made to understand Rb and E2F

dependent transcriptional regulation of cellular machinery. Rb and E2Fs play a central role in the basic mechanism of human cancer and therefore are very useful targets for genetic and drug therapeutic approaches (Chen et al., 1999; Hermiston, 2000; Westphal and Melchner Hv, 2002). Even with these extensive studies about Rb and E2F proteins, we know only a small fraction about the role of pRb and E2F proteins in different cellular processes and therefore the study of DNA tumor viruses serves as a navigation device to reach to those unknown functions.

Figure 5: Cell cycle regulation by Rb and E2F family members.



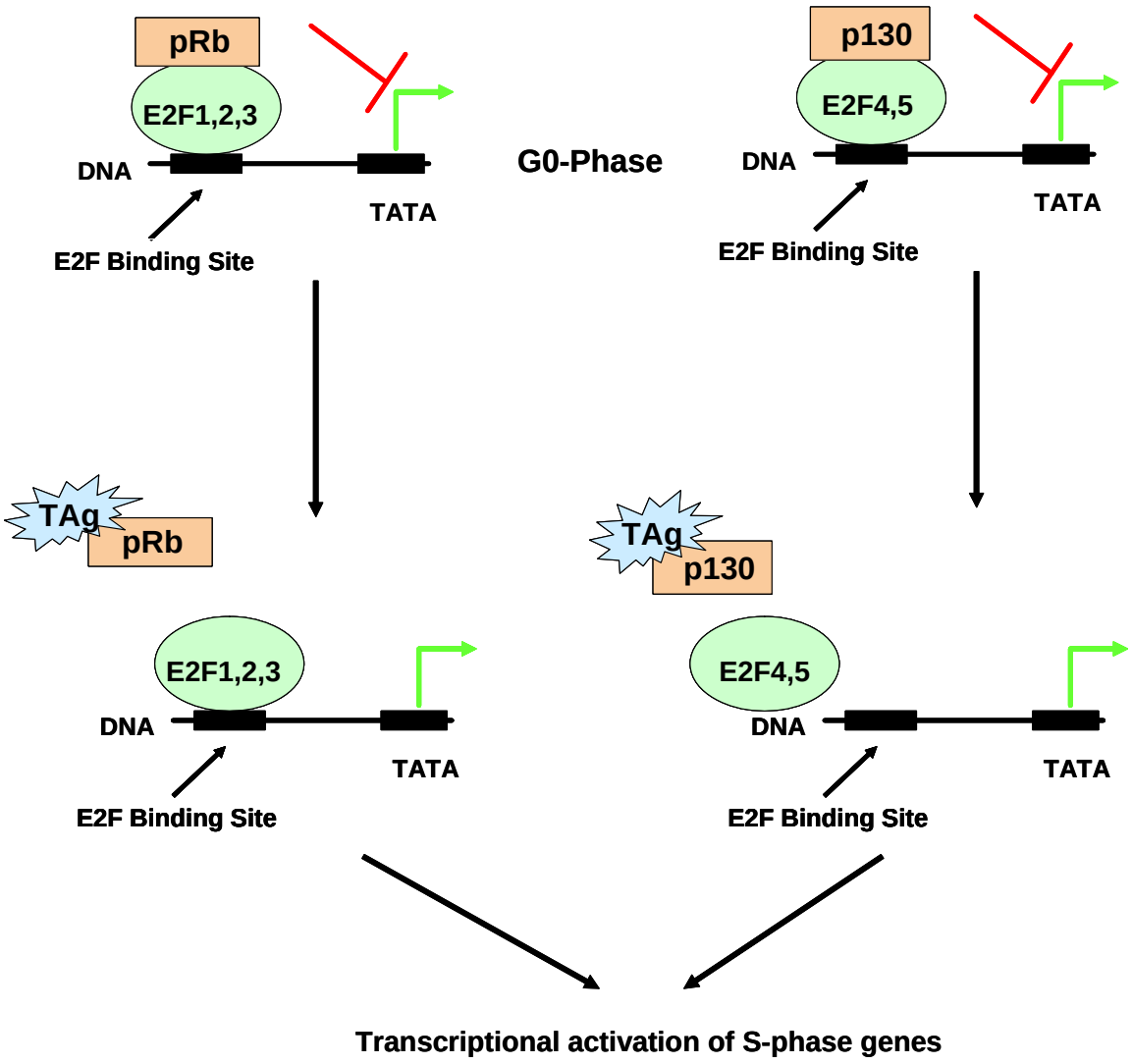
1.3.1.2 TAg interaction with Rb family members

TAg was shown to bind Rb-proteins via the LXCXE motif (DeCaprio et al., 1988; Ewen et al., 1989). Furthermore, mutants of T antigen that are defective for Rb binding also fail to induce transformation (Kalderon and Smith, 1984; Rutila et al., 1986; Srinivasan et al., 1997; Zhu et al., 1992). Binding of TAg to the Rb-proteins via the LXCXE motif in conjunction with the J domain is sufficient to release E2Fs from repressive Rb-protein complexes and to induce S-phase genes (Fig.6). The LXCXE motif is required for growth to a high density in low and high serum concentrations and anchorage independent growth of MEFs both in a normal and Rb null background (Zalvide and DeCaprio, 1995). Similarly, mutations in this sequence render TAg defective in these assays when tested in MEFs lacking both p130 and p107 (Stubdal et al., 1997), suggesting that inactivation of all three Rb family members is necessary for transformation. These data strongly suggest that the Rb-proteins are an important target for TAg mediated transformation. Interestingly, the requirement of the LXCXE motif for anchorage independent growth can be relieved by inactivating mutations of $p19^{ARF}$ (Chao et al., 2000). On the other hand, the LXCXE motif appears to be dispensable for extending the lifespan of primary cells, MEFs and REFs (rat embryo fibroblasts) in culture (Chen and Paucha, 1990; Thompson et al., 1990; Zalvide and DeCaprio, 1995). Furthemore, the binding of TAg to Rb-proteins is not required for SV40 replication (Trifillis et al., 1990). Transgenic mice have been made using wild-type and mutant forms of SV40 TAg to dissect the role of Rb-proteins in tumor development (Bennoun et al., 1998; Chandrasekaran et al., 1996; Chen et al., 1992; Kim et al.,

1994; Saenz Robles et al., 1994). These studies show that the amino terminal region of TAg comprising the J domain and LXCXE motif) is sufficient to induce tumors in various murine tissues and absolutely requires an intact LXCXE motif.

Figure 6: T antigen interaction with RB-family proteins and effects on cell cycle.

In the G0 (growth arrest) phase of the cell cycle, RB-family proteins are bound to E2Fs and repress E2F-responsive gene promoters. Binding of T antigen to RB-family proteins results in the release of E2Fs from RB-proteins which in turn transactivates S-phase genes and induces cell proliferation.

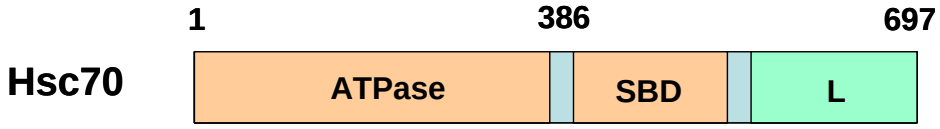


1.3.1.3 Role of J domain in the disruption of Rb-E2F complexes

The J domain of TAg binds to the molecular chaperone Hsc70, the major DnaK homologue present in mammalian cells. This interaction is required for the release of E2Fs from Rb-family protein repression (Sullivan and Pipas, 2002). All Hsc70 family members consist of a large amino terminal ATPase domain, a substrate binding domain, and an extreme carboxy terminal region also known as the variable or "lid" domain (Fig.7). The Hsc70 family of proteins binds to a target substrate and undergoes an ATP-hydrolysis-dependent conformational change, which in turn is transmitted to the bound substrate and alters its conformation. Hsc70 utilizes this mechanism to perform various functions in the cell, such as protein membrane transport, prevention of aggregation of denatured proteins, refolding of denatured proteins, and disruption of multiprotein complexes (Brodsky, 1996; Hartl, 1996; Polissi et al., 1995). Hsc70 by itself has a weak intrinsic ATPase activity. The presence of co-chaperones (such as the DnaJ molecular chaperone) and peptide substrates causes a significant increase in the ATPase activity of Hsc70. The next paragraph describes briefly the cooperative action of DnaK (Hsc70)/ DnaJ (TAg J domain) in the disruption of Rb-E2F complexes.

Figure 7: Domain map of Hsc70.

The ATPase domain (amino acids 1-386) is followed by the substrate binding domain (SBD) and the Lid (L) domain. The opening and closing of the lid domain is dependent on the ATP or ADP-bound status of Hsc70, respectively, and determines the affinity for the substrate.



Several reports suggest the cooperation between the LXCXE motif and J domain for the disruption of Rb-E2F complexes. For example, a p130-E2F complex is found in growth arrested MEFs, but not in MEFs expressing TAg. However, cells expressing J domain mutants or LXCXE mutants are unable to disrupt this complex (Sullivan et al., 2000a; Zalvide et al., 1998). Furthermore, the J domain is required to override the repression of E2F activity mediated by p130 and pRb (Zalvide and DeCaprio, 1995). The amino-terminal mutant N136, consisting of the first 136 amino acids of TAg, is capable of disrupting p130-E2F complexes *in vitro* and *in vivo* (unpublished results). These reports suggest that the J domain and LXCXE motif are sufficient for the disruption of Rb-E2F complexes, but they must reside in *cis*, that is, on the same TAg molecule (Srinivasan et al., 1997). To understand the underlying mechanism of J domain mediated disruption of Rb-E2F complexes, a chaperone model has been proposed (Brodsky and Pipas, 1998) (Fig.8). According to this model TAg forms a complex with Rb/E2F and Hsc70 via the LXCXE motif and the J domain. Then, the J domain stimulates Hsc70 ATP hydrolysis, and the resulting energy is used to release E2F from Rb. Several lines of experimental evidence support this model. For example, TAg does not interfere with E2F binding to Rb since TAg binds to a groove on the surface of the B domain of Rb, while E2Fs bind to a separate surface formed at the intersection of the A and B domain (Kim et al., 2001; Xiao et al., 2003) (Fig.9). Additionally, TAg, Rb, and E2F can form a stable complex (Sullivan et al., 2000a). Furthermore, addition of Hsc70 and an ATP regenerating system releases E2F from p130 in an ATP dependent mechanism (Sullivan et al., 2000a).

Figure 8: The chaperone model for disruption of Rb-E2F complexes.

The Large T antigen J domain recruits hsc70 and stimulates its ATPase activity which in turn releases Rb from E2F. Free E2Fs are now able to transactivate genes required for cell cycle progression.

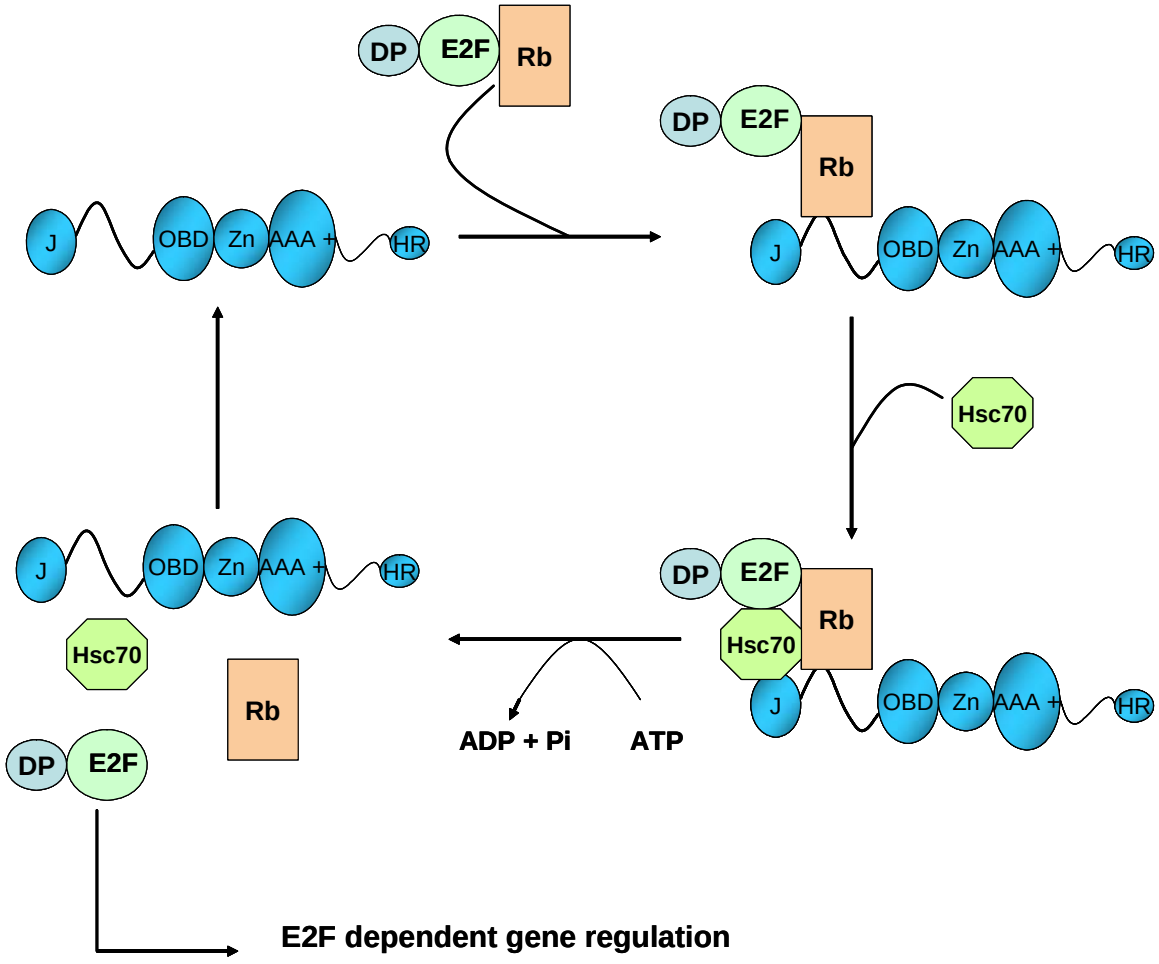
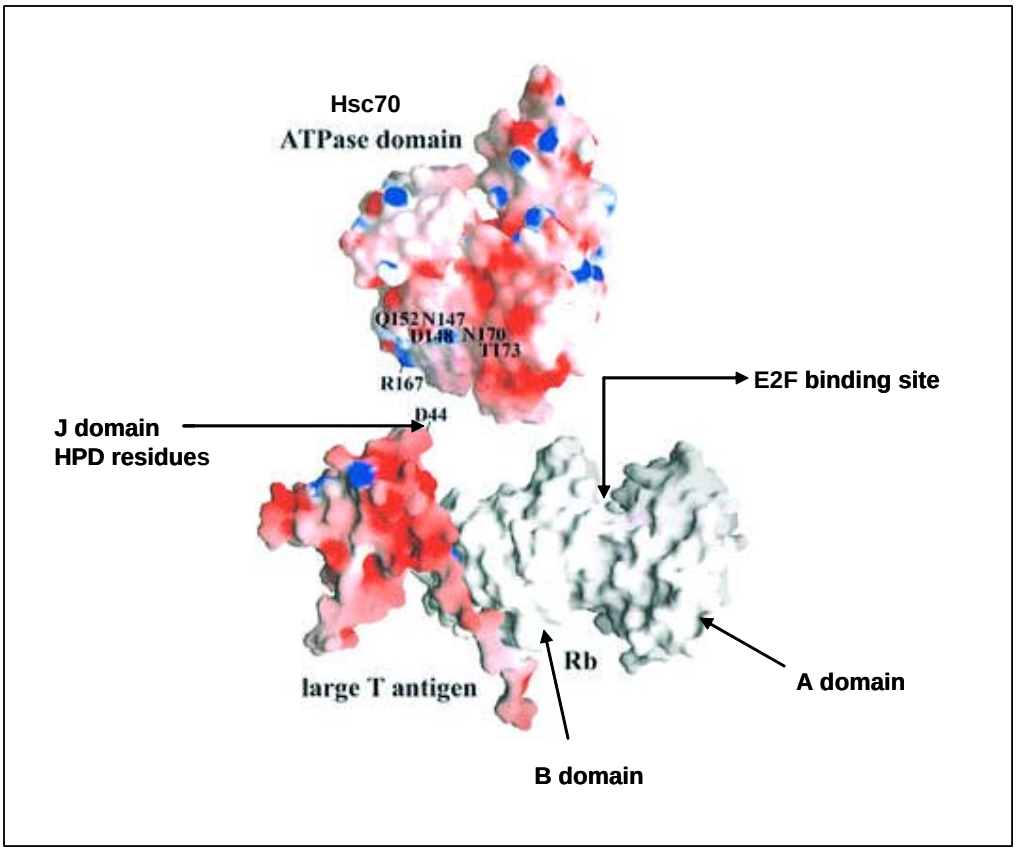


Figure 9: Surface representation of a model for the interaction between Rb, E2F, TAg, and Hsc70.

(Figure modified from Kim et al, 2001).



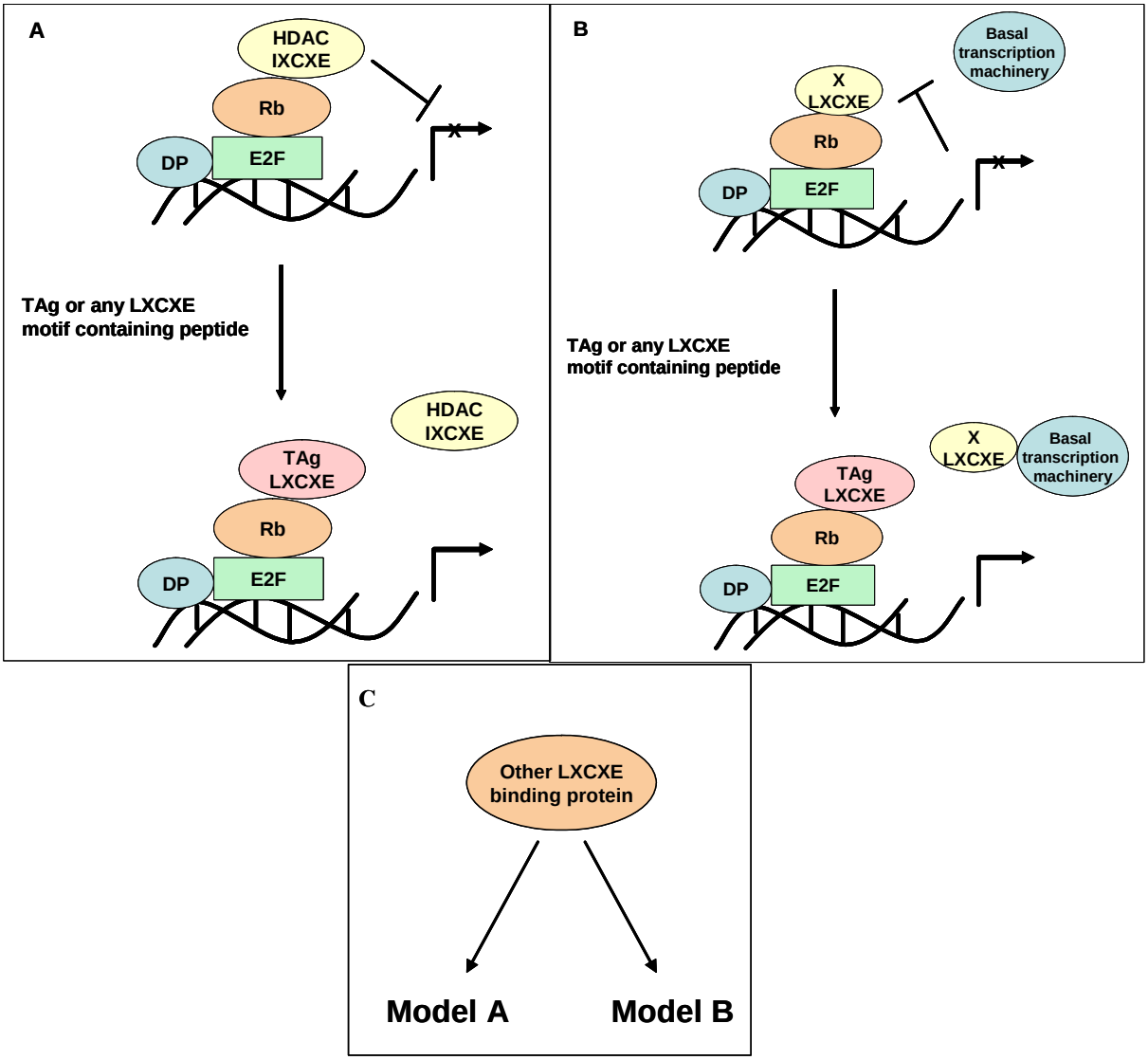
1.3.1.4 J domain independent T antigen effects on the Rb family

The Rb family members perform many functions including repression of activity of E2F and other transcription factors such as MyoD, Pax-3, c-Abl and CDP/cut. The requirement of the J domain in the release of E2F from Rb repression is well established, however the role of the J domain in regulating other transcription factors needs to be determined. Several reports suggest that TAg can alter Rb cellular growth regulation in a J domain independent manner. Stubdal and coworkers reported that induction of soft agar growth by MEFs depends on Rb binding but is independent of the J domain (Stubdal et al., 1997). It has been shown that a fusion protein consisting of the Rb-binding (amino acids 101 to 118) and carboxyl-terminal T-antigen fragments (amino acids 128 to 708) linked through a heterologous sequence is able to induce cells to grow to a high saturation density in high serum (Tevethia et al., 1997), suggesting the contribution of the Rb-binding motif in some growth promoting functions, even in the absence of the J domain. Overexpression of polyomavirus TAg in differentiating C2C12 myoblast cells induces apoptosis. This is dependent on Rb binding but independent of the J domain, since a J domain mutant H42Q induces apoptosis nearly as well as the wild type (Sheng et al., 2000). Moreover, either wild-type TAg or a J domain mutant are capable of restoring growth of a cell line which is growth inhibited by the conditional expression of p53. However, the LXCXE motif mutant is unable to restore growth suggesting that the Rb-binding motif, but not the J domain is required for this activity (Gjoerup et al., 2000; Quartin et al., 1994). Thus the varying requirements for a functional J domain in addition to the LXCXE motif may reflect TAg mediated disruption of Rb interaction with distinct binding partners, each involved in a specific biological response. One plausible hypothesis for these J domain independent, Rb binding dependent biological activities is that the LXCXE motif by itself is able to disrupt HDACs from

pRB (Gjoerup et al., 2000; Sheng et al., 2000), thereby reducing the ability of pRb to inhibit transcription. This hypothesis is supported by the observation that a peptide containing the LXCXE motif of T antigen disrupts complex formation between HDAC1 and pRB *in vitro* (Magnaghi-Jaulin et al., 1998) (Fig.10A). An alternative possibility is that TAg may prevent pRB repression arising from its blockade of interactions between certain transcription factors and the basal transcription machinery (Fig.10B). This hypothesis is supported by the observation that a human papillomavirus E7-derived peptide can disrupt interaction between pRB and upstream binding factor (UBF) and thereby prevent pRB-mediated repression of RNA polymerase I transcription (Cavanaugh et al., 1995). Third possibility is that the LXCXE motif binds some target other than pRb and affects growth control (Fig.10C).

Figure 10: Models for J domain independent regulation of the Rb family.

In this figure "Rb" represent all Rb-family members (pRb, p130 and p107). **(A)** In model A, it is hypothesized that TAg displaces HDAC from Rb because of competition from the LXCXE motif present in TAg. Release of HDAC may result in inhibition of Rb repression activity. HDAC1 contains an IACEE sequence which is similar to the LXCXE or IXCXE motif. **(B)** In model B, it is hypothesized that TAg displaces certain transcription factor(s) from Rb and make it available to interact with the basic transcription machinery to induce transcription which was otherwise repressed by Rb (Figure modified from Gjoerup and Roberts 2000). **(C)** In model C, it is hypothesized that LXCXE motif binds some target other than Rb and can affect growth control by mechanism describe in model A or B.


A
HDAC
IXCXE
Rb
DP
E2F
TAg or any LXCXE
motif containing peptide
HDAC
IXCXE
TAg
LXCXE
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E2F
B
Basal
transcription
machinery
X
LXCXE
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E2F
TAg or any LXCXE
motif containing peptide
X
LXCXE
Basal
transcription
machinery
TAg
LXCXE
Rb
DP
E2F
C
Other LXCXE
binding protein
Model A
Model B

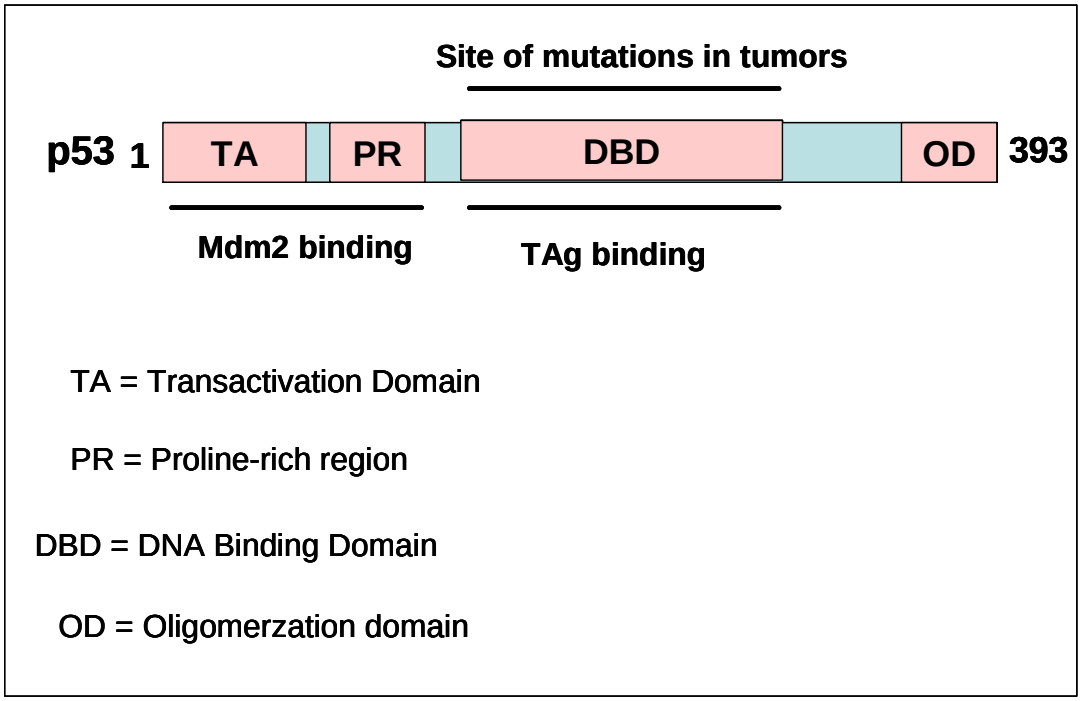
1.3.2 p53

The *p53* tumor suppressor gene encodes one of the most intensively studied proteins, p53, a sequence-specific DNA-binding transcription factor (Fig.11). The very high frequency with which the p53 pathway is inactivated in human cancers (~50%) attests to the crucial and pervasive role of p53 as a tumor suppressor. p53 is so critical because of its involvement in cell-cycle control, in apoptosis and in maintenance of genetic stability-all these fundamental functions of p53 help to protect the organism from cellular damage and disorder and thus p53 is considered as a "guardian of the genome". p53 was initially identified as a protein interacting with TAg in immunoprecipitation assays (Lane and Crawford, 1979; Linzer and Levine, 1979), emphasizing the importance of using DNA tumor viruses as a tool to understand tumorigenesis.

In contrast with pRb, very little p53 protein (almost undetectable) is found in most of the cells of the body under normal conditions. In fact, p53 is not required for normal development, as p53-deficient mice appear normal in all respects although they are prone to a variety of tumors, mostly lymphomas and sarcomas (Donehower et al., 1992). The level of p53 rises in response to genotoxic stresses such as irradiation, chemotoxins, or viral-induced unscheduled DNA synthesis. This increase in p53 levels initiates a cascade of events including stimulation of the transcription of several genes involved in cell cycle arrest and apoptosis. One such gene encodes a Cdk/cyclin kinase inhibitor (CKI) protein called p21, which binds to G1/S-phase Cdks and S-phase Cdks and inhibits their activities, thereby helping to block entry into S phase (Mittnacht, 1998). These observations suggest that p53 has evolved to integrate responses to stress and pathological stimuli.

Figure 11: Functional domains of p53.

The N-terminus of p53 consists of a transactivation domain and a proline-rich region, which are required for apoptotic function. The N-terminus possess interaction sites for components of the transcriptional machinery and Mdm2. T antigen binds to the DNA binding domain of p53 and might inhibit p53 function by blocking its DNA-binding activity. The N-terminus and C-terminus of p53 contains several sites for modifications such as phosphorylation, acetylation, ubiquitylation and sumoylation which influence stability, localization and activity of p53.



Another gene which encodes a E3 ubiquitin ligase (Mdm2) that is activated by p53. Mdm2 then binds to p53 and ubiquitinates it, thus targeting it for proteasome-mediated degradation (Levine, 1997). The down-regulation of p53 function by Mdm2 provides a feedback loop mechanism that allows for restoration of normal cell function when the genomic stress is diminished. On the other hand, phosphorylation of the amino-terminus of p53 reduces its binding to Mdm2 and thus decreases p53 degradation. The decreased binding to Mdm2 enhances the ability of p53 to stimulate gene transcription.

p53 levels are regulated by another feedback loop through $p19^{ARF}$ ($p14^{ARF}$ in humans) (ARF), which stabilizes and activates p53 by binding Mdm2 and blocking its association with p53. On the other hand, p53 downregulates ARF expression (Stott et al., 1998). ARF expression can be upregulated by E2F1, β-catenine, myc, ras and adenovirus E1A (Bates et al., 1998; de Stanchina et al., 1998; Palmero et al., 1998; Stott et al., 1998; Zindy et al., 1998). Beside p21, ARF serves as another link between the RB-E2F and p53 pathways (Fig.12). Overall the activation of the p53 protein and its network of genes sets in motion an elaborate process of autoregulatory-positive or autoregulatory-negative feedback loops, which connect the p53 pathway to other signal transduction pathways in the cell, and this broader communication permits the completion or the reversal of the p53 programmed responses to stress (Harris and Levine, 2005).

A large number of genes directly regulated by p53 contribute to the apoptosis of cells. For instance, Bax, Noxa, and Puma enhance the secretion of cytochrome *c* from the mitochondria into the cytoplasm often in a tissue-specific fashion. Subsequently, cytochrome *c* interacts with APAF-1, another p53 target gene, to initiate a protease cascade, leading to the activation of caspase-9 and caspase-3 followed by apoptosis. This intrinsic pathway is initiated

by a number of stress signals that activate the p53 pathway. In addition, p53 regulates a series of genes (Fas ligand, killer Dr receptor), that initiate an extrinsic apoptotic pathway resulting in the activation of caspase-8 and caspase-3 and apoptosis (Levine et al., 2006; Yu and Zhang, 2005). In contrast to p53-target genes that induce apoptosis, the target genes which bring about senescence are less well characterized (Nakamura, 2004). Besides the traditional role of p53 in cell cycle arrest, senescence and apoptosis, the p53 target genes have been shown to inhibit angiogenesis and metastasis (BAI1, TSP1, Mapsin), to be implicated in DNA repair and damage prevention (Gadd45, Sestrins), or to inhibit the IGF-1/ mTOR pathway (PTEN, TSC2,IGF-BP3) (Levine et al., 2006) (Fig.13).

p73 and p63 are members of the p53 family. These proteins share significant homology with p53 in three functional domains (transactivation domain, DNA binding domain and oligomerization domain) and can induce apoptosis like p53. On the other hand, p73 and p63 give rise to functionally distinct protein isoforms that lack the transactivation domain and function as dominant negative proteins (Irwin and Kaelin, 2001). p63 and p73 share the tumor-suppressor activity of p53 to some extent, but also have essential functions in embryonic development and differentiation control (Stiewe, 2007).

pRb and p53 play a central role in cellular growth control and the crosstalk between these pathways provide a better regulation of different cellular processes. The overlapping nature of these pathways is an important issue to keep in mind when addressing the biology of T antigen, and its interactions with either the Rb or p53 pathway (Fig.12).

Figure 12: Feedback regulation between Rb-E2F and p53 pathways.

E2F 1-3a activates p19ARF and E2F1 activates p53 by binding to their promoters and inducing transcription. p19ARF activates p53 by blocking its degradation by Mdm2. On the other hand, p19ARF binds to E2F1, disrupts E2F1/DP1 DNA binding complexes and targets E2F1 for degradation. p53 can activate transcription of p21 and pRb by binding to their promoters. In turn, p21 blocks cyclin D/ Cdk4/6 complexes that phosphorylate and inactivate pRb which binds E2Fs and repress its transcriptional activity.

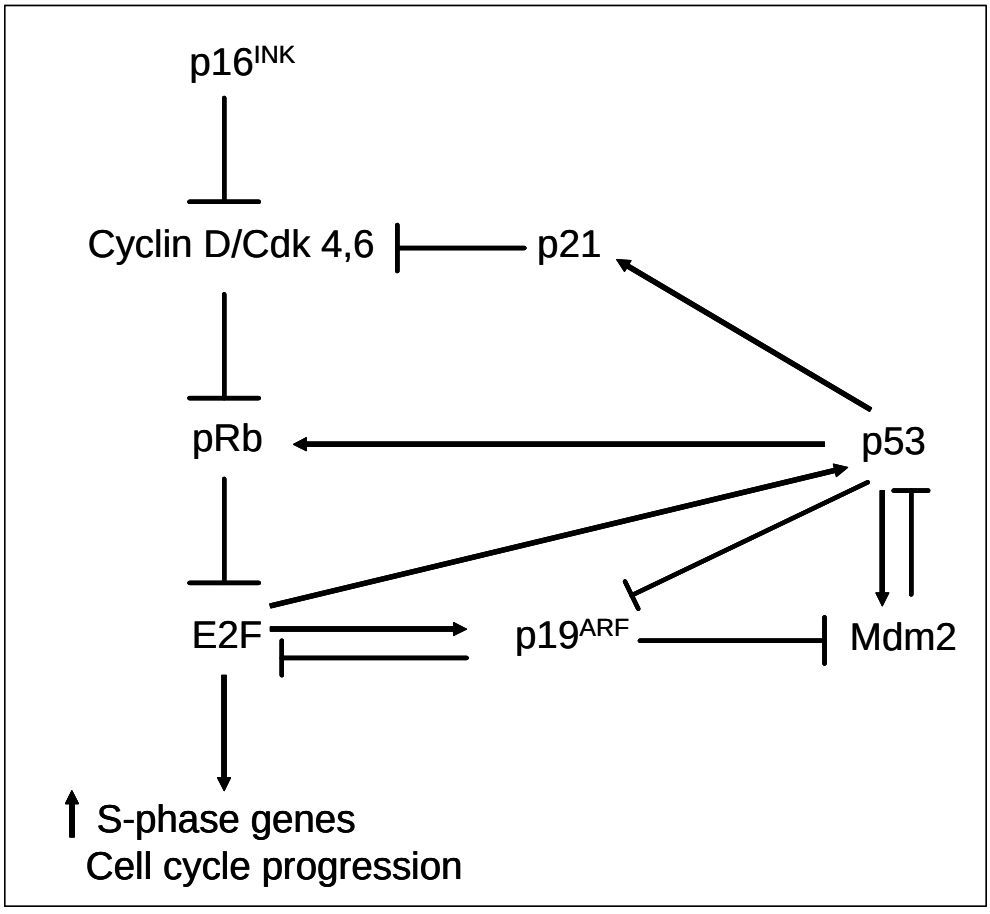
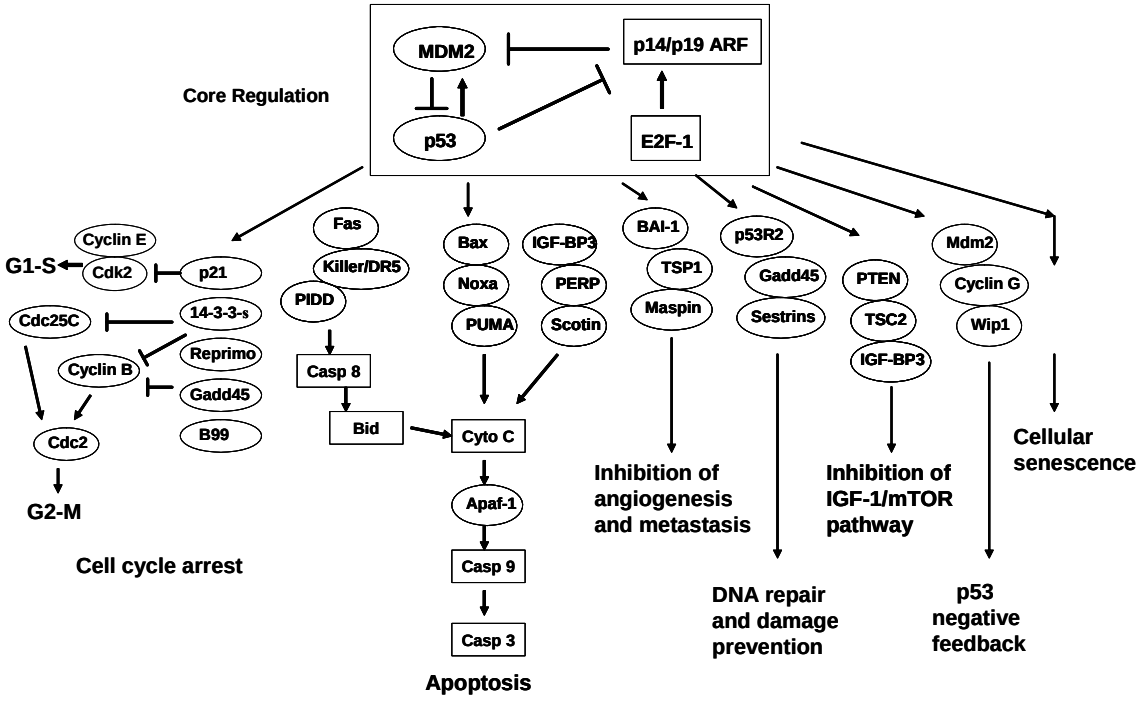


Figure 13: The feedback regulation, the downstream genes and the biological consequences of the p53 pathway.

The p53 protein acts as a transcriptional activator of p53-regulated genes and results in three major biological consequences: cell cycle arrest, senescence or apoptosis. Other functions of p53-target genes include communication with neighboring cells, DNA repair or set up positive and negative feedback loops that enhance or attenuate the function of p53 protein and integrate these stress responses with other signal transduction pathways. Figure modified from Levin *et al.*2006.



1.3.2.1 TAg interaction with the tumor suppressor p53

p53 was first discovered as a cellular protein bound to TAg in SV40 infected or transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). TAg directly binds to the DNA-binding surface of p53 thus blocking its ability to bind promoters and to regulate gene expression (Bargonetti et al., 1993; Jiang et al., 1993). Complex formation between TAg and p53 interferes with the expression of p53-regulated genes involved in several biological processes such as cell cycle (p21, cyclin G1), DNA repair (GADD45), apoptosis (Bax) and signal transduction (IGF-BP3) (Fig.13). For example, TAg blocks the activation of the p53-regulated gene p21 which is a universal Cdk inhibitor and as a result cells are unable to arrest in G1 phase and progress to S phase. Additionally, the binding of p53 with TAg prevents its degradation by Mdm2 and results in p53 stabilization. Therefore, high levels of p53 which are thought to be functionally inactive, are found in SV40 transformed cells (Oren et al., 1981). The classical hypothesis is that TAg creates a p53 null environment in the cell. An alternative hypothesis is that by stabilizing p53, TAg may manipulate its functions to benefit the virus and subsequently cause transformation. Furthermore, several reports suggest that mutant p53 proteins do not represent the simple loss of wild-type p53 activities but instead gain new additional oncogenic functions which contribute to tumor progression (Strano et al., 2007). For example, genetically engineered mice with naturally occurring mutations of p53 knocked into the p53 locus developed unique tumors compared to p53-/- mice, including a variety of carcinomas and more frequent endothelial tumors (Lang et al., 2004; Olive et al., 2004).

SV40 targets p53 in multiple ways. Thus another mechanism by which TAg blocks p53-dependent transcription and growth arrest is through a mechanism that is independent of p53 binding and requires both the J domain and the LXCXE motif (Quartin et al., 1994; Rushton et

al., 1997). These studies suggest that p53 mediated cell cycle arrest can be overcome by formation of TAg with Rb family, p300 or both that could then function to either remove p53-mediated negative growth reagulatory signals or promote a positive cell growth signal. Furthermore small t antigen was also shown to contribute to the inactivation of p53 and may cooperate with TAg to enhance transformation. TAg does not bind to other p53 family members, p63 and p73, which suggests that these proteins are not involved in TAg mediated transformation (Dobbelstein et al., 1999; Higashino et al., 1998).

1.3.3 TAg interaction with the CBP family

p300 and CBP are highly homologous transcriptional coactivators that possess both distinct and overlapping functions. These coactivator proteins are recruited by many different transcription factors such as CREB, nuclear receptors, STAT, NF-κB, and AP-1 and therefore play a broad role in multiple biological processes including cell growth, cell cycle progression and development. In addition, CBP/p300 have been implicated in cancer (Goodman and Smolik, 2000; Iyer et al., 2004). The adenovirus E1A protein binds to p300/CBP directly through an N-terminal CR1 domain, and this region is essential for its transforming activities (Jelsma et al., 1989). Genetic studies demonstrated that SV40 TAg can complement p300/CBP-binding deficient mutants of E1A to restore transformation (Yaciuk et al., 1991). p300/CBP interact with TAg indirectly where p53 serves as a scaffold to bridge the interaction (Poulin et al., 2004) (Fig.14). TAg can enhance the p300/CBP associated histone acetyltransferase activity (Valls et al., 2003). Furthermore, the presence of TAg increases phosphorylation of p53 at S15 which in turn stabilizes the interaction between p53 and p300/CBP (Borger and DeCaprio, 2006). CBP in turn acetylates TAg on K697 in a p53 dependent manner (Poulin et al., 2004). The biological

consequences of this acetylation is not completely understood, however this acetylation site is conserved on the T antigens of JCV, BKV and SA12 (Cantalupo et al., 2005; Poulin et al., 2004). Some experiments suggest that TAg might help to deliver p300/CBP to E2F-dependent promoters, which may enhance transcription of E2F target genes and progression to S phase (Ait-Si-Ali et al., 2000). This hypothesis is supported by the observation where activation of E2F dependent promoters by the murine polyomavirus TAg requires both the inhibition of Rb proteins and an interaction with p300/CBP (Nemethova et al., 2004). On the other hand, it has been reported that stable transfection of NIH3T3 cells with the acetylation-mimicking mutant K697Q had reduced anchorage-independent growth compared to cells expressing wild-type or non-acetylatable K697R TAg. This observation suggests that acetylation destabilizes TAg and regulates the transforming activity of TAg in NIH3T3 cells (Shimazu et al., 2006).

1.3.4 Additional TAg targets

Genetic studies suggest that inactivation of pRb and p53 is not sufficient to induce TAg mediated transformation (Sachsenmeier and Pipas, 2001) thus indicating the presence of additional targets of TAg contributing to transformation. In the past few years several additional targets of TAg have been discovered. Some of them are shown to be important for transformation while others await more studies (Fig.14). Following is the brief description of the additional targets of TAg.

Cul7 is a scaffold protein that forms the core subunit of an E3 ubiquitin ligase complex involved in degradation of proteins (Ali et al., 2004). TAg binds to Cul7 via residues 69-83 which are situated at the carboxy-terminal end of the J domain and upstream from the LXCXE motif. Non-Cul7-binding mutants of TAg are unable to induce anchorage-independent growth of MEFs and fail to support growth in low serum (Ali et al., 2004). However these mutants are

capable of inducing cell division and growth to high densities when expressed in Cul7 deficient MEFs (Kasper et al., 2005). These observations suggest that in addition to p53 and the Rb family of proteins, TAg must inactivate Cul7 to fully transform cells.

Bub1, a mitotic spindle checkpoint protein, was found as a TAg interacting protein in a yeast two hybrid analysis (Cotsiki et al., 2004). TAg mutants W94A or W95A defective in Bub1 binding fail to induce focus formation in Rat-1 cells, but immortalize rat embryo fibroblasts (Cotsiki et al., 2004). The interaction between TAg and Bub1 results in the perturbation of the spindle checkpoint and may induce aneuploidy and genomic instability (Chang et al., 1997; Cotsiki et al., 2004; Woods et al., 1994).

Nbs1 (Nijmegen breakage syndrome protein1) is a component of the MRN (Mre11/Rad50/Nbs1) complex that functions in DNA double-stranded break repair. TAg can bind to Nbs1 via its origin binding domain and this interaction is independent of TAg binding to p53 and pRb and does not require the J domain (Wu et al., 2004). By binding to Nbs1, TAg induces endoreduplication and causes genomic instability. It has been also reported that TAg disturbs the accumulation of Mre11 at sites of DNA damage (Digweed et al., 2002).

Fbw7 is the substrate recognition component of the SCF^{Fbw7} ubiquitin ligase and is a human tumor suppressor. TAg binds to Fbw7 via a decoy phospho-epitope within the host-range domain of TAg that closely mimics the consensus Cdc4 phospho-degron found within Fbw7 substrates (Welcker and Clurman, 2005). The authors report that this interaction can interfere in Fbw7-driven turnover of physiological substrates such as cyclin E in vivo and cause stabilization of these substrates. This leads to a hypothesis that TAg, functioning as a competitive inhibitor, competes with cellular proteins for Fbw7 binding in a substrate-like fashion. How this interaction contributes to TAg mediated transformation is not known yet.

Zhou-Li and co-workers have shown direct association of TAg with Insulin receptor substrate (IRS-1), which is a major substrate for both the insulin receptor and insulin-like growth factor receptor (IGF-IR) (Zhou-Li et al., 1997). The amino terminal 250 amino acids of TAg were shown to be required for this interaction. Only one report suggests the requirement of signaling through IGF-IR for SV40 mediated transformation (DeAngelis et al., 2006).

TAg modulates the transcription of both cellular and viral genes by targeting various components of the eukaryotic transcriptional machinery such as TEF-1, TBP, hTAF, TFIIB and RNApolII (Berger et al., 1996). How these interactions with TAg affect transcription of cellular genes is not yet known. However, since TBP is required by all three RNA polymerases, TAg could potentially affect transcription by all these. TAg binds to transcription enhancing factor -1 (TEF-1) via the OBD domain and inhibits the binding of TEF-1 to target DNA sequences. TEF-1 appears to play a role in the early-to-late switch in viral transcription (Berger et al., 1996). A TEF- binding deficient mutant (S189N) of TAg was shown to be seven to eight fold less efficient than wild-type TAg in focus formation assay (Dickmanns et al., 1994). This may suggest that TAg is involved in transcriptional regulation of cellular genes through their TEF-1 binding sites.

In summary, TAg targets many key cellular regulatory proteins to induce transformation (Fig.15). TAg targets two well characterized tumor suppressors, pRb and p53 and in both cases the primary effect of TAg is to perturb the way the tumor suppressors regulate cellular transcription. On one hand by binding to Rb family members TAg induces proliferation and on the other hand by binding to p53 it blocks apoptosis. Furthermore, it is unclear if TAg recruitment of CBP/p300 via p53 plays a role in SV40-mediated transformation, however modulation of CBP/p300 is necessary for transformation induced by adenovirus E1A. Additional potential targets of the TAg include Bub1, Cul7, Fbw7 and IRS-1. However, their role in TAg

mediate transformation is not clear. TAg also targets the DNA damage sensing and processing complex Mre11-Rad50-Nbs1 (MRN) and may induce genetic instability that contributes to transformation.

Figure 14: Interaction of cellular proteins with different domains/regions of large T antigen.

T antigen binds to RB-family proteins via an LXCXE motif, Hsc70 via the J domain, p53 via the p53 binding site, CBP/p300 via p53, Nbs1 and TEF-1 via the OBD and Fbw7 via the HR domain. The amino-terminus of T antigen interacts with TATA-binding protein (TBP), Oct6 and IRS-1. T antigen was shown to be coimmunoprecipitated with activator protein 2 (AP-2) and various components of the eukaryotic transcriptional machinery such as TAF, TFIIB and RNApolII.

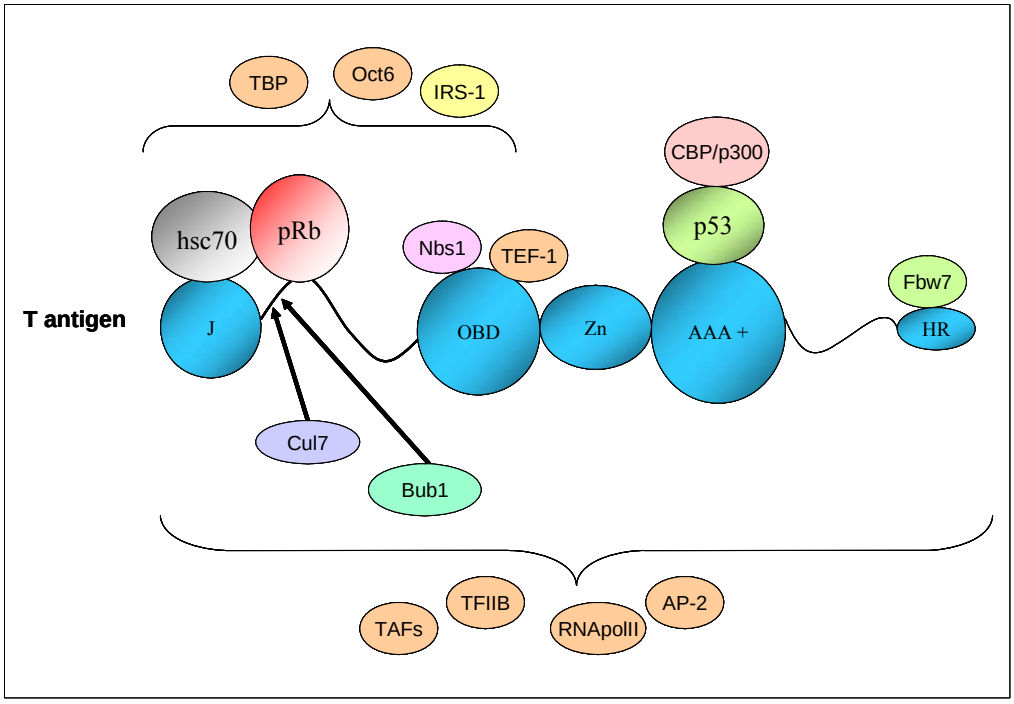
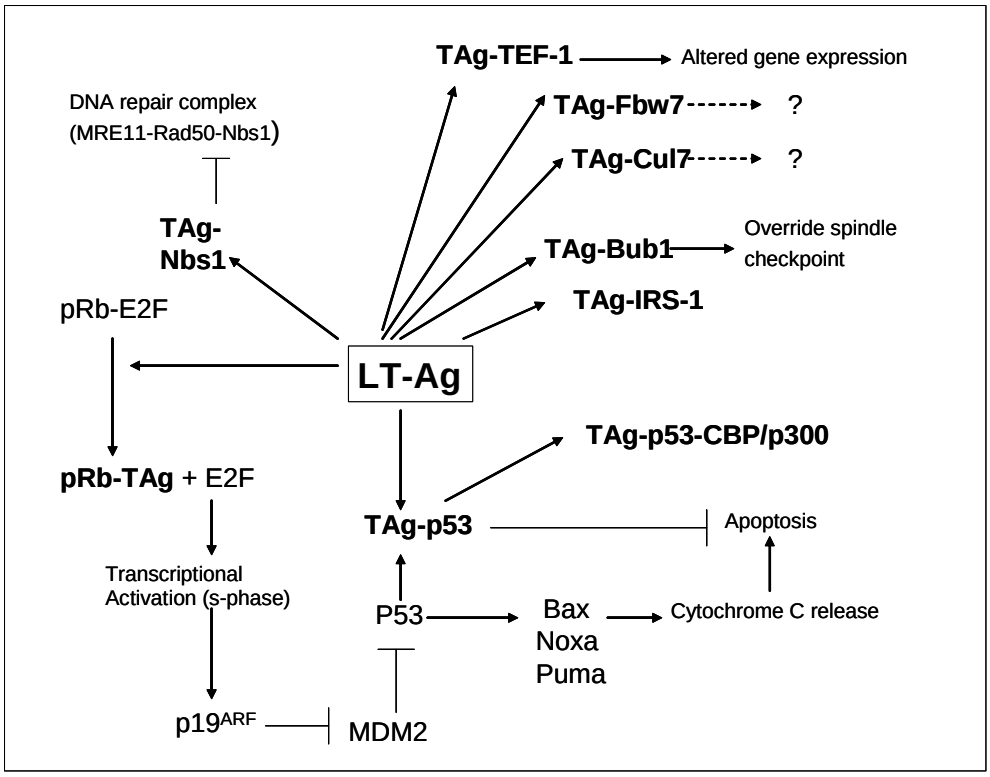


Figure 15: Large T antigen interacts with cellular proteins to elicit transformation.

By blocking pRb dependent response TAg drive cells to re-enter S-phase. By blocking p53 dependent response TAg escape apoptosis. The net result of these actions is cell proliferation. Additional potential targets of the TAg include CBP/p300, Bub1, Cul7, Fbw7 and IRS-1. TAg targets the DNA reapir complex (Mre11-Rad50-Nbs1) and may induce genetic instability that contributes to transformation.



1.4 THE MOUSE INTESTINAL EPITHELIUM AS A MODEL SYSTEM

The epithelial cell renewal process in the mouse intestine makes it a very useful model system to study the regulation of cell division and differentiation. The epithelium is a contiguous, single layer sheet of cells that convolutes and involutes to form two types of structures; the villus that protrudes toward the lumen, and the crypts of Liberkuhn at the base of each villus that invaginate into the underlying basal lamina (Fig.16).

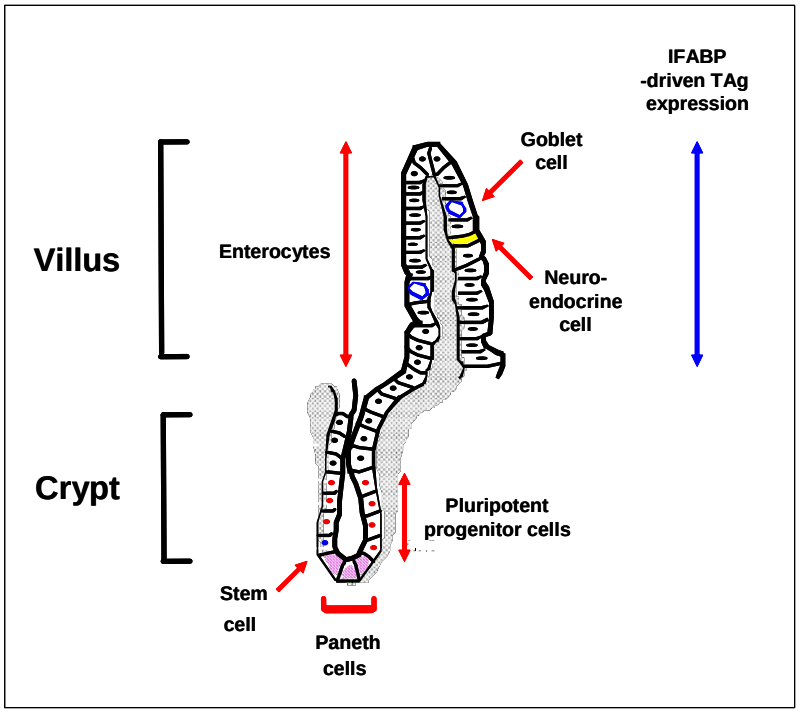
The intestinal epithelium contains four types of differentiated cells: enterocytes, goblet, enteroendocrine, and Paneth cells. These cells are derived from a small number of pluripotent stem cells that reside near the base of each crypt. These stem cells give rise to a zone of proliferating cells, which differentiate as they migrate towards the luminal surface, with the exception of the Paneth cells, which migrate towards the base of the crypt. As a result, villi are mainly composed of enterocytes (95%) with some goblet and enteroendocrine cells (Figure 16). Enterocytes secrete hydrolases and absorb nutrients. Goblet cells, provide a protective mucous lining. The rare enteroendocrine cells secrete hormones (secretin) and neurotransmitters (serotonine) to maintain homeostasis and other neurological mechanisms to stimulate absorption and for distension in the intestine. Paneth cells secrete antimicrobial peptides and enzymes such as cryptidines, defensins, and lysozyme. The differentiated cell types (enterocytes, goblet and enteroendocrine cells) function for only a few days and undergo apoptosis and are then extruded into the intestinal lumen, while Paneth cells are thought to be engulfed by macrophages. As a result, the entire lining of the intestine is replaced approximately every five days (Sancho et al., 2004). Thus, the small intestine represents a dynamic example of self renewing tissue, and one of

the few cases in which the proliferating stem cells and nonproliferating differentiated cell populations reside in discrete compartments.

Intestinal villi and crypts can be readily separated from extraneous tissue, thus allowing the isolation of proteins or nucleic acids from cell populations greatly enriched for non-proliferating, terminally differentiated cells (villi), or from their proliferating, multipotent progenitors (crypts) (Markovics et al., 2005; Whitehead et al., 1987). Overall, the stratification of proliferation and differentiation along the crypt-villus axis makes the intestine an attractive model system for examining the regulation of mediators of the cell cycle.

Figure 16: Diagram of the small intestinal epithelial structures: crypt of lieberkuhn and its associated villus.

Crypts involute into the mesenchyme and represent cell populations enriched for proliferating, multipotent progenitors. Villi protrude into the lumen of the small intestine and represent cell populations greatly enriched for non-proliferating, terminally differentiated cells. Location of intestinal cell types (enterocytes, enteroendocrine, goblet and paneth cells) is indicated. TAg is expressed in terminally differentiated enterocytes by using the intestinal fatty acid binding protein (I-FABP) promoter.



Several paracrine and autocrine signaling pathways regulate stem cell proliferation in the crypts and differentiation in the villus, including the Wnt (Dickkopf, APC, β-catenine, SOX9), Notch-Delta, TGFβ/BMP and Hedgehog pathways (Hauck et al., 2005; Radtke et al., 2006; Sancho et al., 2004; Walters, 2005). Briefly, Wnt signaling is important for stem cell renewal and crypt formation, while BMP signaling suppresses Wnt signaling to ensure a balanced control of stem cell self-renewal (He et al., 2004). The Notch signaling pathway regulates differentiation choices between more specialized secretory cell types. Sonic hedgehog (SHH) and Indian hedgehog (IHH) signaling is required for embryonic villus formation and for cell fate specification.

A number of correlations have been made between targeted genetic manipulations or mutations of the above mentioned signaling pathways and the resulting intestinal phenotypes (Hauck et al., 2005). Tumorigenesis in the small intestine is actually quite low, and most of the transformation resulting from mutations in these pathways occurs in the colon, which appears to be similarly regulated to the small intestine. The difference in tumorigenic frequency between the small and large intestine has been attributed to the higher propensity of the small intestinal epithelial stem cell's to commit apoptosis when damaged, and the higher genetic fidelity achieved by regular retention of the template strands of DNA (Potten et al., 2003). One of the important aspects to study cancer is to understand a fine balance between the cell proliferation and differentiation. Therefore the small intestine becomes a very useful model system because it harbors both proliferating stem cells and nonproliferating differentiated cell populations in discrete compartments.

1.5 T ANTIGEN MUTANTS AND THEIR PHENOTYPES IN CELL CULTURE AND IN TRANSGENIC MICE

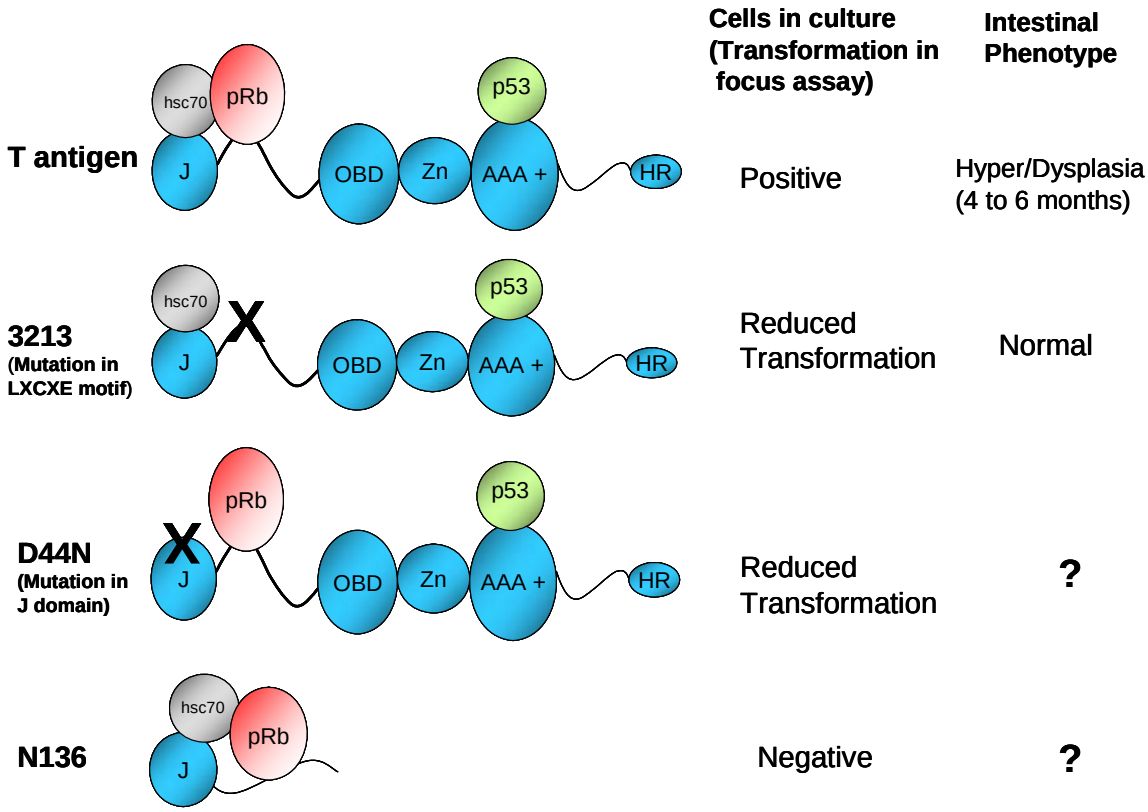
In the present study we have used MEF cell lines as well as transgenic mice expressing wild-type TAg or TAg mutants (N136, 3213, or D44N) (Figure 17). In MEFs, expression of wild-type TAg results in a transformed phenotype, while cells expressing N136 (comprised of the J domain and LXCXE motif) shows transformation defective phenotype as assessed by focus formation and soft-agar assays (Thompson et al., 1990). It has been reported that the LXCXE mutant K1 (E107K) is unable to induce focus formation and anchorage independent growth in MEFs (Zalvide and DeCaprio, 1995), while 3213 (E107K, E108K) show reduced frequency of focus formation. Additionally, the J domain mutant D44N induces focus formation and anchorage independent growth, although at a somewhat reduced frequency compared to wild-type TAg (Hahn et al., 2002; Markovics et al., 2005; Stubdal et al., 1997). The phenotype induced by these mutants of TAg is cell–type specific. For example, 3213 and D44N show an intermediate phenotype with reduced transformation in MEFs, while they are transformation defective when expressed in REF52 cells. Therefore, one should be cautious when interpreting phenotypes induced by these mutants in cell culture and keep in mind the cell type specificity and type of mutation.

Our lab has generated a series of transgenic mice expressing wild-type or a TAg mutant (N136 or 3213 or D44N) in terminally differentiated enterocytes using the intestinal fatty acid binding protein (I-FABP) promoter (Fig.16). Previously it has been shown that these promoter elements could drive expression of a reporter construct exclusively in the villus compartment of the small intestinal epithelium with the highest expression in the distal jejunum, then decreasing

both proximally towards the duodenum and distally towards the colon, similar to the pattern of expression of the endogenous gene (Cohn et al., 1992). Expression of TAg in I-FABP driven transgenic mice extends from the uppermost portion of the crypts at the base of the villus to the apical extrusion zone of the villus (Hauft et al., 1992). Expression of wild-type TAg in murine enterocytes results in intestinal hyperplasia that progresses to dysplasia by 4 to 6 months of age (Hauft et al., 1992; Kim et al., 1993). Expression of 3213 which is unable to bind to the Rb family of proteins in the intestinal epithelium of transgenic mice does not result in hyperplasia and dysplasia (Kim et al., 1994). One of the goals of my thesis is to determine the requirement of the J domain for the induction of intestinal hyperplasia. Furthermore, we aimed to determine whether the amino truncation mutant of TAg (N136) is capable of inducing enterocyte proliferation and intestinal hyperplasia/dysplasia.

Figure 17: Large T antigen mutants and their phenotypes in cell culture and in transgenic mice.

Large T antigen and its mutants were expressed in mouse embryo fibroblasts and in mouse intestinal villus enterocytes. The capability of inducing transformation in cell culture was assessed by focus formation assay and intestinal phenotypes were scored as hyperplasia and dysplasia.



1.6 DISSERTATION OVERVIEW

According to the current model, TAg inactivates Rb proteins and thus induces E2F dependent transcription, which consequently leads to cell proliferation/transformation. In addition, TAg stabilizes and inactivates p53, thus it blocks apoptosis and results in reduced cell death. The aim of this dissertation is to test this model and explore new targets and/or functions of TAg which may contribute to transformation.

Chapter 3 describes studies to determine the role of the J domain in the induction of intestinal hyperplasia. Several cell culture studies suggested that cooperation of the J domain with the Rb binding motif (LXCXE) is required to inactivate the growth suppressive functions of p130, p107 and pRb. However, the requirement of the J domain for transformation in cell culture is not clear, due to an intermediate phenotype. Therefore, we decided to determine the role of the J domain in a normal tissue setting (mouse intestinal system) for induction of hyperplasia.

Chapters 4 describe experiments aimed to determine whether the amino truncation mutant of TAg (N136) is capable of inducing enterocyte proliferation and intestinal hyperplasia/dysplasia. Based on previous studies we hypothesized that inactivation of Rb-proteins by TAg is sufficient to induce enterocyte proliferation, but that an activity mapping to the C-terminus and/or expression levels and/or localization of TAg is required for progression to dysplasia. To test this we have examined several lines of transgenic mice expressing the amino-terminal truncation mutant, N136, in villus enterocytes.

Chapter 5 and 6 analyzes the global change in the host cell gene expression after SV40 abortive infection or after expression of TAg in either cell culture or in an animal model. Additionally, we aimed to investigate whether the Rb and p53 pathways are the only players of TAg induced transformation or if additional factors contribute to it. Our study identified

additional sets of genes altered by TAg expression either belonging to the interferon pathway or to other important biological classes.

2.0 MATERIAL AND METHODS

2.1 PRODUCTION AND MAINTENANCE OF TRANSGENIC MICE.

A BamHI/XhoI fragment (2.7Kb) from pRSVBneoT (Srinivasan et al., 1997), encoding full length T antigen, was ligated to the 3.9Kb BclI/XhoI vector portion of pIFABP.GFP (Saenz-Robles et al., 2007). The resulting plasmid (pIFABP.T) was the target for site-directed mutagenesis (QuickChange kit, Stratagene) to introduce the D44N mutation into TAg^{wt}. The resulting construct (pIAFBP.D44N) was confirmed by sequence analysis and digested with SacII/MluI to render a 4.2Kb fragment containing the IFABP promoter, the coding sequences for SV40 large T antigen mutant D44N and the SV40 polyadenylation signals. Pronuclear microinjection of this fragment resulted in transgenic mice (Fabpi-SV40 TAg^{D44N}) expressing TAg^{D44N} and small t antigen in the enterocytes of the small intestine. Three different transgenic lines were established (A, C and G), all of which showed similar phenotypes. Unless noted, TAg^{D44N} animals used in this report were from pedigree C. TAg^{N136} and TAg^{3213} transgenic mice were made as described by Sáenz Robles et al (Saenz-Robles et al., 2007).Transgenic mice expressing SV40 TAg^{wt} under the control of the rat intestinal fatty acid binding protein promoter (pedigree 103) were obtained from Jeff Gordon (Kim et al., 1993). Murine viral pathogens (hepatitis, minute, lymphocytic, choriomeningitis, ectromelia, polyoma, Sendai, pneumonia, and mouse adenoviruses, enteric bacterial pathogens) and parasites were absent from the mouse

colony. All lines were maintained by crosses to nontransgenic FVB. The genotype of each mouse was determined by PCR amplification as described (Saenz-Robles et al., 2007).

2.2 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 previously (Markovics et al., 2005) and grown in in DMEM (Mediatech, cat# 10-013-CV) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, cat# SH30071.03), 100 U/ml penicillin and 100 ug/ml streptomycin (Invitrogen, cat# 15140-122). MEFs were grown at 37^0C in 5% CO_2. Stable MEF cell lines were made with pRSVB-neo plasmid that expresses a Geneticin (G418) resistance gene and a cDNA or genomic version of the wild-type or mutant T antigens (N136, 3213 (E107K, E108K) and 5110 (D44N)) under the control of the Rouse sarcoma virus (RSV) promoter (Srinivasan et al., 1997). Primary cultures of MEFs were transfected with above plasmids using Lipofectamine reagent (Life Technologies), according to the manufacturer's instructions. Upon selection in culture with 0.4 mg/ml of G418 (Invitrogen, cat# 11811-031), transformed colonies (foci) were selected and individually grown and several independent cell lines were established.

2.3 SV40 INFECTION

For the abortive SV40 infections, confluent MEFs were infected with SV40 at a multiplicity of infection 10 viral particles per cell (M.O.I = 10) as described (Tremblay et al., 2001). Cells were incubated for an additional 3 to 4 days before the cells were harvested with trypsin. The cell pellet was washed three times with cold PBS/EDTA and frozen at -80C. RNA extraction from SV40 infected MEFs is described in section 2.8 of this chapter. Three independent mock and SV40 infections were conducted.

2.4 PREPARATION OF INTESTINAL VILLI SAMPLES BY LASER CAPTURE MICROSCOPY (LCM)

The intestine was removed in its entirety from the abdominal cavity, from the exit of the stomach to the entry into the large intestine. The intestines were then opened along their cephalocaudal axis, washed with phosphate-buffered saline, and rolled from the duodenum to the ileum. Rolls from wild-type, TAg and TAg mutant transgenic mice were then processed to isolate villi cell types by LCM. Briefly, the rolls were quickly frozen and stored at -80^{0}C. Frozen sections were cut and immediately dehydrated in graded alcohol solutions. The dried sections were examined microscopically and the top 2/3rds of 50 villous segments from each section were collected using a laser capture microscope (Pix Cell II, Arcturus, Mt. View, CA). The captured tissue was extracted in Pico Pure extraction buffer (Arcturus) according to the manufacturer's directions and stored at -80C.

2.5 IMMUNOBLOT ANALYSIS

Material enriched in intestinal villi was prepared and analyzed by western blot as described elsewhere (Markovics et al., 2005). Appropriate dilutions of the following primary antibodies were used: T antigen-mouse monoclonal PAb416 (Harlow et al., 1981); anti-pRb-mouse IgG (G-3245) (BD-Pharmingen); 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*); anti-E2F3-rabbit IgG (C-18) (sc-878) (all from Santa Cruz Biotechnologies). Goat anti-mouse A2554 and goat anti-rabbit A0545 (Sigma) were used as secondary antibodies.

2.6 IMMUNOHISTOCHEMISTRY

Assessment of the proliferative status was obtained after mice were given an intraperitoneal injection of 5-bromo-2'deoxyuridine (BrdU; Sigma) (100 mg/kg body weight) two hours prior to their sacrifice. Sections were stained with monoclonal Rat BU1/75 (dilution, 1:2000) (Accurate Scientific, Westbury, NY). Antigen-antibody complexes were detected with ABC Elite kit Rat (Vector Labs, Burlingame, CA) and development of the peroxidase reaction was performed with DAB substrate (DAKO). Stained sections of murine intestines were photographed under a Nikon FXA microscope (original magnification x200).

Detection of T antigen proteins was performed in 5-μm-thick histological sections using appropriate dilutions of the anti-TAg antibody PAb419 (for TAg^{wt}) (Harlow et al., 1981) and PAb416 (for TAg^{N136}) as the primary antibody and biotinilated anti-mouse-Fab as the secondary

antibody, followed by streptavidin-peroxidase ARK kit plus development of the peroxidase reaction with DAB substrate (DAKO), according to the manufacturer's instructions.

2.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA).

Equal amounts of top and bottom strands of a oligonucleotide containing an E2F binding site (5' — ATTTAAGTTTCGCGCCCTTTCTCAA — 3') were annealed and 28pmol of the double-stranded oligo was then end-labeled with γ^{32}P-ATP as described, with modifications (Sullivan et al., 2000a). To purify the end-labeled oligo from unlabeled γ^{32}P-ATP, the oligo was subjected to centrifugation through the "Centri-spinTM-20" columns as per the manufacturer's protocol (Princeton Separations). 30µg of protein extracts with or without antibodies were diluted in gel shift buffer (10mM Hepes KOH, pH 7.9, 20mM KCI, 3mM MgCl2, 0.5mM EGTA, 0.5mM DTT, 0.5mM PMSF, 0.05%NP40), plus an additional 100mM DTT, 1.5mg/mL BSA, 50µg/mL salmon sperm DNA, 10% glycerol and incubated with 2µg of labeled probe for 30 minutes at room temperature. Antibodies used for supershift were pRb-mouse IgG (G-3245) (14001A)(BD-Pharmingen); anti-p130- rabbit IgG (C-20) (sc-317), anti-p107-rabbit IgG (C- 18) (sc-318) , anti-E2F 1-mouse IgG_{2a} (KH95) (sc-251) or anti-E2F1-rabbit IgG (C-20) (sc-l93), anti-E2F2-mouse IgG_1 (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-ill) (sc-I 699) (all from Santa Cruz Biotechnologies, Inc.). The p130 antibody shows residual cross reactivity with the p107 protein, therefore partially shifting p107 containing complexes. The samples were then subjected to gel electrophoresis through a non-denaturing polyacrylamide gel (4% bis-acrylamide, 0.25X TBE, 0.1% ammonium persulfate

(APS), 0.001% TEMED) in 0.25X TBE running buffer at 4^{O}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^{O}C.

2.8 RNA EXTRACTION

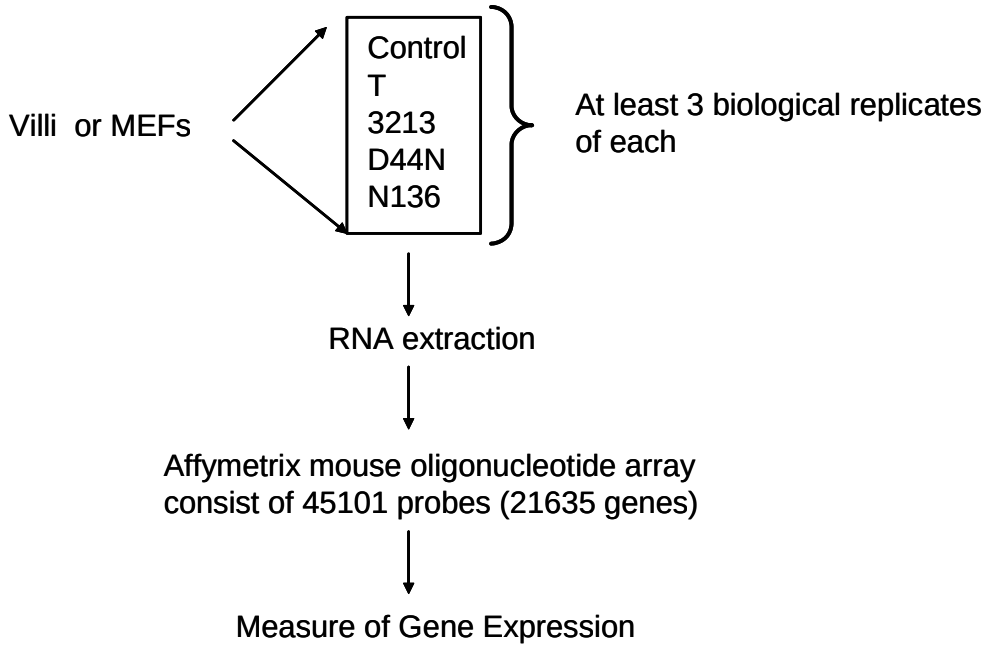
Wild-type MEFs and MEFs expressing TAg and TAg mutants were grown to confluence and fed with fresh medium. After 2 days, the cells were harvested and the cell pellet was washed with cold PBS/EDTA three times. Two independent wild-type MEF and three independent MEF T and MEF mutant RNA preparations were prepared. Total RNA from transfected or infected MEFs was isolated using the RNeasy kit according to the manufacture's protocol (Qiagen Inc.). Genomic DNA contaimination was elimainated by digesting the RNA with RNase-free DNase. The yield (at absorbance A260) and purity (A260/A280 ratio) of each RNA sample were determined by using an spectrophotometer (Eppendorf).

For microarray experiment, total RNA was isolated from LCM villi sample with the PicoPure kit (Arcturus) and amplified twice with the RiboAmp kit (Arcturus). Independent RNA preparations were made from three wild-type, D44N, and 3213 mice, four TAg mice and five N136 transgenic mice. All mice were between the ages of 2.4 to 5 months old.

For D44N project, whole intestine or intestinal fractions were collected, lysed and homogenized in buffer containing guanidine isothiocyanate, an RNase inhibitor, and the total RNA was extracted using the RNeasy kit (Qiagen).

2.9 MICROARRAYS

Total RNA was sent to the Genomics and Proteomics Core Laboratories (University of Pittsburgh) for hybridization to the Mouse 430 2.0 whole genome array (Affymetrix) containing 45101 probesets representing 21635 unique genes. CEL files for each array were converted into RMA expression values using BRB-Array Tools (Rich Simon, National Cancer Institute, http://linus.nci.nih.gov/BRB-ArrayTools.html). An average fold change ratio (Experimental/Control) and a one-sample T-test was calculated for each probe set.



2.10 REVERSE TRANSCRIPTION PCR (RT-PCR) ANALYSIS

cDNA synthesis from 1 ug of total RNA was performed using Superscript II Reverse Transcriptase (Invitrogen). PCR was performed with 1ul cDNA using GoTAq polymerase (Promega) for 25 cycles with specific primers for the different transcripts. Amplification with primers for the Adh5 transcript was used as a normalizing control. PCR reaction products were resolved through a 2% agarose gel in 1X TAE and stained with GelStar (Cambrex Bio Science). The following primers were used for different transcripts in this study:

Table 1: Primers used in this study

Gene	Forward or Reverse	Primer Sequence (5' to 3')	Product size (bp)	Annealing temp. (°C)	Extention time (sec)
Adh	F	TGCACCACCAACTGCTTAG	152	58	30
	R	GATGCAGGGATGATGTTC			
BRCA1	F	GCTTGACCCCCAAAGAAGTAATGAC	343	56	30
	R	GGCTCACAACAATAGACCTGTAGGC			
RRM2	F	TTGCCTGCCTGATGTTCAAGCACC	436	56.3	30
	R	GTGTAGCCAGTTGGTTGTTTTTGC			
DHFR	F	TGGTTTGGATAGTCGGAGGCAG	261	55	30
	R	GGGGAGCAGAGAACTTGAAAGC			
B-myb	F	CTCTCCAGCTCCAGGGTATC	350	58	30
	R	GCACTGCAGTCATCCCAGCA			
TS	F	TCAGTTCTATGTGGTGAATGGGG	235	55.5	30
	R	TGGGAAAGGTCTTGGTTCTCGC			
Cyclin E	F	GCAGAAGGTCTCAGGTTATC	608	55	45
	R	GTGGCCTCCTTAACTTCAAG			
Lrdd	F	GAGAGCAGGTTGCCCATACTTG	151	55.7	30
	R	GAGCCATCAGGTAGGTAGAGGAAAC			
p21	F	GAACTTTGACTTCGTCACGGAGAC	339	60.1	30
	R	CTTCAGGGTTTTCTCTTGCAGAAG			
NOXA	F	TTGTTTGGAGACAAGGGTCCC	189	52.8	30
	R	GACCATAAATACCCATTGGGCAAG			
Mdm2	F	TTGGAACCCCCAGGAAGAGTGAAC	238	55.4	30
	R	CCACACCCCTATGACAAAAAGC			
Cyclin G1	F	AGGAAAGGAATGTCCCACTGGC	188	54.4	30
	R	CCTCTCAAATGGCAAGGTGTCG			
Fas	F	GCCGAATGTCGCAGAACCTTAG	204	54.4	30
	R	CAGGAGTTGCCAATGTCAATACAG			
PERP	F	AGGGCTACAGAGGAGGAAAGTCAC	377	53.9	30
	R	CGGTCTACGCTGGTTCTAAGCATAC			
Pten	F	ATTTGGTCACCCTTTGAGTCCTC	231	52.9	30
	R	AGTTTGTCTTGTTGTTAGCCCACC			
Rprm	F	ATGTGTGTGCTCTCGCTCACTGTG	131	55.6	30
	R	ACCACTGCCTCCACCTCTTTAGAC			
GD15	F	CAGGCAACTCTTGAAGACTTGGG	323	60.6	30
	R	AGCATCCTTGAAGGGGAGTGTAGG			
LIF	F	GGACCAGAGAAAAAGGGATGGG	140	53.6	30
	R	AAATAGACAAGAGTGGGGGGAGGG			
BMP8a	F	ATGCTAACTGAGAGGTCTGGGGTC	162	55.6	30
	R	CAACTGCCACAATGAATGGTGC			
PDGF-α	F	AAGTGGAGTATGTCAGGAAGAAGCC	191	57.3	30
	R	TGTTCAGGAATGTCACACGCC			
Areg	F	CGACAAGAAAACGGGACTGTGC	153	55.3	30
	R	TGACAACTGGGCATCTGGAACC			
Proliferin	F	AAGCCCTCTGGACGATCTAGTGAG	204	54.3	30
	R	CGAGCATCTTCATTGTCAGATTGC			

Gene	Forward or Reverse	Primer Sequence (5' to 3')	Product size (bp)	Annealing temp. (°C)	Extention time (sec)
Ifi44	F	GAGAGAACAGGGAATGAAGAAGGC	134	52.4	30
	R	CCAACAGAATTGCGATTGGTCC			
Ifi27	F	CCATAGCAGCCAAGATGATGTCTG	121	55	30
	R	GCATTTGTTGATGTGGAGAGTCC			
Oas2	F	AAAACCAACCGCTCCCAGTTCGTC	488	57.1	30
	R	GCAATGTCAAAGTCATCTGTGCC			
GTPase2	F	CTTCCACCTGCTTGTTCTTTGG	266	55.4	30
	R	TCACAGTTTCCTCAGTGCTGGG			
Rsad2	F	CAATCACACCCAGCAGCAGTTAG	209	54.4	30
	R	AGCGATGCCTCAGAACACAGTG			
Mx1	F	CAGCACCTGAAAGCCTACTACCAG	135	53.6	30
	R	GGTGTCCTGTAAAAGCTGAAGCATC			
Oasl2	F	TTACAGAACAGCCAGAGCTATACGG	548	56.1	40
	R	CAAGGGAGATAGATTTACGTCCACG			
IRF7	F	ACACCATCTACCTGGGTTTTG	243	54	60
	R	TTGGGATTCTGAGTCAAGGC			
H60	F	TGCTGACAGATAGGTGTTGGCG	278	53.8	30
	R	CCCTCTGGAATTTCTTCTCACCC			
Cyclin D1	F	CCAACAACTTCCTCTCCTGCTACC	596	57.9	45
	R	TCCTTCTCAAGACTTCCCCTGTG			
IGF-BP3	F	TCTTGGGGTCCTTCTCAAACTCTC	190	52.2	30
	R	CAGACACAGGCTCCTTTCCTTG			
Elastin	F	TCCATCCGTCCATCTTGACTGC	148	58.3	30
	R	AACCAGCCCACACAACCCTTTG			
Mafb	F	CAGGCTTTGCGTCCTAAGAAGTC	157	56.1	30
	R	TGGCTGTTTGGCTCAATGGG			
Dner	F	CAAGGATGGGCTTTTTTGACAG	553	53.3	40
	R	ACAGAAGGGACTGGCACCTCTTAC			
E2F1	F	TTGCCTGTCTGTTTGCTGAGCC	420	60	30
	R	CGGAGATTTTCACACCTTTCCCTG			
E2F2	F	TTCGCTTTACACGCAGACGG	289	60	30
	R	AATGAACTTCTTGGTCAGGAGCC			
E2F3a	F	AGCCTCTACACCACGCCACAAG	309	60	30
	R	ATCCAGGACCCCATCAGGAGAC			
RB	F	TCACACAACCCAGCAGTGCG	270	56	30
	R	CTATCCGAGCGCTCCTGTTC			
p107	F	TAGATATCTTTCAAAATCCATATGAAGAGC	2250	56	120
	R	ATTGTACACCACGAC TCC			
p130	F	TTGGGACTCTGTCTCGGTGTCTAAG	499	57	30
	R	AATGCGTCATGCTCCAGAACACCAG			

3.0 ENTEROCYTE PROLIFERATION AND INTESTINAL HYPERPLASIA INDUCED BY SIMIAN VIRUS 40 T ANTIGEN REQUIRES A FUNCTIONAL J DOMAIN

3.1 INTRODUCTION

Observations in cell culture strongly suggest that the J domain of TAg is required to overcome the p130 and/or p107 mediated repression of E2F-dependent transcription, and thus for the transformation induced by TAg (Zalvide and DeCaprio, 1995). To determine the J domain requirements in a normal tissue model we have generated transgenic mice using the intestinal fatty acid binding protein promoter to direct expression of the T antigen mutant D44N (TAg^{D44N}) to villus enterocytes. Our results indicate that an intact J domain is required to induce intestinal proliferation mediated by TAg, and suggest that different tissues require different signals to undergo tumorigenic proliferation.

3.2 RESULTS

3.2.1 Transgenic mice expressing the J-domain mutant D44N in enterocytes show normal intestinal phenotype.

Mice expressing the wild-type T antigen (TAg^{wt}) in the intestinal enterocytes develop hyperplasia by 6 weeks after birth and progress to dysplasia with age (Hauft et al., 1992; Kim et al., 1994; Kim et al., 1993; Markovics et al., 2005). Additionally, these mice show a significant increase in the size of intestinal crypts (Markovics et al., 2005). However, mice expressing a single amino acid substitution J-domain mutant TAg (TAg^{D44N}) in enterocytes posses normal intestines and do not show any signs of hyperplasia (Fig.18A).

Expression of TAg^{wt} under the control of the IFABP promoter was previously shown to be restricted to villus enterocytes (Kim et al., 1993). We confirmed the expression patterns of TAg in TAg^{wt} and TAg^{D44N} mice by immunostaining. Paraffin sections stained with anti-TAg antibody revealed that expression of either TAg^{wt} or TAg^{D44N} was restricted to villus enterocytes (Fig.18A). Western blot analysis confirmed that similar amounts of wild-type and mutant proteins were expressed in the intestines of the transgenic mice (Fig.18B). To assess the proliferative status of transgenic intestines, histological sections were stained with anti-BrdU. In agreement with the morphological phenotype observed, we found numerous BrdU positive cells in villus enterocytes of mice expressing TAg^{wt} (Fig.19B), while none were present in the mice expressing TAg^{D44N} (Fig.19C). No signs of ectopic proliferation were found in mice expressing TAg^{D44N} and proliferation was restricted to the crypts as in non-transgenic mice (Fig. 19, compare A and C). We have analyzed three independent TAg^{D44N} transgenic lines and observed the same phenotype described above.

As previously reported, TAg^{wt} mice show a significant increase in the total length of the small intestine, at some times doubling the length of the control littermates (Markovics et al., 2005). In contrast, the intestines of TAg^{D44N} mice do not show a significant difference in intestinal length in comparison to nontransgenic littermates (Fig. 20), either in male or female specimens.

Figure 18: Expression of both TAg^{wt} and TAg^{D44N} is restricted to villus enterocytes.

(A) Intestinal tissues were embedded in paraffin and 5um sections stained with an anti-TAg antibody. Brown color reflects the presence of TAg in nuclei. **(B)** Expression levels of TAg in TAg^{wt} and TAg^{D44N} villi samples. Protein extracts from villi of non-transgenic, TAg^{wt} and TAg^{D44N} transgenic mice were subjected to immunoblots for TAg. Protein levels for ß tubulin were used as loading controls.

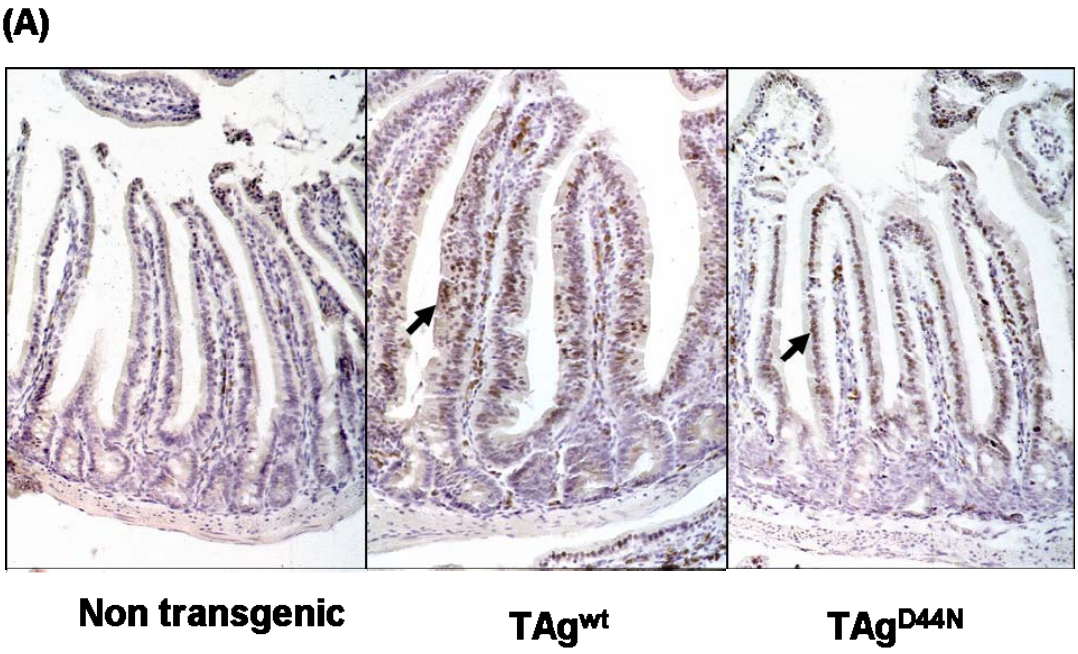


Figure 19: Ectopic proliferation of enterocytes requires a functional J domain.

Proliferation in TAg^{D44N} mice is restricted to the crypts as in non-transgenic mice. Intestinal sections stained with anti-BrdU and show numerous BrdU positive cells in villus enterocytes of mice expressing TAg^{wt}, but none present in the mice expressing TAg^{D44N} or non-transgenic.

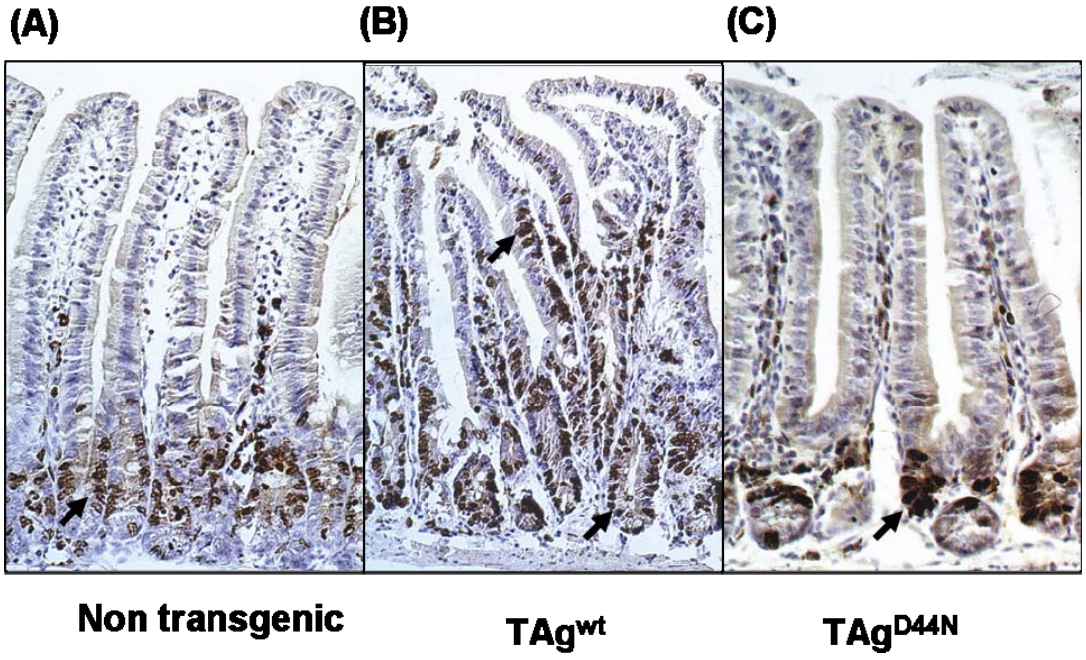
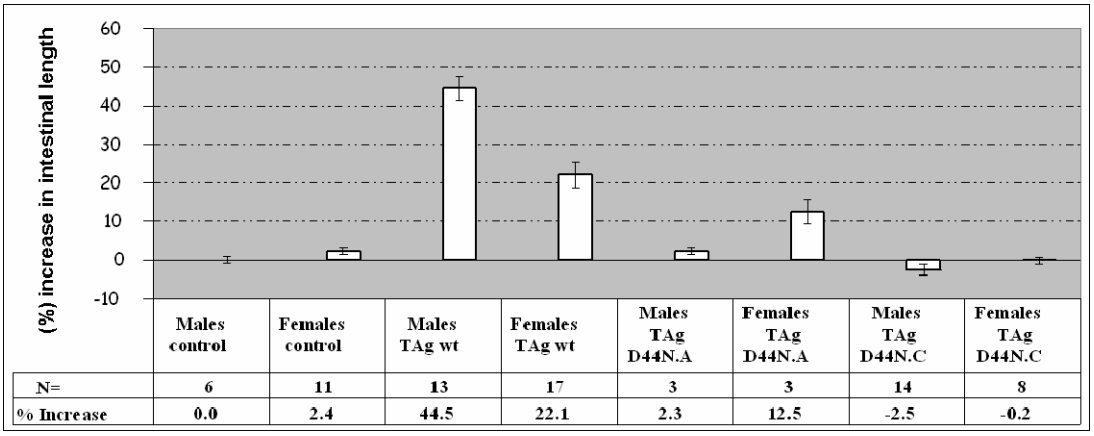


Figure 20: Expression of TAg^{D44N} does not increase intestinal length in mice.

The total length of the small intestine in adult male or female mice was measured and the average length of each group (control, TAg^{wt} and TAg^{D44N}) is represented. The error bars indicate the standard deviation in the group. Intestines expressing TAg^{D44N} show similar length to those in control mice.



	Males control	Females control	Males TAg wt	Females TAg wt	Males TAg D44N.A	Females TAg D44N.A	Males TAg D44N.C	Females TAg D44N.C
N=	6	11	13	17	3	3	14	8
% Increase	0.0	2.4	44.5	22.1	2.3	12.5	-2.5	-0.2

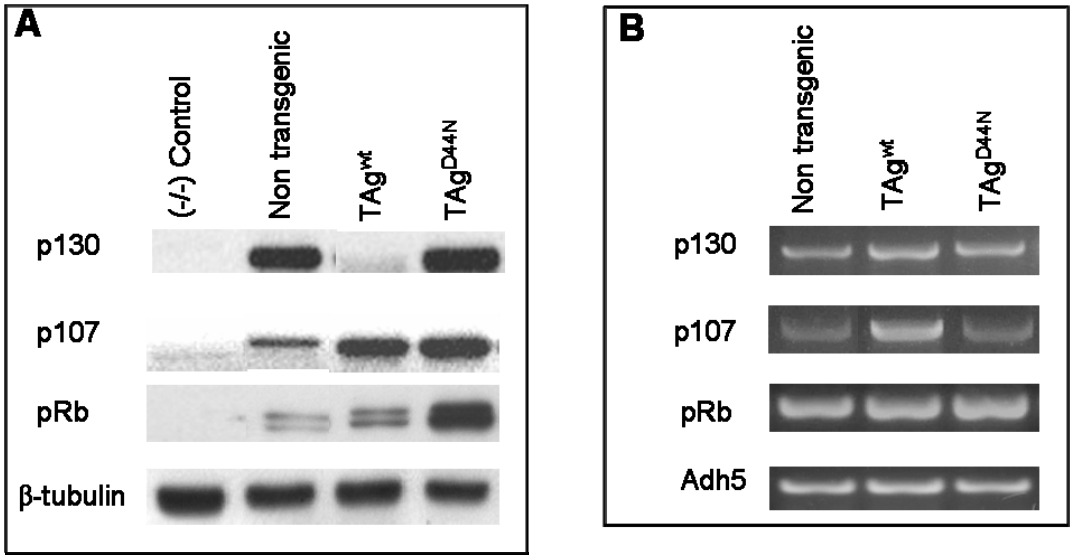
3.2.2 Expression of TAg^{D44N} results in increased pRb and p107 protein levels in villus enterocytes.

To assess the steady state levels of Rb family proteins (p130, p107 and pRb) we used intestinal extracts enriched for villi cells from TAg^{wt} and TAg^{D44N} mice or from their nontransgenic littermates (Fig. 21A). TAg^{D44N} mice were able to upregulate p107 protein expression to the same degree as mice expressing TAg^{wt}. As expected, extracts from mice expressing TAg^{wt} show reduced p130 protein levels in comparison to nontransgenic littermates. However, in agreement with observations in cell culture experiments (Stubdal et al., 1997), TAg^{D44N} mice were unable trigger a reduction in the steady state levels of p130. Interestingly, a profound upregulation of pRb protein levels was found in TAg^{D44N} mice compared to non-transgenic and TAg^{wt} mice.

To determine whether the change in protein levels were paralleled by a change in the steady state levels of the corresponding messenger, we performed semiquantitative RT-PCR analysis (Fig.21B). As previously reported, TAg^{wt} mice were able to upregulate p107 but exhibited no change in the pRb and p130 mRNA levels in comparison to nontransgenic littermates (Saenz-Robles et al., 2007). Though TAg^{D44N} mice show increased levels of p107 and pRb proteins, we did not find any changes in their mRNA levels and conclude that the increased levels of these proteins probably occurs posttranscriptionally.

Figure 21: TAg^{D44N} expression in villus enterocytes alters the pRb protein levels but does not affect their transcript levels.

(A) Steady-state protein levels of Rb-family members in non-transgenic, TAg^{wt} and TAg^{D44N} villi samples. Whole cell extracts from villi of non-transgenic, TAg^{wt} and TAg^{D44N} transgenic mice as well as knock-out (-/-) intestinal controls, were subjected to immunoblots for p130, p107 and pRb. Protein levels for ß tubulin were used as loading controls. **(B)** Steady-state transcript levels of Rb-family members in non-transgenic, TAg^{wt} and TAg^{D44N} villi samples. cDNAs were reverse-transcribed from equal amounts of total RNA extracts and subjected to PCR using specific primers. Transcript levels of Alcohal dehydrogenase 5 (Adh5) were used as loading controls.



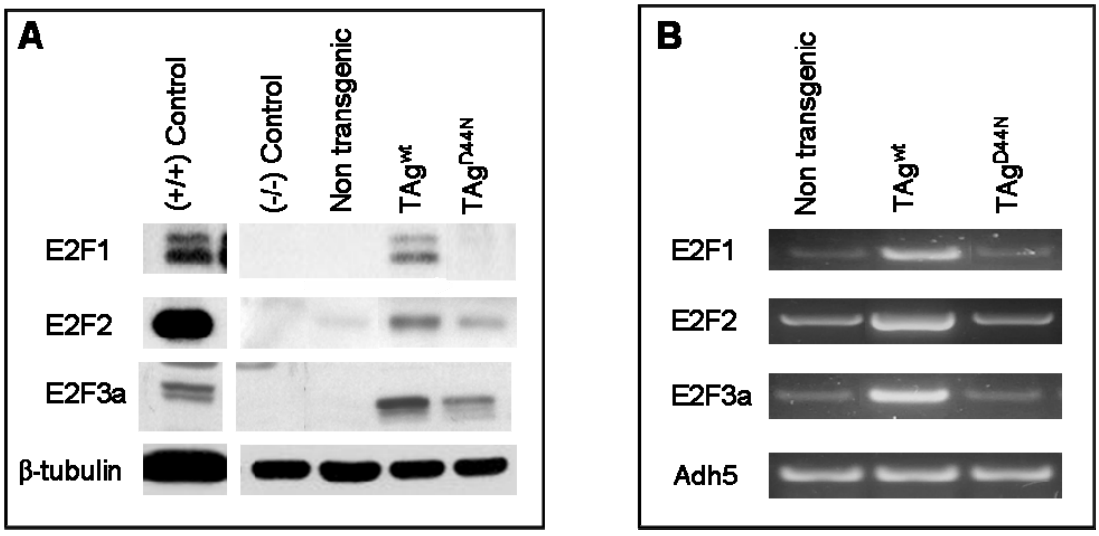
3.2.3 TAg^{D44N} affect activator E2F protein levels differentially.

Extracts from TAg^{wt} mice show upregulation of all activator E2Fs- E2F1, E2F2, E2F3a protein levels (Saenz-Robles et al., 2007), which in turn activate S-phase genes and induce cell proliferation. To determine the effect on steady state levels of activator E2Fs in TAg^{D44N} mice we performed western blot analysis using intestinal extracts enriched for villi cells (Fig.22A). We found a modest but consistent upregulation of E2F2 and E2F3a in TAg^{D44N} intestines in comparison to non-transgenic intestines. In contrast, no change in E2F1 protein levels in TAg^{D44N} mice compared to non-transgenic was observed.

To determine whether these changes were reflected in the messenger for the E2Fs, we performed semiquantitative RT-PCR (Fig.22B). As expected, TAg^{wt} mice were able to upregulate E2F1, E2F2 and E2F3a mRNA levels. However, no change in mRNA levels was observed in TAg^{D44N} intestines. Thus, the increased protein levels of E2F2 and E2F3a probably occurs posttranscriptionally.

Figure 22: Expression of TAg^{D44N} in villus enterocytes affects activator E2F protein levels differentially without altering their transcript levels.

(A) Protein extracts from villi of non-transgenic, TAg^{wt} and TAg^{D44N} mice were subjected to immunoblot analysis. Nuclear extract from S phase synchronized normal MEFs was used as a positive control for E2F1. Whole cell extracts from 293 cells over expressing E2F2 were used as positive control for E2F2 and whole cell extracts from mammary gland tumor were used as positive control for E2F3a. **(B)** RNA from villi of non-transgenic, TAg^{wt} and TAg^{D44N} mice was subjected to RT-PCR analysis using specific primers. Transcript levels of Adh5 were used as loading controls.

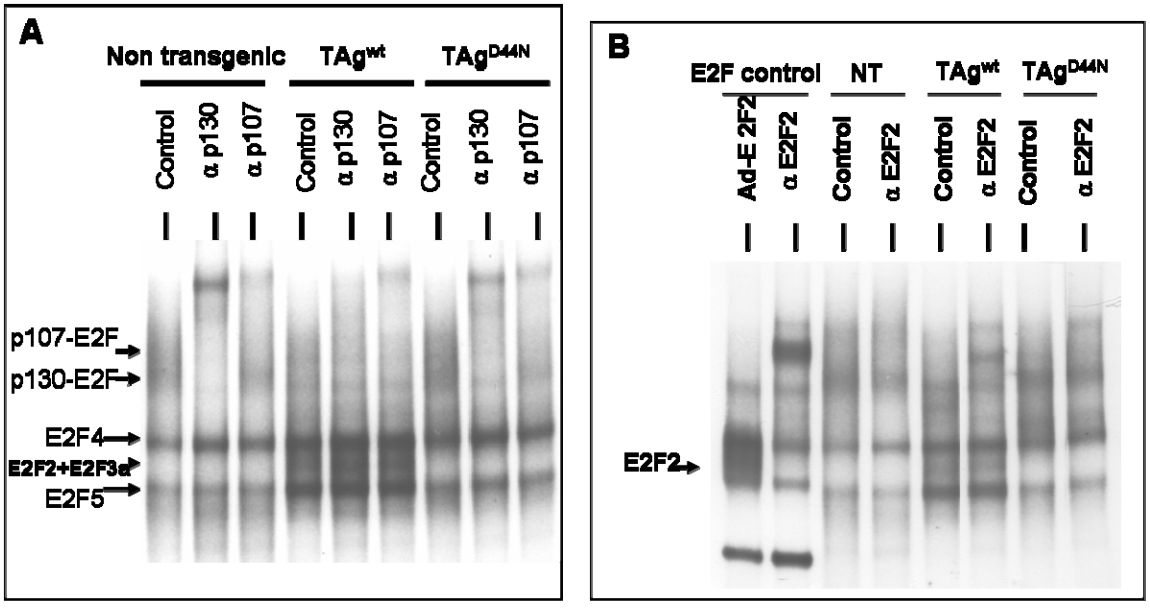


3.2.4 TAg^{D44N} is unable to disrupt p130/E2F complexes.

Rb-proteins exert their effects in part by binding to and regulating the E2F family of transcription factors. To examine the effects of TAg^{D44N} expression on Rb-E2F DNA binding complexes we performed a series of EMSA experiments. A radiolabeled oligonucleotide probe with consensus E2F binding sites was incubated with extracts from nontransgenic, TAg^{wt} and TAg^{D44N} growth-arrested enterocytes (villi) and the products were resolved on a nondenaturing gel. Antibody supershift experiments were performed to determine which Rb-protein constituted those Rb-E2F DNA binding complexes. We did not observe any differences in DNA binding complexes between nontransgenic and TAg^{D44N}, a fact consistent with the normal morphological phenotype observed (Fig.23A). We found three major bands in both nontransgenic and TAg^{D44N} mice. The slowest migrating band represents a p130-E2F complex. Two faster migrating bands do not contain Rb proteins and represent E2F4-DNA and E2F5-DNA complexes. Additionally, we found a minor slow migrating band in nontransgenic, TAg^{wt} and TAg^{D44N} mice which represents a p107-E2F complex. In contrast, no p130-E2F complexes were detected in enterocytes expressing TAg^{wt}. As reported previously, TAg^{wt} induces the formation of Rb-free E2F2-DNA and E2F3a-DNA binding complexes (Saenz-Robles et al., 2007) which are absent in both nontransgenic and TAg^{D44N} mice. Furthermore, supershifts with antibodies against pRb, E2F2 and E2F3a failed to detect any pRb-E2F2 (Fig 23B) or pRb-E2F3a DNA binding complexes in TAg^{D44N} intestines. Thus, despite the modest upregulation in E2F2 and E2F3a protein levels in TAg^{D44N} mice, there is no evidence that the DNA binding activity of these transcription factors is increased.

Figure 23: Analysis of RB-E2F DNA binding complexes in non-transgenic, TAg^{wt} and TAg^{D44N} mice.

(A) An intact J domain is required to disrupt p130-E2F DNA binding complexes in villus enterocytes. Gel shift analysis with a radiolabeled nucleotide probe containing a consensus E2F binding site was performed on protein extracts isolated from villi of non-transgenic, TAg^{wt} and TAg^{D44N} mice. The composition of each migrating complex was determined by supershift analysis with specific antibodies. **(B)** E2F2 DNA binding complexes are not induced in TAg^{D44N} mice.

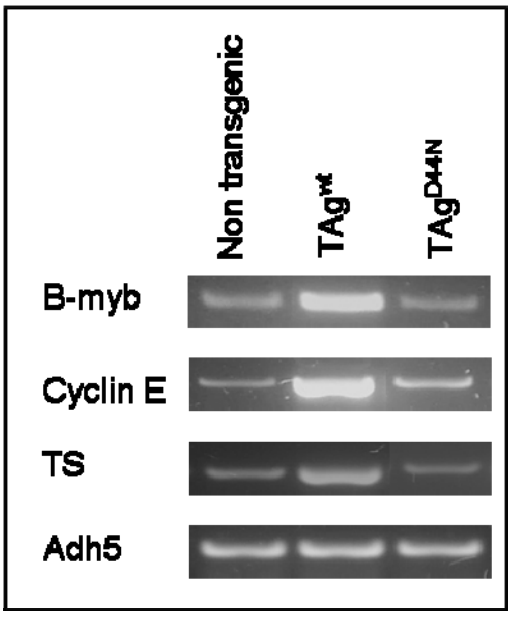


3.2.5 TAg^{D44N} is defective for the regulation of E2F target genes.

To further confirm that there is no ectopic E2F activity present in TAg^{D44N} mice, we analyzed the expression of some of the genes regulated by E2Fs (DeGregori, 2002; Ishida et al., 2001; Stevaux and Dyson, 2002). We used semiquantitative RT-PCR (Fig.24) and in some cases (cyclin E1 and thymidylate synthase, data not shown) real time PCR to assess the mRNA levels of b-myb, cyclin E1 and thymidylate synthase. In contrast to TAg^{wt} mice, samples from TAg^{D44N} intestines did not show any upregulation of these genes (Fig.24). These results agree with our previous EMSA experiments suggesting that no ectopic E2F activity is present in TAg^{D44N} mice.

Figure 24: TAg^{D44N} is unable to upregulate E2F target genes in intestinal enterocytes.

Steady-state transcript levels of E2F target genes were determined in non-transgenic, TAg^{wt} and TAg^{D44N} villi samples. cDNAs were reverse-transcribed from equal amounts of total RNA extracts and subjected to PCR using specific primers for b-myb, cyclin E and thymidylate synthase (TS). Transcript levels of Adh5 were used as loading controls.



3.2.6 TAg^{D44N} is unable to induce hyperplasia in p130 (-/-) background.

Unlike TAg^{wt}, the expression of TAg^{D44N} in enterocytes did not downregulate p130 protein levels and was unable to dissociate p130-E2F complexes. To determine if inactivation of p130 by TAg is sufficient to induce hyperplasia, we crossed TAg^{D44N} mice into the p130 (-/-) background. The proliferative status of these transgenic intestines was assessed in intestinal sections stained with anti-BrdU. No signs of ectopic proliferation were found in TAg^{D44N}; p130 (-/-) mice, and proliferation was restricted to the crypts as found in p130 (-/-) and TAg^{D44N} mice (Fig.25). To determine the steady state levels of Rb family members and activator E2Fs in TAg^{D44N}; p130 (-/-) mice, we performed Western blot analysis using intestinal extracts. We found that the absence of p130 had no effect on the ability of TAg^{D44N} to regulate the protein levels of Rb and E2F family members (Fig.26A and 26B).

Figure 25: Loss of p130 alone in not sufficient to induce ectopic proliferation of enterocytes in TAg^{D44N} mice.

Proliferation in TAg^{D44N}; p130 (-/-) mice is restricted to the crypts as in TAg^{D44N} mice. Intestinal sections stained with anti-BrdU and show numerous BrdU positive cells (indicated by arrows) in crypts of p130 (-/-), TAg^{D44N} and TAg^{D44N}; p130 (-/-) mice.

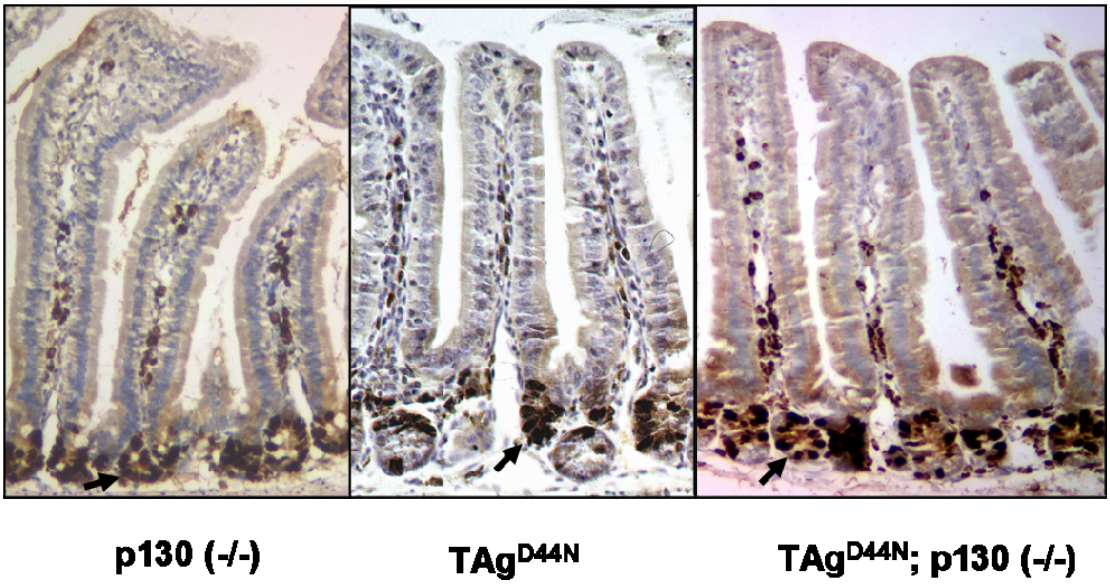
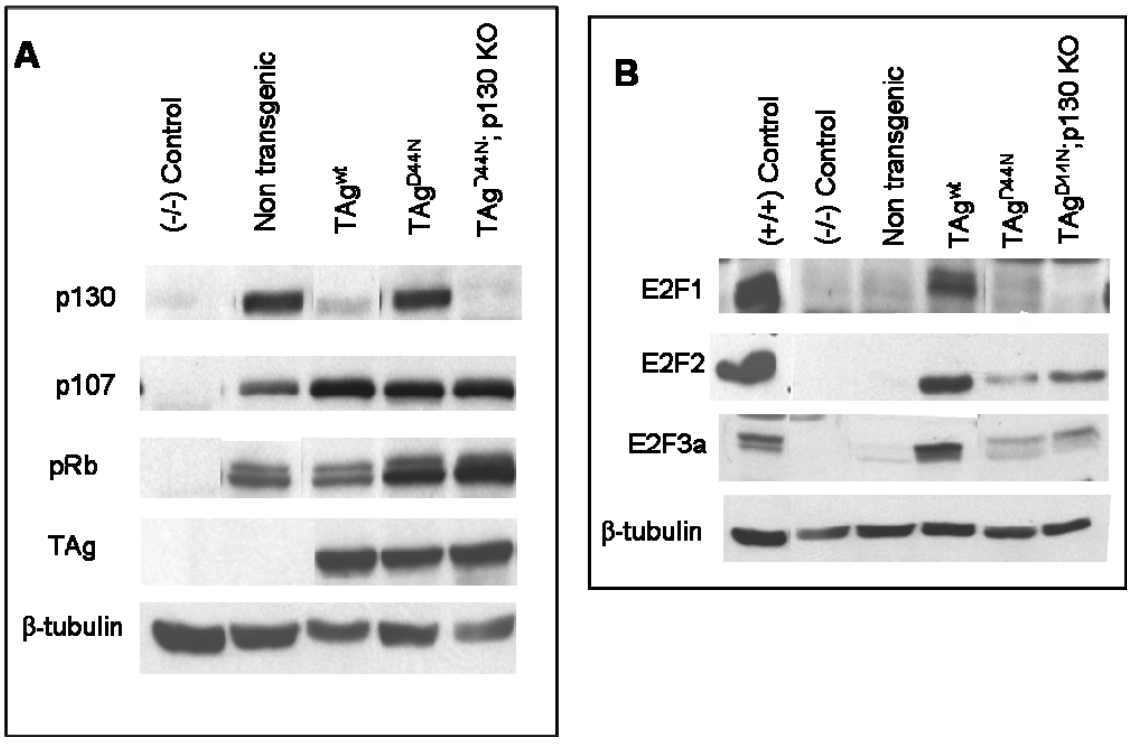


Figure 26: Loss of p130 does not affect the steady state levels of pRb and E2F family members in TAg^{D44N}; p130 (-/-) mice.

Whole cell extracts from middle intestines of non-transgenic, TAg^{wt}, TAg^{D44N} and TAg^{D44N}; p130 (-/-) transgenic mice as well as knock-out (-/-) intestinal controls, were subjected to immunoblots for **(A)** p130, p107, pRb and **(B)** E2F1, E2F2 and E2F3a. Protein levels for ß tubulin was used as loading control.



3.2.7 Conclusions

We found that mice expressing the J domain mutant have normal intestines without loss of growth control. Unlike mice expressing wild-type TAg, mice expressing D44N do not reduce the protein levels of p130 and are also unable to dissociate p130-E2F DNA binding complexes. We found increased protein levels of pRb and p107 in TAg^{D44N} mice. Morover, we observed a modest but consistant upregulation of E2F2 and E2F3a protein levels. However we did not find regulation of E2F target genes in TAg^{D44N} mice. Furthermore, mice expressing D44N in a null p130 background are still unable to develop hyperplasia. These studies demonstrate that the ectopic proliferation of enterocytes by T antigen requires a functional J domain and suggest that the J domain is necessary to inactivate all three pRb family members.

4.0 AMINO-TERMINAL MUTANT OF T ANTIGEN INDUCES ENTEROCYTE PROLIFERATION AND INTESTINAL HYPERPLASIA/DYSPLASIA

4.1 INTRODUCTION

TAg carries multiple biochemical activities that act by themselves or in a concerted manner to control various aspects of viral infection and cellular transformation. Full length TAg expression in the mouse intestine results in enterocyte proliferation, hyperplasia and dysplasia. The present study is aimed to determine whether the inactivation of Rb-proteins by TAg is sufficient to induce enterocyte proliferation or if additional activity/ies mapping to the C-terminus of TAg is/are required for progression to dysplasia. To test this we have generated transgenic mice using the I-FABP promoter to drive expression of the T antigen mutant N136 (TAg^{N136}) to villus enterocytes. This mutant produces a form of TAg (amino acids 1 to 136) that includes the J domain and LXCXE motif (required for Rb-family inactivation) but lacks the p53-binding region. Our results indicated that the inactivation of Rb-proteins is sufficient to induce hyperplasia, however progression to dysplasia is significantly delayed. We further discuss plausible mechanisms for this delayed progression to dysplasia observed in TAg^{N136} mice.

4.2 RESULTS

4.2.1 Transgenic mice expressing N136 in enterocytes develop hyperplasia and show signs of dysplasia.

Intestinal hyperplasia is characterized by hyperproliferation of the villus enterocytes resulting in longer and thicker villi with more densely packed cells. Dysplasia is a much more severe phenotype characterized by gross morphological changes in the tissue architecture such as branching of the villi and multi-layering of cells in the epithelium. Mice expressing the wild-type T antigen (TAg^{wt}) in the intestinal enterocytes develop hyperplasia by 6 weeks after birth and progress to dysplasia with age (6 to 9 months) (Hauft et al., 1992; Kim et al., 1994; Markovics et al., 2005). Additionally, these mice show a significant increase in the size of intestinal crypts (Markovics et al., 2005). Mice expressing an amino-terminal fragment N136 (TAg^{N136}) in enterocytes develop hyperplasia as well but not as early as TAg^{wt} (4 to 6 months). Furthermore, they show characteristics of dysplasia later in life (10 to 12 months) but full dysplasia doesn't take place except in occasions where the founder expresses very high levels of N136 protein (Table 2).

Table 2: Phenotypic evaluation of transgenic mice expressing N136.

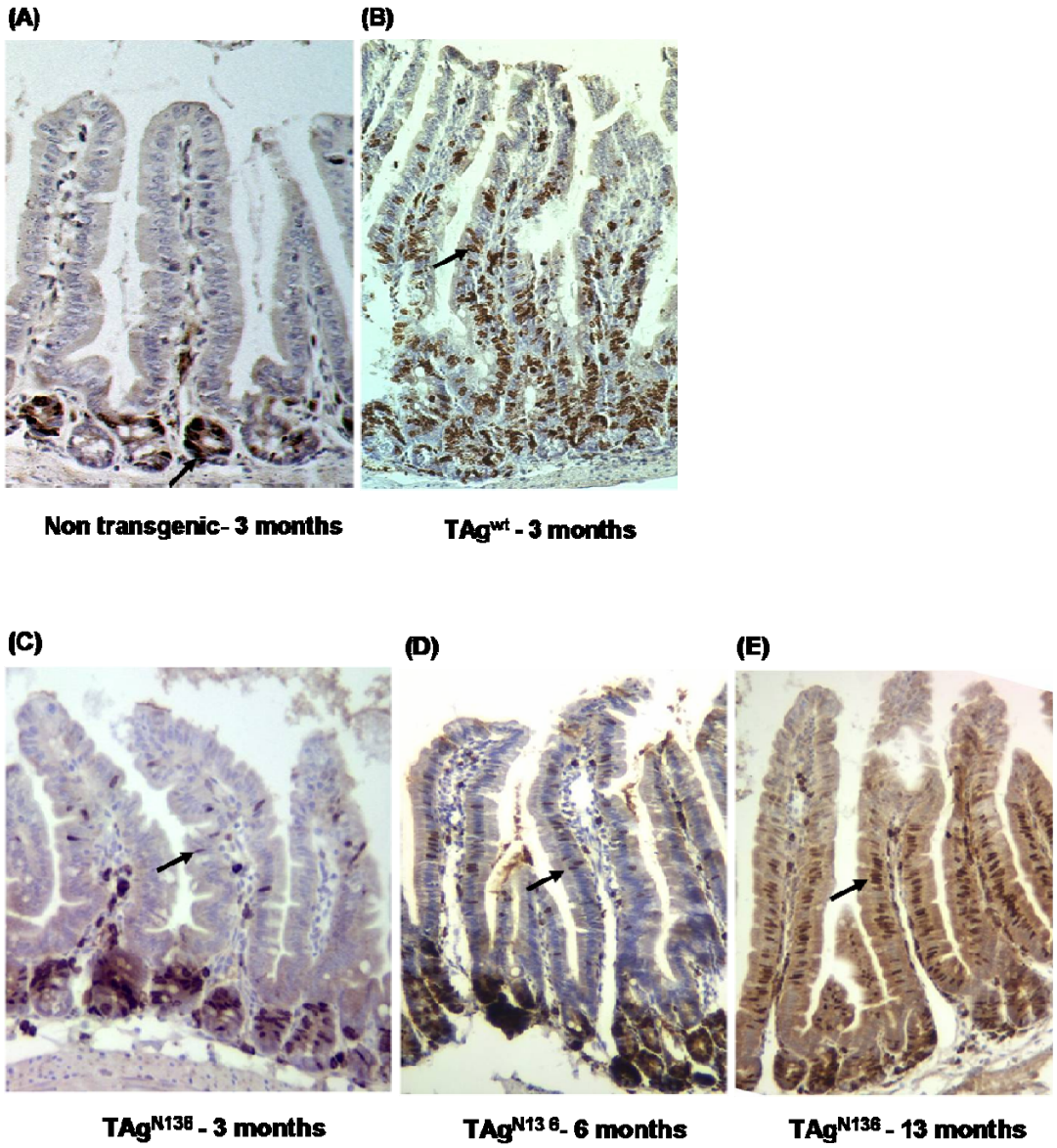
HYP= Hyperplasia, DYS-BRANCH= showing signs of dysplasia like branching of villi, N= number of mice evaluated

Age range	N= (tested)	Phenotype
0-3 months	11	36% HYP, 36% mild HYP; 27% normal
3-6 months	10	67% HYP; 33% mild HYP
6-9 months	13	43% HYP-DYS-BRANCH; 57% HYP
9-12 months	11	45% HYP-DYS-BRANCH; 36% HYP; 18% normal
12-18 months	9	33% HYP-DYS-BRANCH; 44% HYP; 22% normal

To assess the proliferative status of transgenic intestines, histological sections were stained with anti-BrdU. We found numerous BrdU positive cells in villus enterocytes of mice expressing either TAg^{wt} or TAg^{N136} (Fig.27). To determine whether cells were progressing through the cell cycle at early ages, TAg^{N136} mice at various ages were analyzed (Fig.27C-E). Our results show an increase in BrdU incorporation that correlates with age. Three month old TAg^{N136} mice have very few BrdU positive cells in comparison to 6 month old mice. Similarly, 6 month old TAg^{N136} mice have less BrdU positive cells in comparison to 13 month old.

Figure 27: N136 induces ectopic proliferation in villus enterocytes.

(A, B) TAg^{wt} mice show ectopic proliferation of enterocytes while proliferation is restricted to the crypts in non-transgenic mice. **(C-E)** Increased ectopic proliferation in TAg^{N136} mice correlates with age. Intestinal sections were stained with anti-BrdU and show numerous BrdU positive cells (indicated by arrow) in villus enterocytes of mice expressing TAg^{wt} and $TAg^{N136.}$



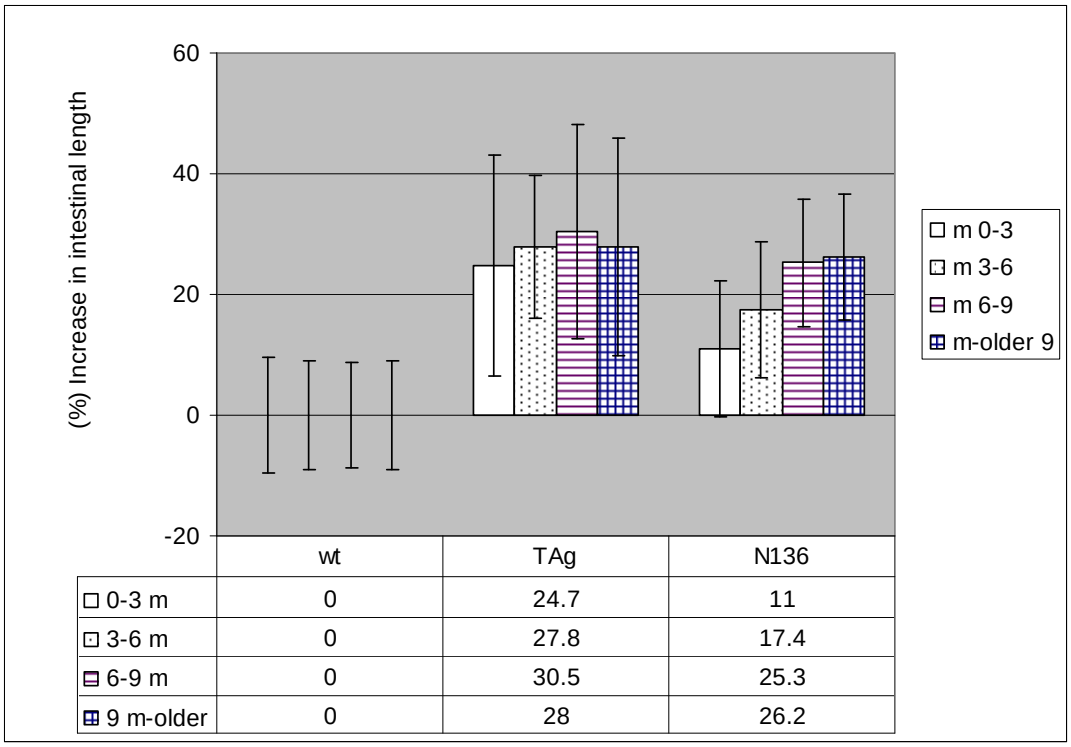
As previously reported, TAg^{wt} mice showed a significant increase in the total length of their small intestines (Markovics et al., 2005). The average intestinal length of TAg^{wt} mice ranges from 55 to 60 cm. The intestines of TAg^{N136} mice also showed a significant increase in intestinal length in comparison to nontransgenic littermates (Fig.28), either in male or female specimens. For instance, 3 to 6 month old TAg^{wt} mice show a 28% and TAg^{N136} mice show a 20% increase in intestinal length in comparison to nontransgenic mice. A total of 68 TAg^{N136} mice with different age groups (such as 0-3 months, 3-6 months, 6-9 months, older than 9 months) were included for this analysis.

4.2.2 Expression levels of N136 do not change significantly through development

To determine if there is any relation between TAg^{N136} protein levels and the age of mice, we examined the levels of TAg^{N136} at different ages (Fig.29). We did not find any-age related increase in TAg^{N136} protein levels. However, we observed a direct relationship between the presence of oncoprotein and tumorigenic phenotype. Furthermore, to determine if levels of protein is responsible for the delayed phenotype in TAg^{N136} mice, we examined two independent N136 transgenic lines expressing similar levels of protein like wild-type TAg. These N136 mice were also unable to induce dysplasia as early as TAg mice.

Figure 28: Expression of N136 increases the intestinal length in mice.

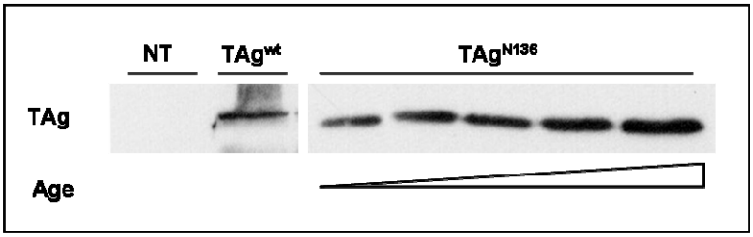
The total length of the small intestine was measured and the average length of each group (control, TAg^{wt} and TAg^{N136}) is represented. The error bars indicate the standard deviation in the group.



	wt	TAg	N136
0-3 m	0	24.7	11
3-6 m	0	27.8	17.4
6-9 m	0	30.5	25.3
9 m-older	0	28	26.2

Figure 29: Expression levels of N136 protein through development.

Protein extracts from the middle intestines of non-transgenic (NT), TAg^{wt} and TAg^{N136} transgenic mice were subjected to immunoblots for TAg. TAg^{N136} mice of various ages (4, 9, 11, 12 and 18 month) were used.

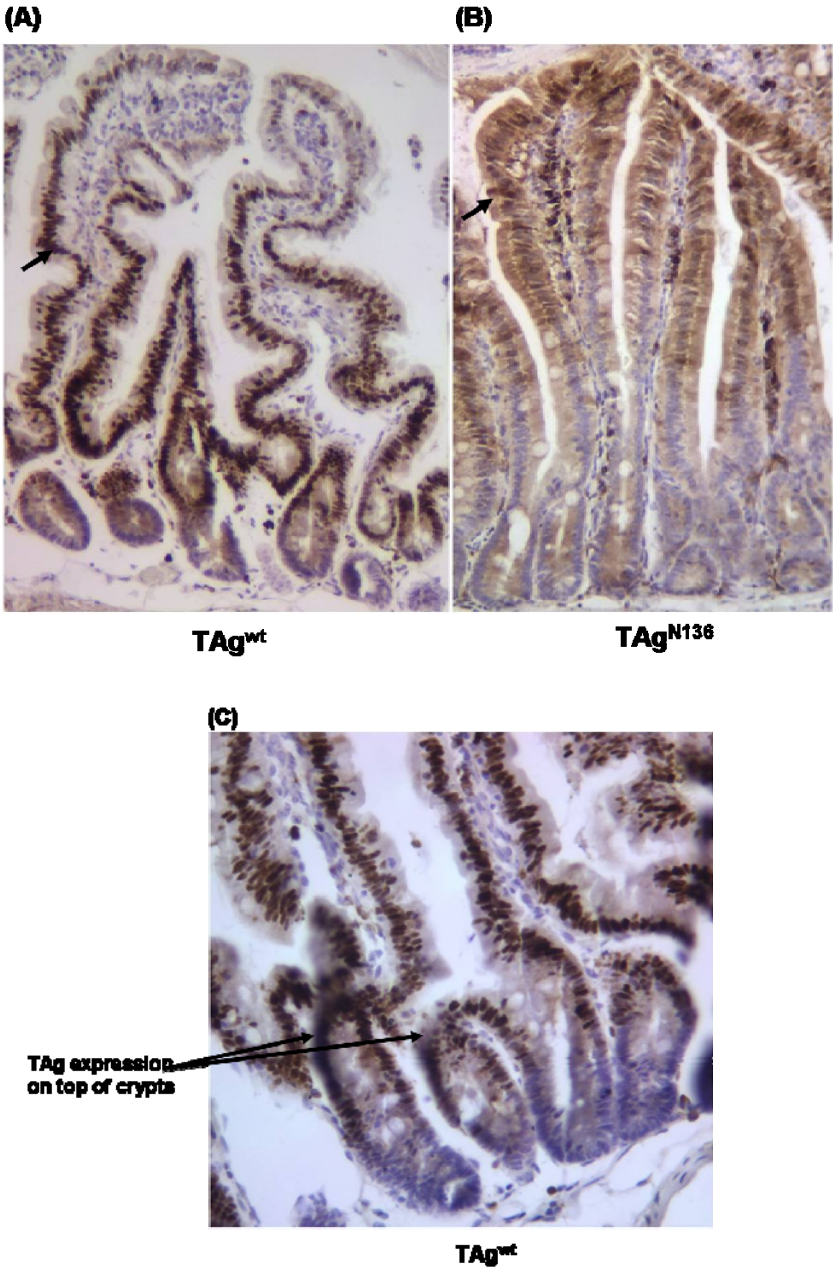


4.2.3 N136 expression is restricted to villus enterocytes

Expression of TAg^{wt} under the control of the IFABP promoter was previously shown to be restricted to villus enterocytes (Kim et al., 1993). We confirmed the expression patterns of TAg in TAg^{wt} and TAg^{N136} mice by immunostaining. Paraffin sections stained with anti-TAg antibody revealed significant differences in expression patterns between TAg^{wt} and TAg^{N136} villus enterocytes (Fig.30A). TAg^{wt} mice used in this study show TAg expression not only in villi but also in the villi/crypt junction as well as on top of the crypts (Fig.30B). However, TAg^{N136} expression is restricted to top $3/4^{th}$ of the villi and was not observed in the villi/crypt junction nor on the top of crypts.

Figure 30: Expression patterns of TAg and N136 in villus enterocytes.

Intestinal tissues were embedded in paraffin and 5um sections stained with anti-TAg antibodies. Brown color reflects the presence of TAg.**(A)** TAg^{wt} mice expressing TAg protein in nucleus of villus enterocytes (bottom ¾ th) and extends to the top of the crypts **(B)** TAg^{N136} mice expressing N136 protein in nucleus and cytoplasm of villus enterocytes (top ¾ th). **(C)** Higher magnification showing expression of TAg on the top of the crypts.



4.2.4 Conclusions

We found that mice expressing N136 develop hyperplasia and show signs of dysplasia after a prolonged latency period. Like TAg^{wt}, mice expressing N136 show a significant increase in intestinal length in comarision to nontrasngenic littermates. Furthermore, we found increase BrdU incorparoation with the age of TAg^{N136} mice, however we did not find corresponding increase in N136 protein levels. Interestingly, we observed significant differences in expression patterns between TAg^{wt} and TAg^{N136} villus enterocytes. TAg^{wt} mice show concentrated nuclear expression of TAg not only in villi but also in the villi/crypt junction. However, TAg^{N136} mice show nuclear as well as cytoplsmic expression of N136 in only top $3/4^{th}$ of the villi.

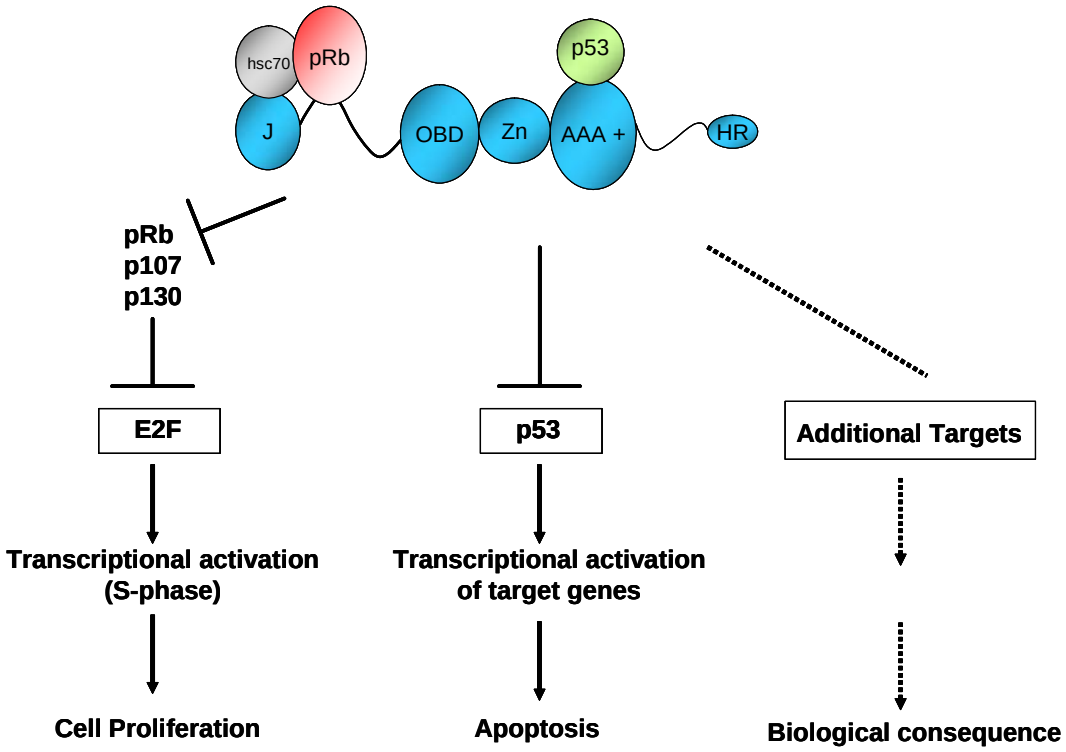
5.0 ROLE OF MULTIPLE DOMAINS OF T ANTIGEN IN GENE REGULATION AND TRANSFORMATION IN MOUSE EMBRYO FIBROBLASTS

5.1 INTRODUCTION

To analyze global changes in the host cell gene expression and to determine whether Rb and p53 pathways are the only main players involved in TAg induced transformation, we employed oligonucleotide microarrays following infection with wild-type SV40 virus or transfection with DNA expressing the early region of SV40 (wild-type or carrying some mutation) in MEFs. In the present study we have used MEF cell lines expressing wild-type TAg or mutants (N136, 3213 or D44N). We have taken advantage of these mutants to discern the role of different domains/regions of TAg in governing/regulating different biological processes.

Our effort is to link the changes in gene expression with the known transforming functions or novel activity/ies of TAg by testing three hypothesis: (a) TAg inactivates RB-family proteins and induce E2F dependent transcription by an LXCXE and J domain dependent manner (b) TAg inactivates p53 and blocks p53 dependent transcription (c) TAg interacts with other proteins / factors such as the transcription factors TBP, TEF-1, TAFs, TFIIB, and RNAPolII and regulates gene expression involved in novel activities of TAg (Fig.31)

Figure 31: Three hypothesis for microarray experiments.



5.2 RESULTS

5.2.1 Global patterns of cellular gene expression in MEFs expressing wild-type or mutant T antigens.

Microarray technology serves as an important tool to analyze changes in RNA content of a cell in response to treatment such as chemicals or viruses. Alteration in gene expression may reflect modifications in transcription, processing, and stability. In the present study we analyzed the RNA profile in cultured two day post-confluent MEFs expressing wild-type or mutant T antigens. At this stage, normal MEFs reach a growth arrest phase, however MEFs expressing TAg do not growth arrest and continue proliferating. The Affymetrix mouse whole genome chip which consists of 21,635 unique genes was used for microarray analysis. Genes three fold up or down were selected for gross analysis of gene expression. We found that MEFs expressing TAg show upregulation of 445 genes and downregulation of 557 genes in comparison to normal MEFs. Furthermore, MEFs expressing N136 show upregulation of 592 genes and downregulation of 511 genes. Noticeably, N136 upregulates more genes than wild-type TAg. Mutation of the LXCXE motif (3213) or the J domain (D44N) results in a significant reduction in the number of genes upregulated by TAg, suggesting the importance of these domains in gene regulation. MEFs expressing 3213 show upregulation of 118 genes and downregulation of 398 genes. Additionally, MEFs expressing D44N show upregulation of 116 genes and downregulation of 469 genes. The number of genes downregulated by TAg were not affected significantly by these mutations (Table 3).

Table 3: Number of upregulated or downregulated genes in MEFs expressing TAg or mutants.

Genes with 3 fold or more upregaultion or downregulation were selected for this analysis. Upregulated genes must have 0 absent calls in experimental samples and downregulated genes must have 0 absent calls in control sample. We used the Affymetrix mouse whole genome chip which consists of 21,635 genes.

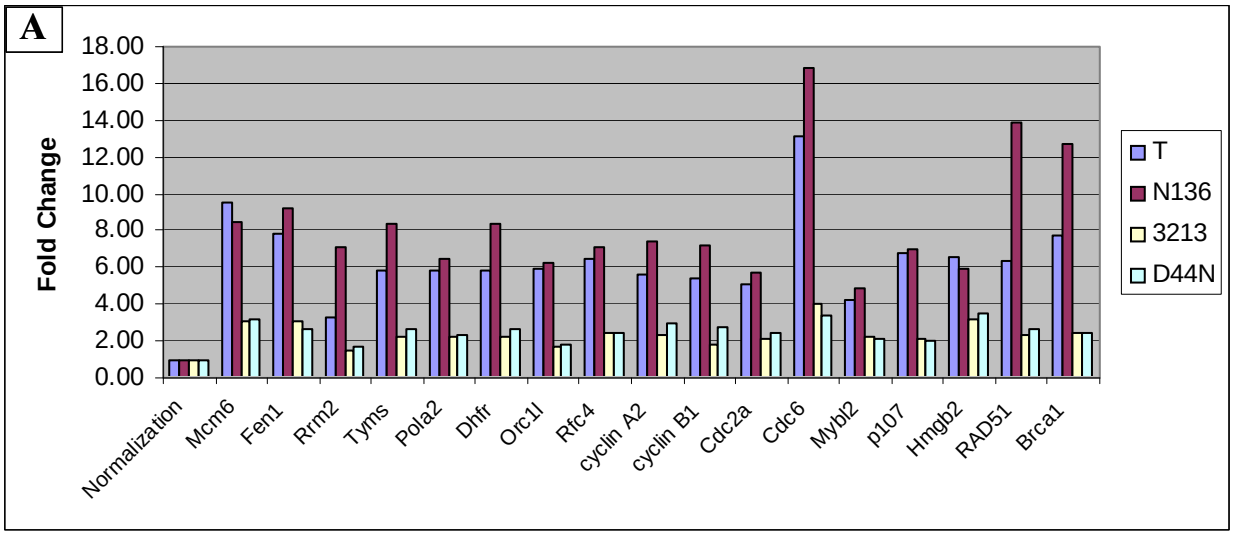
	Upregulated Genes	Downregulated Genes
T	445	557
N136	592	511
3213	118	398
D44N	116	469

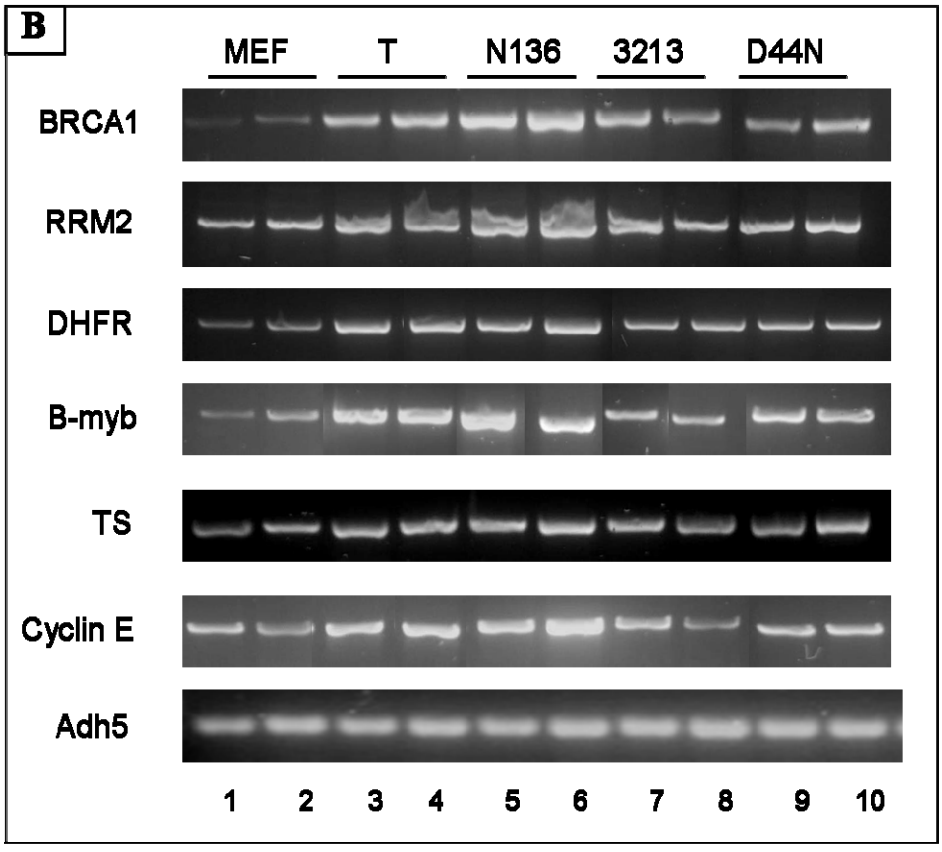
5.2.2 TAg regulates gene expression by inactivating RB-family proteins.

The LXCXE motif and J domain are required for the complete inactivation of Rb-family members and thus for the induction E2F target genes. Therefore we hypothesize that N136 should be able to regulate the same set of E2F-target genes as full-length TAg while 3213 and D44N should not. Consistent with this hypothesis our microarray data show profound upregulation of E2F target genes by TAg and N136, however 3213 and D44N show an intermediate level of upregulation (~35% of TAg or N136) for these genes (Fig.32A). To further confirm the expression levels of some of the E2F-target genes (such as BRCA1, RRM2, DHFR, B-myb, TS and Cyclin E), we performed semiquantative RT-PCR analysis (Fig.32B). All these genes are significantly upregulated by TAg and N136 (lanes 3-6) in comparison to normal MEFs (lanes 1-2). Furthermore, 3213 and D44N also show increased levels of mRNA for these genes (lanes 7-10) in comparison to normal MEFs (lanes 1-2), however this upregulation is less than TAg and N136 (compare lanes 3-6 with lanes 7-10).

Figure 32: Regulation of E2F-target genes by TAg and mutants.

TAg and N136 show significant upregulation while 3213 and D44N show intermediate upregulation of E2F target genes. **(A)** Average fold change of the specified genes were determined by microarray analysis of each category (n=3): T, N136, 3213 and D44N. "Normalization" represents fold change of 1 (not regulated) in each category. **(B)** RT-PCR analysis was carried out using Adh5 levels as controls in normal MEFs, T, N136, 3213 and D44N.


A
Fold Change
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Normalization
Mcm6
Fen1
Rrm2
Tyms
Pola2
Dhfr
Orc1l
Rfc4
cyclin A2
cyclin B1
Cdc2a
Cdc6
Mybl2
p107
Hmgb2
RAD51
Brca1
T
N136
3213
D44N

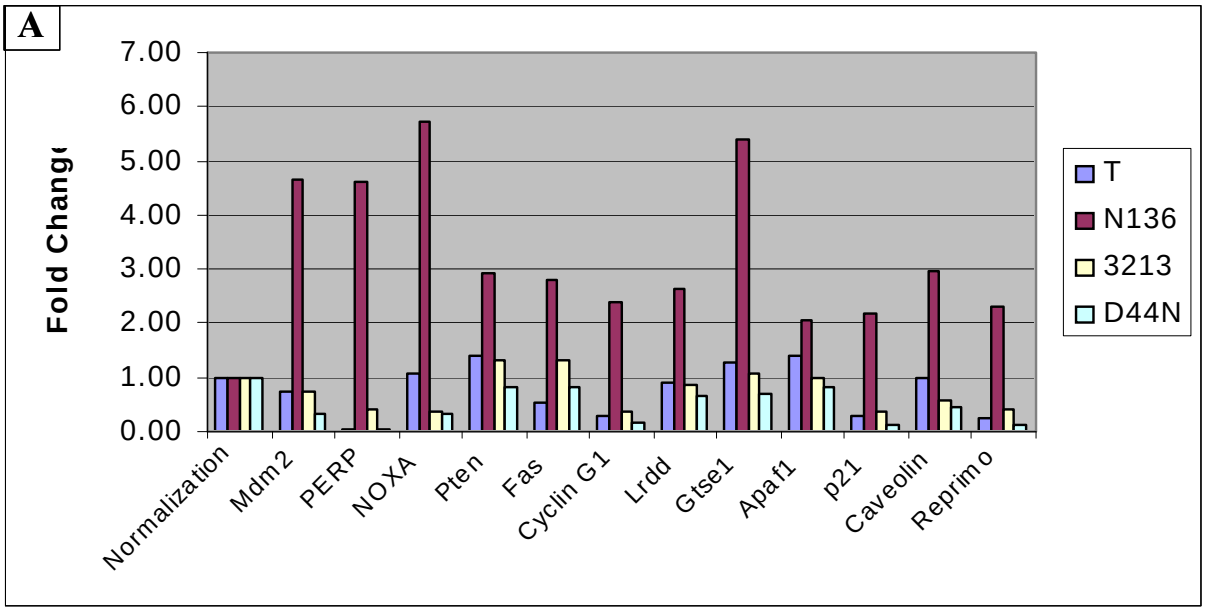

B
MEF
T
N136
3213
D44N
BRCA1
RRM2
DHFR
B-myb
TS
Cyclin E
Adh5
1
2
3
4
5
6
7
8
9
10

5.2.3 TAg regulates gene expression by blocking p53 dependent transcription

TAg binds to p53 and blocks p53 dependent transcription. Therefore we hypothesize that TAg, 3213 and D44N should be able to block transcription of p53 target genes, while N136 should not. Consistent with this hypothesis our microarray data show significant upregulation of p53 target genes by N136 (Fig.33A and Table 5 on page 128). To confirm the expression levels of some of the p53-target genes (such as Lrdd, p21, NOXA, Mdm2, Cyclin G1, Fas, PERP, Pten and Rprm) we performed semiquantative RT-PCR analysis (Fig.33B). All these genes are significantly upregulated by N136 (lanes 5-6) in comparison to normal MEFs (lanes 1-2), TAg (lanes 3-4), 3213 (lanes 6-7) and D44N (lanes 9-10). To further confirm that this upregulation is due to the inability to bind to p53, we have used another mutant, Patch1, which is a four amino acid substitution mutant on the p53 binding surface of TAg and is therefore defective for p53 binding. Patch1 was able to upregulate the same set of p53 target genes as N136 (Fig 33B, compare lanes 5-6 with lanes 11-12). Interestingly, some of the genes such as p21, Cyclin G1 and PERP are downregulated by TAg but upregulated by N136.

Figure 33: N136 upregulates p53 target genes.

(A) Average fold change of the specified genes were determined by microarray analysis of each category (n=3): T, N136, 3213 and D44N. "Normalization" represents fold change of 1 (not regulated) in each category. **(B)** RT-PCR analysis was carried out using Adh5 levels as controls in normal MEFs, T, N136, 3213 and D44N. Patch1 is a four amino acid substitution mutant on the p53 binding surface of TAg and is therefore defective for p53 binding.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Fold Change
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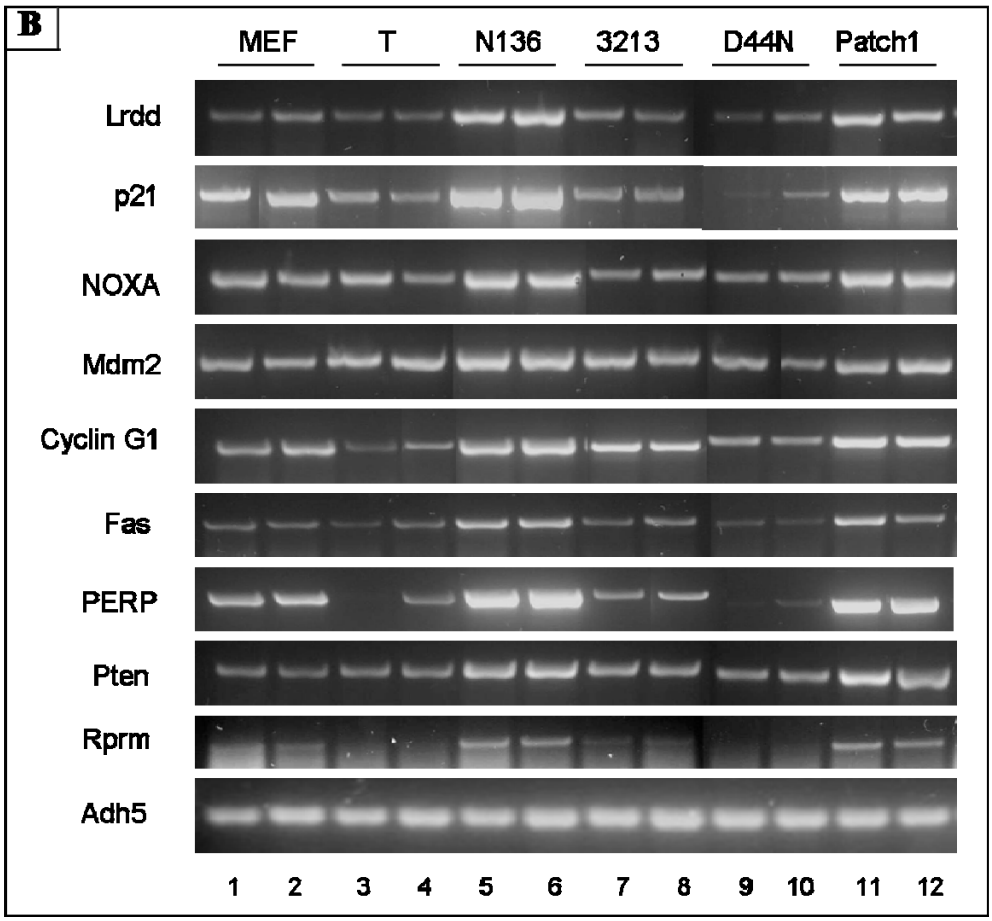
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Normalization
Mdm2
PERP
NOXA
Pten
Fas
Cyclin G1
Lrdd
Gtse1
Apaf1
p21
Caveolin
Reprimo
T
N136
3213
D44N


B
MEF
T
N136
3213
D44N
Patch1
Lrdd
p21
NOXA
Mdm2
Cyclin G1
Fas
PERP
Pten
Rprm
Adh5
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3
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7
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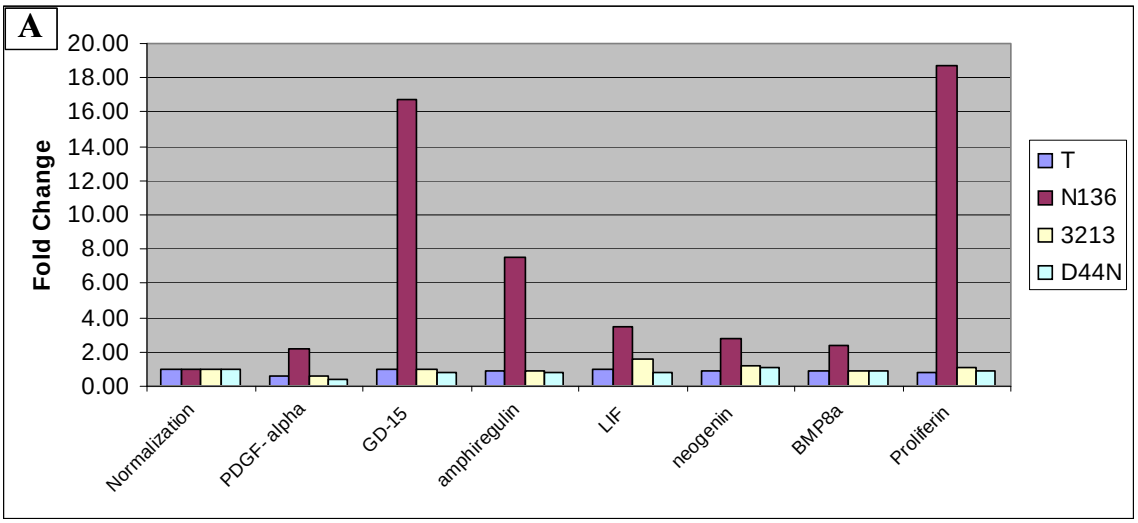
12

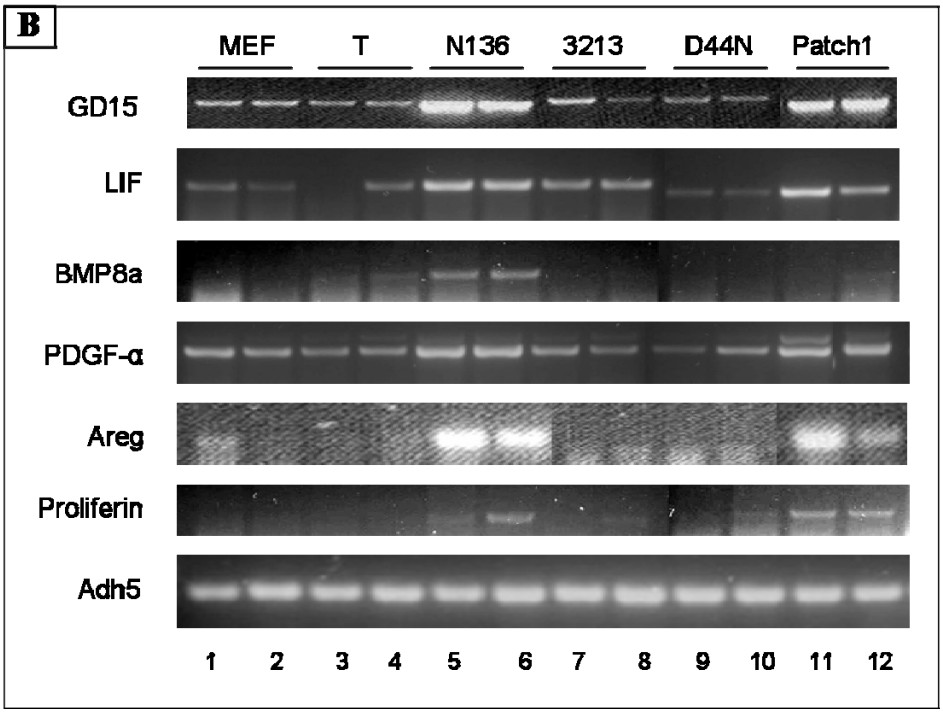
5.2.4 Regulation of growth factors by N136

N136 upregulates genes that belong to a class of growth factors (Fig.34A). We have confirmed the expression levels of some of these genes (such as GD15, LIF, BMP8a, PDGF-α, amphiregulin and proliferin) by semiquantitative RT-PCR analysis (Fig.34B). All these genes are significantly upregulated by N136 (lanes 5-6). Some of these genes (such as GD-15, proliferin, amphiregulin and PDGF-α) are upregulated by Patch1 also (lanes 11-12).

Figure 34: N136 upregulates growth factors.

(A) Average fold change of the specified genes were determined by microarray analysis of each category (n=3): T, N136, 3213 and D44N. "Normalization" represents fold change of 1 (not regulated) in each category. **(B)** RT-PCR analysis was carried out using Adh5 levels as controls in normal MEFs, T, N136, 3213 and D44N.



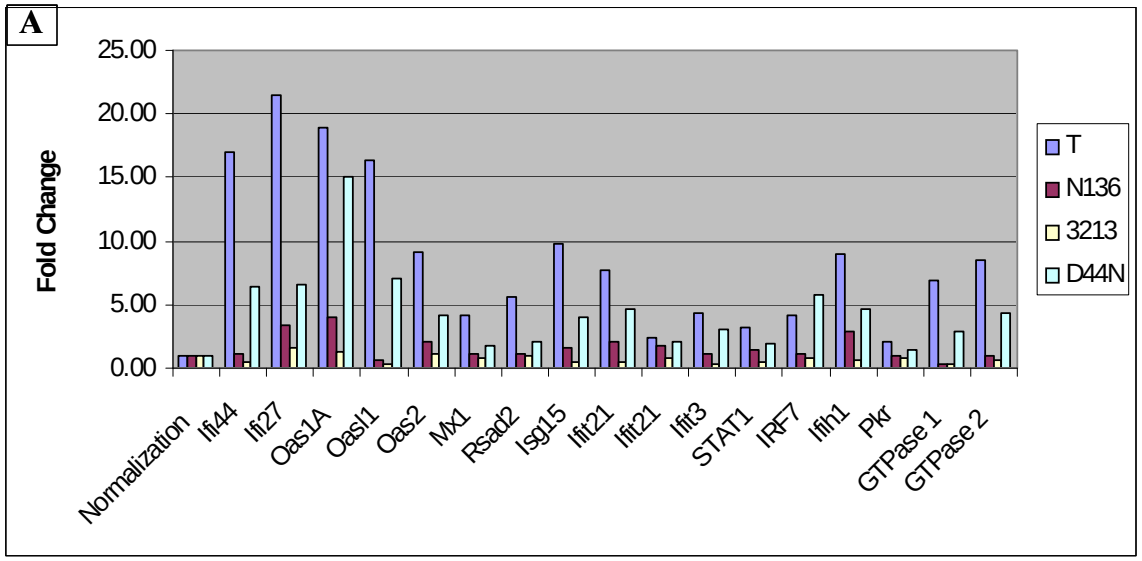


5.2.5 TAg regulates expression of immune response genes

We found upregulation of immune response genes by TAg (Fig.35A). So far there has been no report connecting the regulation of immune response genes by TAg to classical interactions with Rb-family proteins and p53. This may lead to the identification of a novel regulatory mechanism by TAg. We confirmed the expression levels of some of the interferon-responsive genes such as Ifi44, Ifi27, Oas2, GTPase2, Rsad2, Mx1, Oasl2, and IRF7 (Fig 35B). We found profound upregulation of these genes by TAg and D44N (lanes 3-4 and 9-10) but not by N136 and 3213 (lanes 5-6 and 7-8). On the other hand, an important histocompatibilty gene, H60, was downregulated by TAg and D44N (lanes 3-4 and 9-10) but not by N136 and 3213 (lanes 5-6 and 7-8).

Figure 35: TAg and D44N upregulates interferon-induced genes expression.

(A) Average fold change of the specified genes were determined by microarray analysis of each category (n=3): T, N136, 3213 and D44N. "Normalization" represents fold change of 1 (not regulated) in each category. **(B)** RT-PCR analysis was carried out using Adh5 levels as controls in normal MEFs, T, N136, 3213 and D44N.


A
25.00
20.00
15.00
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Fold Change
T
N136
3213
D44N
Normalization
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Ifi27
Oas1A
Oasl1
Oas2
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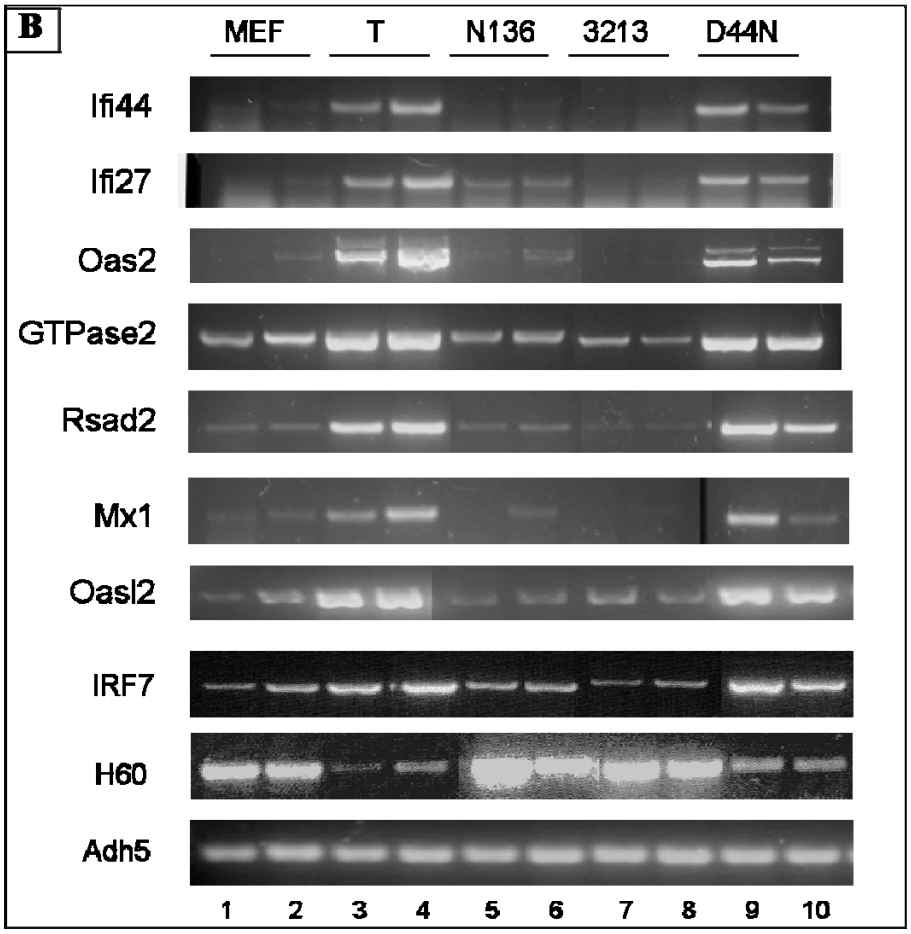
Ifih1
Pkr
GTPase 1
GTPase 2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MEF
T
N136
3213
D44N
Ifi44
Ifi27
Oas2
GTPase2
Rsad2
Mx1
Oasl2
IRF7
H60
Adh5
1
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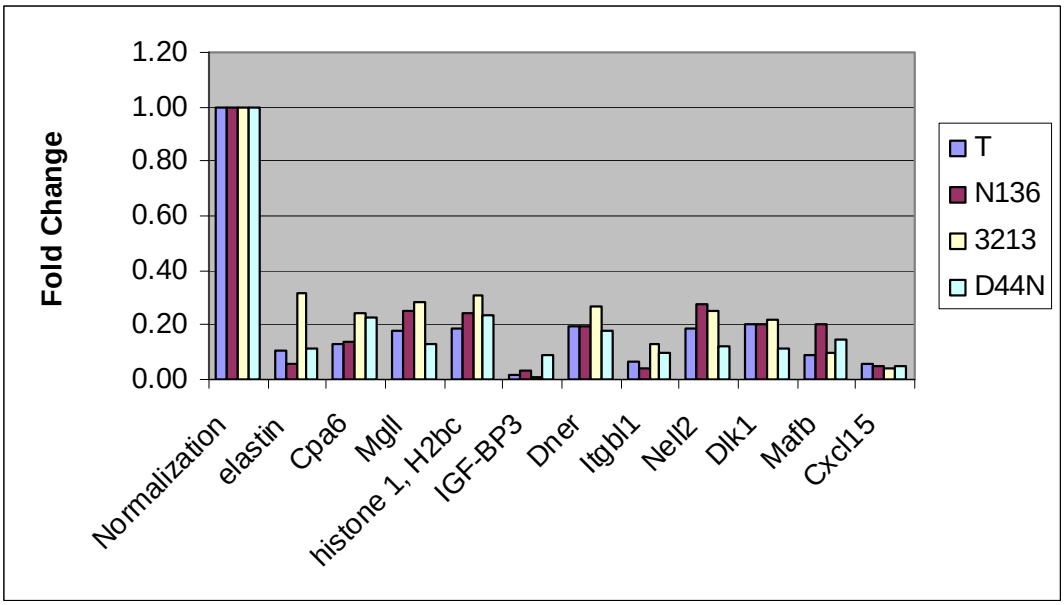
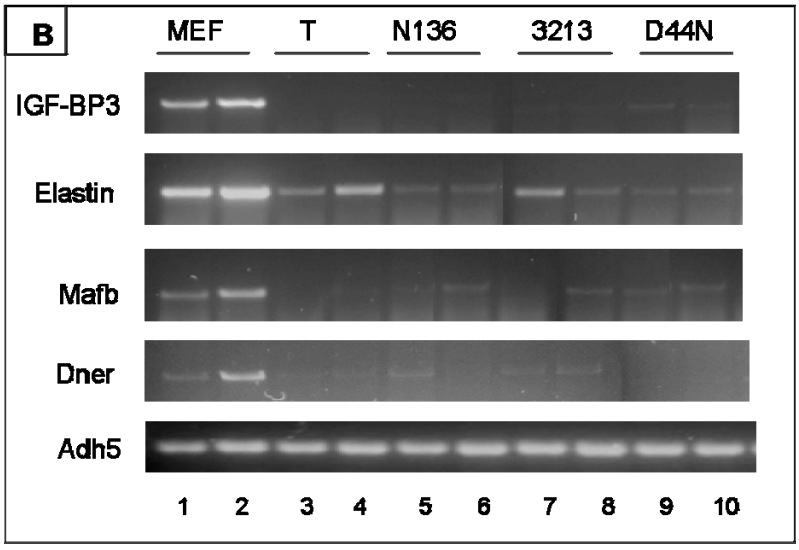
5.2.6 TAg regulates genes expression independent of the LXCXE motif, J domain, and p53 binding domain

We found a large number of genes downregulated by TAg and mutants (Fig 36A) that belong to significant biological classes such as cadherin, cytokine-cytokine receptor interaction, chromatin and neuroactive ligand receptor interaction. We have confirmed the expression level of some of these genes (such as IGF-BP3, elastin, Mafb, and Dner) (Fig.36B). We found significant downregulation of these genes by TAg and all mutants (lanes 3-10) in comparison to normal MEFs (lanes 1-2).

Figure 36: TAg downregulates genes independent of the LXCXE motif, J domain and p53 binding domain.

(A) Average fold change of the specified genes were determined by microarray analysis of each category (n=3): T, N136, 3213 and D44N. "Normalization" represents fold change of 1 (not regulated) in each category. **(B)** RT-PCR analysis was carried out using Adh5 levels as controls in normal MEFs, T, N136, 3213 and D44N.



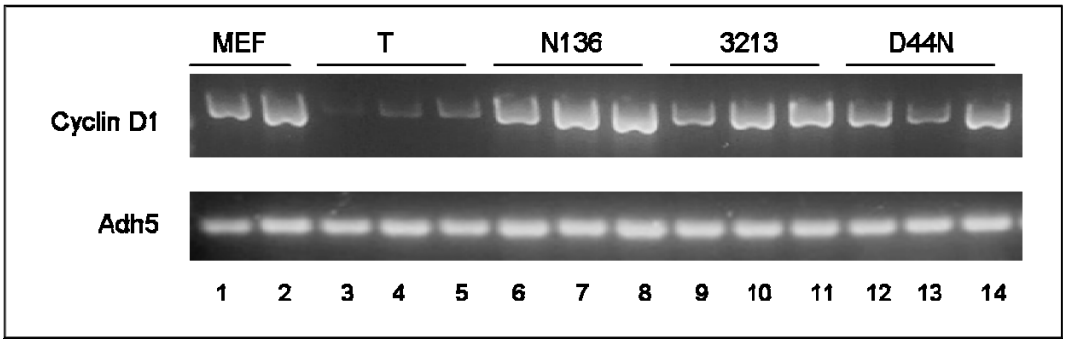


5.2.7 Cooperation between different domains of TAg to regulate gene expression

Cyclin D1 encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates pRb and promotes progression through the G1-S phase of the cell cycle. Cyclin D1 associates with, and regulates the activity of transcription factors, coactivators and corepressors that govern histone acetylation and chromatin remodeling proteins. Regulation of Cyclin D1 presents a unique example of cooperation/crosstalk between different domains of TAg. Cyclin D1 was downregulated by TAg only (Fig.37, lanes 3-5). None of the mutants were able to downregulate Cyclin D1 (lanes 6-14).

Figure 37: Cooperation between different domains of TAg to regulate gene expression.

RT-PCR analysis was carried out using Adh5 levels as controls in normal MEFs, T, N136, 3213 and D44N. Downregulation of Cyclin D1 is dependent on the C-terminus, LXCXE motif and J domain of TAg.



5.2.8 Genetic classification of genes regulated by different domains of TAg

To determine the role of different domains or regions of TAg in gene regulation, we have divided genes into 9 genetic classes depending on their regulation by wild-type TAg and/or mutants (Table 4). Each class represents the number of genes regulated by a single domain/region or combination of other domain(s)/regions(s). Genes with 3 or more fold up/down regulation were considered as regulated genes by TAg or different mutants (N136, 3213, D44N). Genes which are equal to or greater than 0.8 fold (for determining downregulated genes) and equal to or less than 1.2 fold (for determining upregulated genes) were considered as not regulated. The criteria for genetic class 7 was relaxed from 3 fold to 2 fold to include genes belonging to the immune response.

Genetic class 1, 2, 3, and 4 represent genes regulated only by TAg or N136 or 3213 or D44N, respectively. Genetic class 5, 6 and 7 represent genes regulated by TAg and N136, N136 and 3213, TAg and D44N, respectively. Genetic class 8 represents genes regulated by TAg and all the mutants. Finally genetic class 9 represents genes, which are not regulated by TAg or mutants. Genetic classes that contain more than 10 regulated genes were selected for further analysis. For example, genetic class 2 represents genes uniquely regulated by N136 and consists of 76 up and 20 down-regulated genes. Genetic class 7 represents genes regulated by T and D44N which consists of 24 up and 3 down-regulated genes. Genetic class 8 is the biggest class and represents genes regulated by T, N136, 3213 and D44N which consists of 44 up and 127 down-regulated genes.

Table 4: Genetic classification of genes regulated by different domains of TAg.

Plus sign (+): represents regulation of genes by 3-fold or more by TAg or different mutants (N136, 3213, D44N). Minus sign (-): represents lack of regulation of genes. See text for details.

Genetic class	T	N136	3213	D44N	No. of upregulated genes	No. of downregulated genes
1	+	-	-	-	2	0
2	-	+	-	-	76	20
3	-	-	+	-	5	1
4	-	-	-	+	4	3
5	+	+	-	-	7	3
6	-	+	+	-	2	2
7	+	-	-	+	24	3
8	+	+	+	+	44	127
9	-	-	-	-	9000	

5.2.9 Comparisons of gene expression patterns between TAg and its mutants (N136 or 3213 or D44N)

To determine the affect of individual domain/region of TAg on gene expression we have compared each mutant individually with wild-type TAg (Venn diagrams in Fig.38 and 39). To identify genes uniquely upregulated by TAg but not by the mutant, a filter is set where the TAg ratio was greater than or equal to 3 fold and the mutant ratio was less than 1.2 fold and similarly for identifying uniquely upregulated genes by the mutant. To identify genes uniquely downregulated by TAg but not by the mutant, a filter is set where the TAg ratio was less than or equal to 0.33 fold and the mutant ratio was greater than 0.8 fold and similarly for identifying uniquely downregulated genes by the mutant. We have used DAVID 2.0 bioinformatics resource (http://david.abcc.ncifcrf.gov/home.jsp) to group genes into different biological classes. The bioinformatics programs conveniently classify the genes into various biological classes and therefore give an overview of biological pathways. However, the relevance of these biological classes can only be proved by experimentation. The following section describes the classification of the genes as obtained by the bioinformatics program.

(i) *Comparison of the gene expression patterns between TAg and N136:*

There is a large overlap (338) of genes upregulated by both TAg and N136 (Fig.38A). These genes are associated with E2F-dependent transcription, DNA replication and repair, cell-cycle regulation, chromatin assembly/modification and microtubule cytoskeleton. When interpreting this data one need to be aware with the fact that the regulation of these genes can be direct or indirect effect of expression of TAg or mutant in the cell.

A total of 31 genes uniquely upregulated by TAg belong to one major biological class: response to virus/immune response/antiviral defense (Fig.38A). In contrast, N136 is unable to induce immune response genes. On the other hand, N136 uniquely induces 111 genes belonging to biological classes such as apoptosis (p53-target genes), growth factors, metabolism, cytokine and neuron differentiation (Fig.38A).

There is a large overlap (277) of genes downregulated by both TAg and N136 (Fig.39A). These genes are part of different biological classes such as Cadherins (family of procadherins, cadherin 11, cadherin 4) focal adhesion and cytokine-cytokine receptor interaction and so on. TAg uniquely downregulates 110 genes while N136 uniquely downegulates 45 genes (Fig.39A). Interestingly, biological classes such as glucuronosyl trasnferase activity, apoptosis, neuron differentiation and transmembrane are upregulated by N136 but downregulated by T (compare Fig.38A to Fig.39A). Genes which are uniquely downregulated by N136 belong to the class of metal binding and cell adhesion (VCAM1, Scarf2, cadherin 3).

(ii) Comparison of the gene expression patterns between TAg and 3213:

There is not a big overlap (71) of genes upregulated by both TAg and 3213 (Fig. 1B). However these genes are associated with E2F-dependent transcription, DNA replication and repair, nucleotide metabolism and microtubule cytoskeleton. Comparisons between genes upregulated by both TAg and N136 (338 genes) with TAg and 3213 (71 genes) (Fig.38A and 38B) shows a significant reduction in the total number of genes regulated. This can be explained by the intermediate levels of gene regulation displayed by 3213 and therefore even they show some upregulation but failed to meet the 3 fold criteria. For example, TAg and N136 upregulate an

E2F-target gene, p107, by 7 fold, however 3213 upregulates p107 transcription by 2 fold (Fig.32).

When comparing the upregulation of genes between TAg and 3213 (Fig.38B), we found that 71 genes are uniquely upregulated by TAg which belong to immune response, chromosomal proteins and antigen processing. In contrast, 3213 is unable to induce the above classes of genes. On the other hand, 15 genes are uniquely upregulated by 3213 and do not belong to any specific biological class (Fig.38B). Due to the variability in the raw microarray data between three clones of 3213, the existence of this class is questionable.

A total of 58 genes are uniquely downregulated by TAg (Fig.39B). These genes belong to the class of cell adhesion and enzyme linked receptor signaling pathway (IGF1, latent TGFb1). A total of 16 genes are uniquely downregulated by 3213 and belong to the class of glycoproteins. Noticeably, there is a big overlap (269) between genes downregulated by both TAg and 3213. These genes represent a variety of biological classes (Fig.39B).

(iii) Comparison of the gene expression patterns between TAg and D44N:

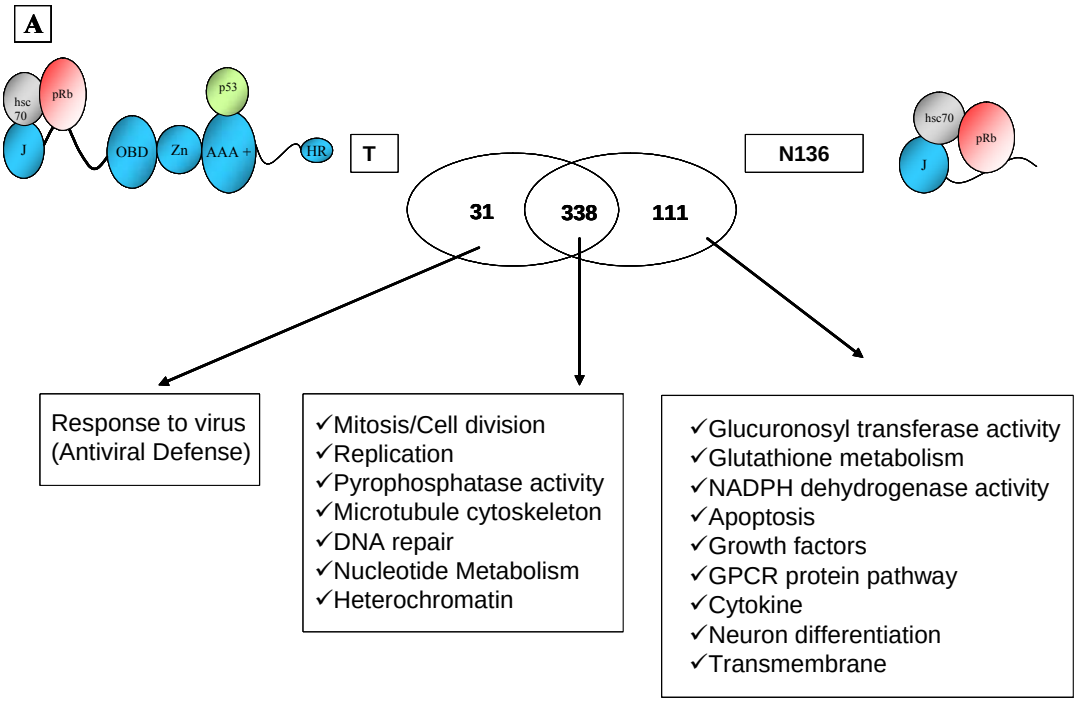
A total of 106 genes are upregulated by both TAg and D44N (Fig.38C). These genes belong to the same biological classes like TAg vs. 3213 and similarly show an intermediate upregulation of genes that are required for cell proliferation (Fig.32). On the other hand, the pattern of gene upregulation between TAg and D44N shows some significant differences in comparison to the TAg and 3213. For example, the class of immune response and antigen processing are upregulated by both TAg and D44N (Fig38C).

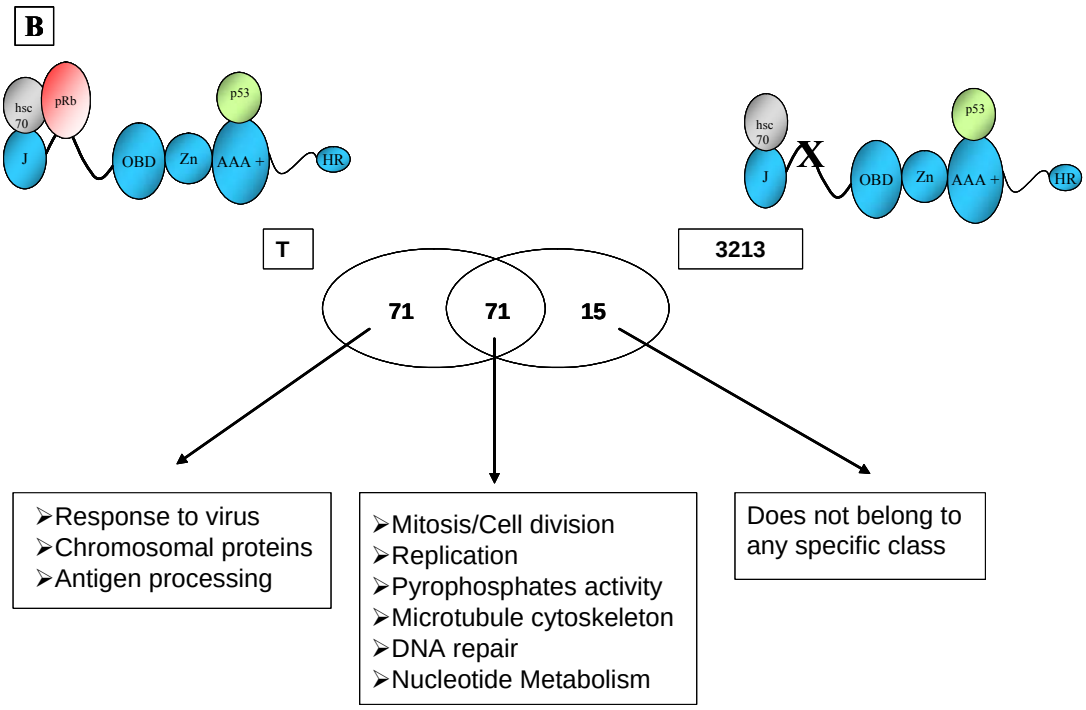
We found that 23 genes are uniquely upregulated by TAg which belong to the class of DNA damage/repair (Fig.38C). Only 4 genes are uniquely upregulated by D44N and do not belong to any specific biological class (Fig.38C).

There is a large overlap (310) between genes downregulated by both TAg and D44N (Fig.39C). These genes belong to the same biological classes as the commonly downregulated genes by TAg and 3213. A total of 27 genes are uniquely downregulated by TAg. These genes belong to the class of solute carrier family (detoxification pathway) and homeobox (Fig.39C). 10 genes are uniquely downregulated by D44N and belong to the class of nuclear proteins (Fig.39C).

Figure 38: Upregulation of genes by TAg and mutants in MEFs.

In each Venn diagram the intersection represents the number of genes that are commonly upregulated 3-fold or more by TAg and different mutants (N136, 3213, D44N). Left circle represents genes which are uniquely upregulated by TAg while the right circle represent genes which are uniquely upregulated by the mutant. See text for details. Genes were classified in different biological classes using the DAVID 2.0 bioinformatics resource. Biological classes are sorted so that the genes with low p values (high enrichment score) come first in the list.




B
hsc 70
pRb
p53
J
OBD
Zn
AAA +
HR
X
T
3213
71
71
15
➢Response to virus
➢Chromosomal proteins
➢Antigen processing
➢Mitosis/Cell division
➢Replication
➢Pyrophosphates activity
➢Microtubule cytoskeleton
➢DNA repair
➢Nucleotide Metabolism
Does not belong to any specific class

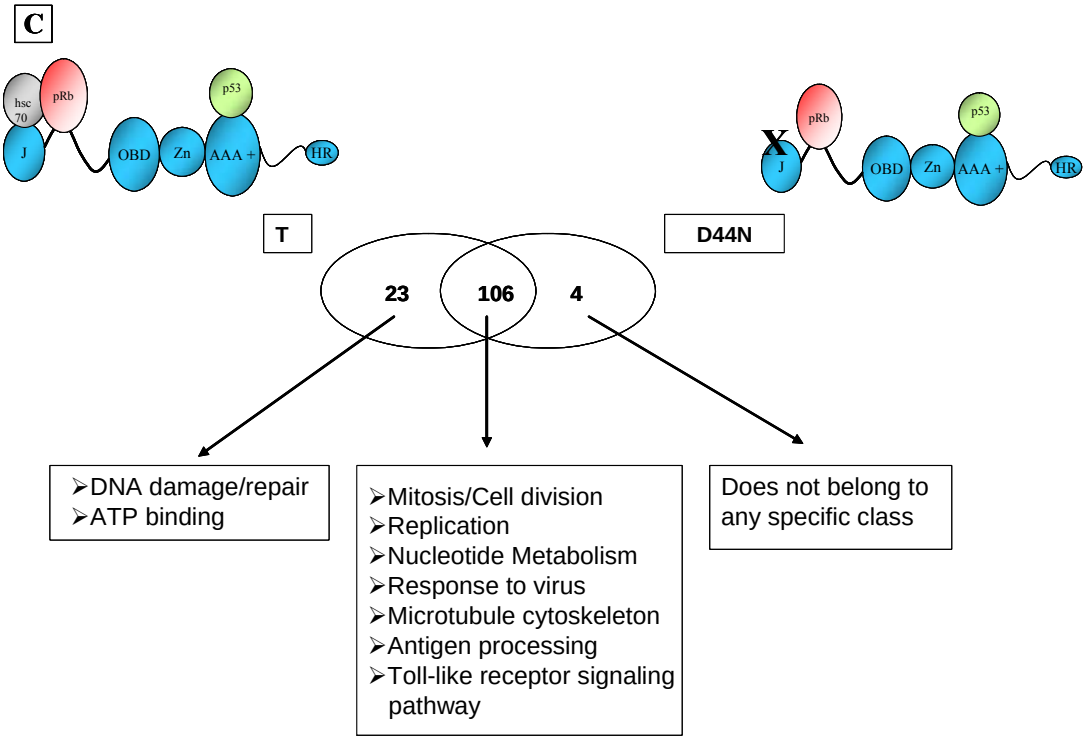
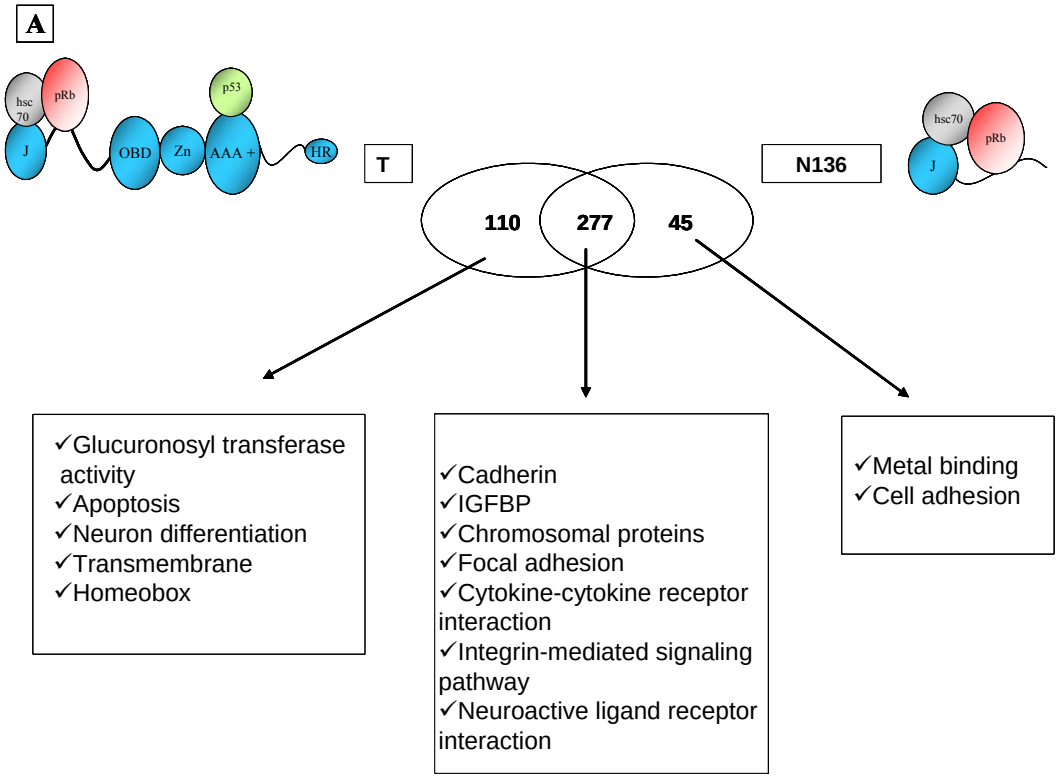
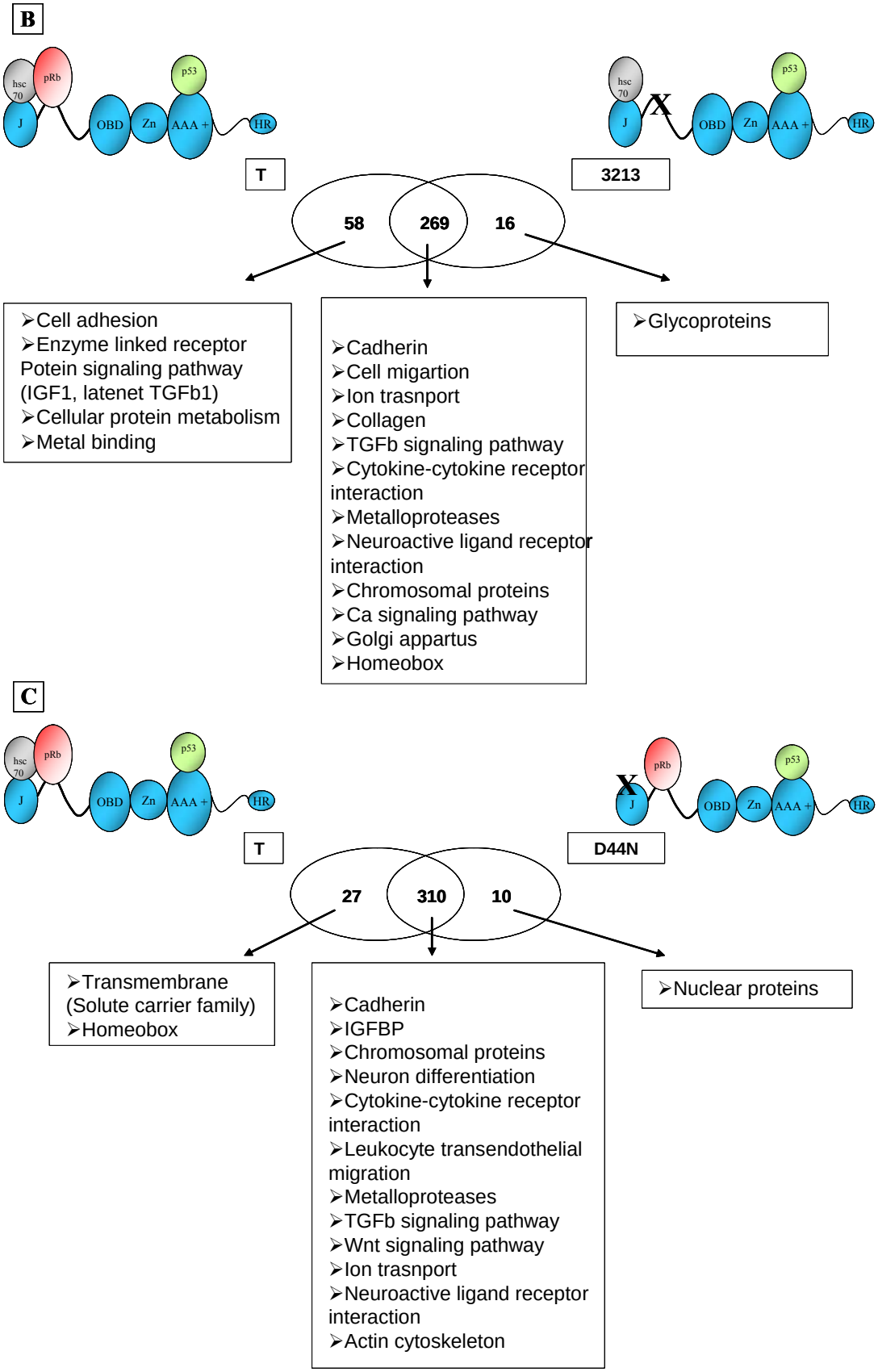

C
hsc 70
pRb
p53
J
OBD
Zn
AAA +
HR
X
T
D44N
23
106
4
➢DNA damage/repair
➢ATP binding
➢Mitosis/Cell division
➢Replication
➢Nucleotide Metabolism
➢Response to virus
➢Microtubule cytoskeleton
➢Antigen processing
➢Toll-like receptor signaling pathway
Does not belong to any specific class

Figure 39: Downregulation of genes by TAg and mutants in MEFs.

In each Venn diagram the intersection represents the number of genes that are commonly downregulated 3-fold or more by TAg and different mutants (N136, 3213, D44N). Left circle represents genes which are uniquely downregulated by TAg while the right circle represent genes which are uniquely downregulated by the mutant. See text for details. Genes were classified in different biological classes using the DAVID 2.0 bioinformatics resource. Biological classes are sorted so that the genes with low p values (high enrichment score) come first in the list.




B
hsc 70
pRb
p53
J
OBD
Zn
AAA +
HR
T
3213
58
269
16
➢Cell adhesion
➢Enzyme linked receptor Potein signaling pathway (IGF1, latenet TGFb1)
➢Cellular protein metabolism
➢Metal binding
➢Cadherin
➢Cell migartion
➢Ion trasnport
➢Collagen
➢TGFb signaling pathway
➢Cytokine-cytokine receptor interaction
➢Metalloproteases
➢Neuroactive ligand receptor interaction
➢Chromosomal proteins
➢Ca signaling pathway
➢Golgi appartus
➢Homeobox
➢Glycoproteins
C
T
D44N
27
310
10
➢Transmembrane (Solute carrier family)
➢Homeobox
➢Cadherin
➢IGFBP
➢Chromosomal proteins
➢Neuron differentiation
➢Cytokine-cytokine receptor interaction
➢Leukocyte transendothelial migration
➢Metalloproteases
➢TGFb signaling pathway
➢Wnt signaling pathway
➢Ion trasnport
➢Neuroactive ligand receptor interaction
➢Actin cytoskeleton
➢Nuclear proteins

5.2.10 SV40 infected MEF microarry

We infected MEFs with SV40 virions to ensure that alterations to gene expression were not nonspecific events caused by transfection of cells with SV40 DNA. Overall, 391 genes were upregulated 3 fold or more. These genes belong to several significant biological classes such as cell cycle, nucleotide metabolism, response to virus, antigen processing, microtubule cytoskeleton, cytokine-cytokine receptor interaction, natural killer cell mediated cytotoxicity, ubiquitin protein ligase activity and serine protease inhibitor. On the other hand, 90 genes were downregulated 3 fold or more by SV40 infection. These genes belong to biological classes such as chromatin (histones), EGF-like (Dner, Dlk1, Itgbl1, Nell2), actin cytoskeleton, Golgi apparatus (St8sia2, Galntl4, Copq2) and zinc ion binding (Dpyd, Cpa6, Kcnd2, Unc13c). Comparison between SV40 infection and MEF-T cell line shows that there is an overlap of 75% of the upregulated genes and 60% of the downregulated genes. To avoid any nonspecific alteration in gene expression due to the process of cell line establishment, we have selected only those genes that show similar regulation by both SV40 infected and MEF-T cell line for RT-PCR analysis.

5.2.11 Conclusions from MEFs Microarray

Our microarray findings suggest that TAg regulates gene expression by activating the transcriptional circuits controlled by Rb as reflected by induction of E2F-target genes by TAg and N136. Furthermore, TAg can regulate gene expression by inactivating the transcriptional circuit controlled by p53 which is reflected by the ability of N136, but not by TAg, 3213 and D44N to induce p53-target genes. Additionally, N136 upregulates genes that belong to a class of

growth factors. On the other hand, we found upregulation of a large number of immune response genes by TAg and D44N but not by N136 and 3213. Finally, a set of genes (such as Dner, Mafb, IGF-BP3 and Elastin) were downregulated by TAg and its mutants, suggesting Rb and p53 independent interactions of TAg in gene regulation.

6.0 ROLE OF MULTIPLE DOMAINS OF T ANTIGEN IN GENE REGULATION AND TRANSFORMATION IN MOUSE INTESTINAL EPITHELIUM

6.1 INTRODUCTION

Much of our understanding of the mechanism of transformation by SV40 stems from work with established and primary cell cultures or from *in vitro* molecular studies using purified or partially purified proteins. These approaches have proved to be very successful and have resulted in the identification of at least some of the cellular targets of the large and small T antigens, and in an initial understanding of the biochemical basis for their action. Cell culture studies are particularly valuable because they are amenable to a combined genetic biochemical approach whereby individual viral functions can be associated with the regulation of specific cellular pathways. However, many of the cellular regulatory pathways can be perturbed during cell line establishment. Therefore mouse models offer a tool for studying growth control pathways in a natural tissue setting. In the present study we have used mouse intestinal epithelial cells as a model to understand how SV40 induces neoplastic transformation. Transgenic mice expressing wild-type TAg or TAg mutants (N136 or 3213 or D44N) in terminally differentiated enterocytes are used to determine if the Rb and p53 pathways play a major role in the induction of intestinal hyperplasia and dysplasia or if there is a contribution by other factors/pathways.

6.2 RESULTS

6.2.1 Global patterns of cellular gene expression in villus enterocytes of transgenic mice expressing wild-type or mutant T antigens.

In the present study we analyzed the RNA profile of villus enterocytes of transgenic mice expressing TAg or mutants using microarray technology. The villus enterocytes are enriched for nonproliferating, terminally differentiated cells. Expression of full-length TAg in murine enterocytes forces a nonproliferating cell population to restart the cell cycle and results in intestinal hyperplasia that progress to dysplasia with the age. The Affymetrix mouse whole genome chip which consists of 21,635 unique genes was used for microarray analysis. Genes three fold up or down were selected for gross analysis of gene expression. TAg^{wt} mice show upregulation of 291 genes and downregulation of 140 genes in comparison to their nontransgenic littermate. Furthermore, TAg^{N136} mice show upregulation of 274 genes and downregulation of 197 genes. Mutation of the LXCXE motif (3213) or the J domain (D44N) results in a large reduction in the number of genes upregulated by TAg, suggesting the important contribution of these domains in gene regulation. TAg^{3213} mice show upregulation of 19 genes and downregulation of 28 genes. Additionally, TAg^{D44N} mice show upregulation of 20 genes and downregulation of 61 genes (Table 5).

Table 5: Number of upregulated or downregulated genes in villi expressing TAg or mutants.

Genes with 3 fold or more upregaultion or downregulation were selected for this analysis. Upregulated genes must have 0 absent calls in experimental samples and downregulated genes must have 0 absent calls in control sample). We used the Affymetrix mouse whole genome chip which consists of 21,635 genes.

	Upregulated Genes	Downregulated Genes
T	291	140
N136	274	197
3213	19	28
D44N	20	61

6.2.2 TAg regulates gene expression by inactivating RB-family proteins in villus enterocytes

We hypothesize that TAg and N136 inactivates Rb-family members and cause an upregulation of E2F-target genes. Consistent with this hypothesis, our microarray data shows profound upregulation of E2F target genes by TAg and N136. However, 3213 and D44N, which have a mutation in the LXCXE motif and J domain respectively, are unable to upregulate these genes (Fig.40).

6.2.3 TAg regulates gene expression independent of p53 in villus enterocytes

We do not find upregulation of p53-target genes by N136 in villus enterocytes (Table 6). This result is consistent with the previous observation that villus enterocytes lack p53 (Markovics et al., 2005).

Figure 40: TAg and N136 upregulate E2F target genes in villi.

Average fold change of the specified genes were determined by microarray analysis of each category (n=3): T, N136, 3213 and D44N. "Normalization" represents fold change of 1 (not regulated) in each category.

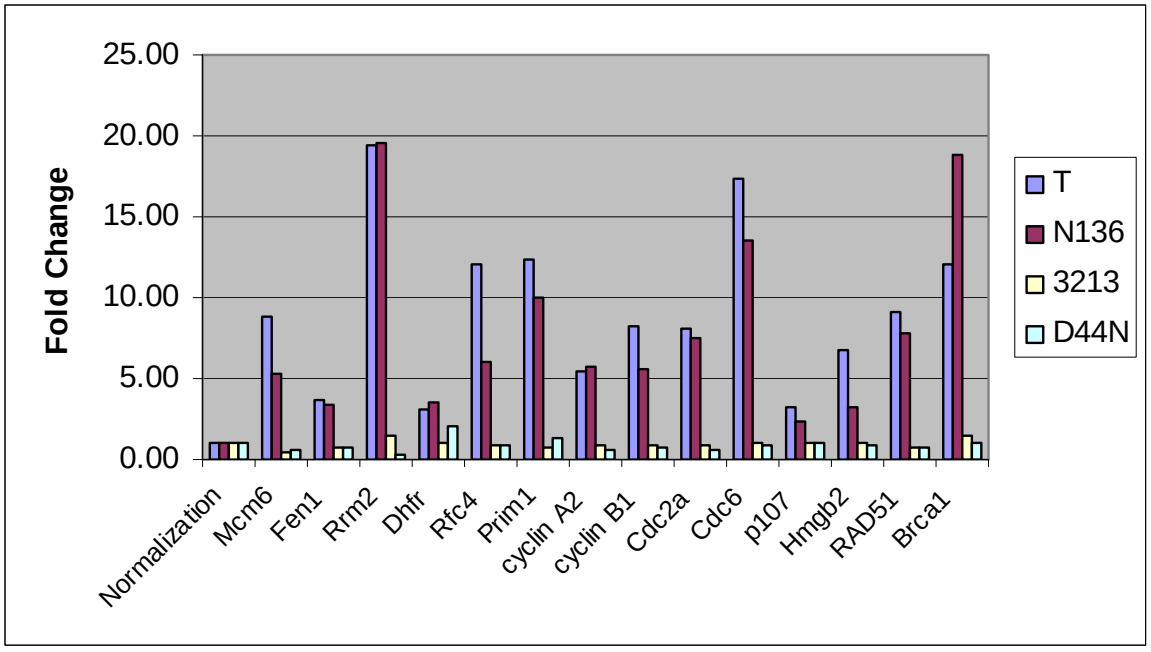


Table 6: p53 target genes are upregulated only in MEFs expressing N136.

Numbers indicate fold upregulation of the transcript level of p53 target genes. Villi expressing N136 do not upregulate p53 target genes consistent with the fact that in mouse intestine there is no p53.

Target Genes	Fold Regulation in MEFs		Fold Regulation in Villi	
	T	N136	T	N136
Mdm2	0.6	5.3	1.0	1.0
PERP	.03	4.6	1.1	1.1
NOXA	1.0	5.7	1.6	1.7
Pten	1.3	2.9	1.0	1.0
Fas	0.5	2.8	0.8	0.9
Cyclin G1	0.3	2.6	0.6	1.2
PIDD/Lrdd	0.8	2.6	0.8	0.9
Gtse1	1.2	5.3	1.0	1.2
Apaf1	1.4	2.0	1.1	0.8
p21	0.2	2.7	1.7	2.1
Caveolin	0.9	2.9	1.1	0.5
Reprimo	0.2	2.3	1.0	1.0

6.2.4 Genetic classification of genes regulated by different domains of TAg

To determine the role of different domains or regions of TAg in gene regulation, we have divided genes into 10 genetic classes (Table 7). Each class represents the number of genes regulated by a single domain/region or combination of other domain(s)/regions(s). Genes with 3 or more fold up/down regulation were considered as regulated genes by TAg or different mutants (N136, 3213, D44N). Genes which are equal to or greater than 0.8 fold (for determining downregulated genes) and equal to or less than 1.2 fold (for determining upregulated genes) were considered as not regulated. Genetic classes that contain more than 10 genes were selected for further analysis. We have classified genes into 10 genetic classes depending on their regulation by wild-type TAg and/or mutants. Genetic classes 1-9 are the same as MEFs micoarrays (Table 4). Genetic class 10 represents genes regulated by TAg, N136 and D44N but not by 3213. Genetic class 5, consisting of 152 up and 6 down-regulated genes, is the biggest class and represents genes regulated by T and N136 but not by 3213 and D44N.

Table 7: Genetic classification of genes regulated by different domains of TAg in villi.

Plus sign (+): represents regulation of genes by 3-fold or more by TAg or different mutants (N136, 3213, D44N). Minus sign (-): represents lack of regulation of genes. See text for details.

Genetic class	T	N136	3213	D44N	No. of upregulated genes	No. of downregulated genes
1	+	-	-	-	**0**	**4**
2	-	+	-	-	**3**	**0**
3	-	-	+	-	**1**	**2**
4	-	-	-	+	**4**	**2**
5	+	+	-	-	**152**	**6**
6	-	+	+	-	**0**	**0**
7	+	-	-	+	**0**	**0**
8	+	+	+	+	**1**	**1**
9	-	-	-	-	**12500**	
10	+	+	-	+	**0**	**7**

6.2.5 Comparisons of gene expression patterns between TAg and its mutants (N136 or 3213 or D44N)

To determine the affect of individual domains/regions of TAg on gene expression we have compared each mutant individually with wild-type TAg (Venn diagrams in Fig.41 and 42). To identify genes uniquely upregulated by TAg but not by the mutant, a filter is set where the TAg ratio was greater than or equal to 3 fold and the mutant ratio was less than 1.2 fold and similarly for identifying uniquely upregulated genes by the mutant. On the other hand to identify genes uniquely downregulated by TAg but not by the mutant, a filter is set where the TAg ratio was less than or equal to 0.33 fold and the mutant ratio was greater than 0.8 fold and similarly for identifying uniquely downregulated genes by the mutant. We have used DAVID 2.0 bioinformatics resource (http://david.abcc.ncifcrf.gov/home.jsp) to group genes into different biological classes.

(i) Comparison of the gene expression patterns between TAg and N136:

All of the genes upregulated by TAg (199) are also upregulated by N136 (Fig.41A). These genes are associated with E2F-dependent transcription, DNA replication and repair, cell-cycle regulation, chromatin assembly/modification and microtubule cytoskeleton. None of the genes were uniquely upregulated by TAg. On the other hand, N136 uniquely induces 11 genes that belong to biological classes such as carbohydrate metabolism and angiogenesis.

We did not find any specific class of genes uniquely downregulated by TAg or N136 (Fig.42A). However, there is a large overlap (93) of genes downregulated by both TAg and N136. These genes belong to different biological classes such as transmembrane proteins, chromosomal proteins, immune response/ antigen processing and protein kinases.

(ii) Comparison of the gene expression patterns between TAg and 3213:

3213 mice lose the expression of all genes (251) upregulated by TAg (Fig. 41B). Therefore we do not find any significant overlap of genes between TAg and 3213. We did not find any specific class of genes uniquely upregulated by 3213. Therefore all genes upregulated by TAg in enterocyte require an intact LXCXE motif.

A total of 76 genes are uniquely downregulated by TAg (Fig. 42B). These genes belong to the class of immunoglobulins, immune response/antigen processing, chromatin/histones, ion transport and fatty acid metabolism. Genes uniquely downregulated by 3213 belong to the family of cytochrome p450 (detoxification pathway). Like the upregulated genes, there is no significant overlap between TAg and 3213.

(iii) Comparison of the gene expression patterns between TAg and D44N:

Comparison of gene upregulation between TAg and D44N shows the same pattern as the comparison between TAg and 3213. D44N mice loose the expression of all genes (238) upregulated by TAg (Fig.41C). Therefore we do not find any significant overlap of genes between TAg and D44N nor find any specific class of genes uniquely upregulated by D44N. Hence all genes upregulated by TAg in enterocytes require an intact J domain.

Genes commonly downregulated by both TAg and D44N belong to the class of major histocompatibility complex and chromatin/histones. Total of 57 genes are uniquely downregulated by TAg (Fig.42C). These genes belong to the class of peroxisomal proteins, transmembrane proteins, immunoglobulins, oxidoreductase, carboxylic acid metabolism and

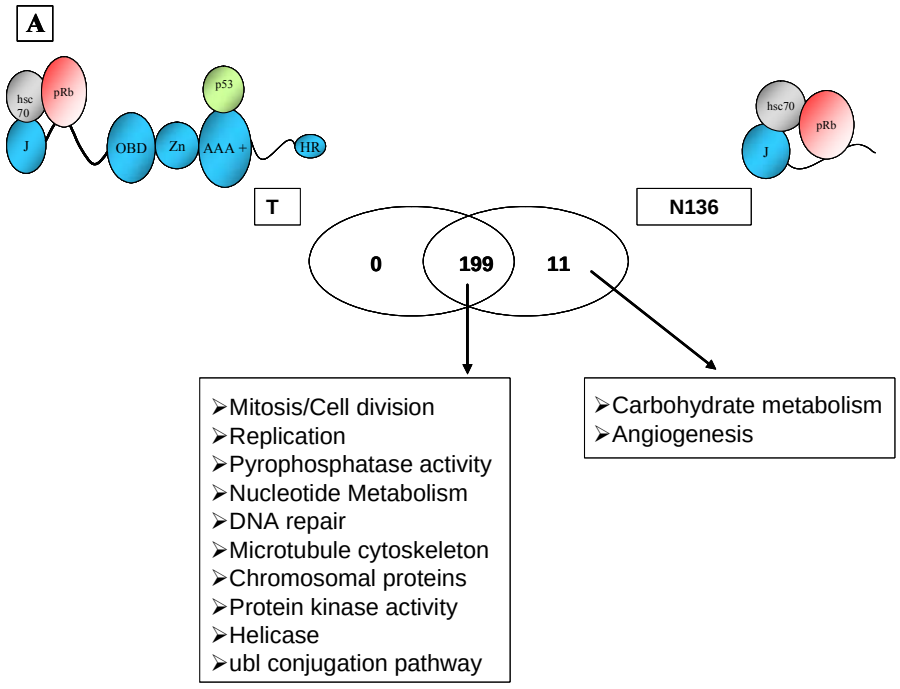
protein kinases. Additionally, 13 genes are uniquely downregulated by D44N and belong to the class of amino-acid biosynthesis and cell cycle.

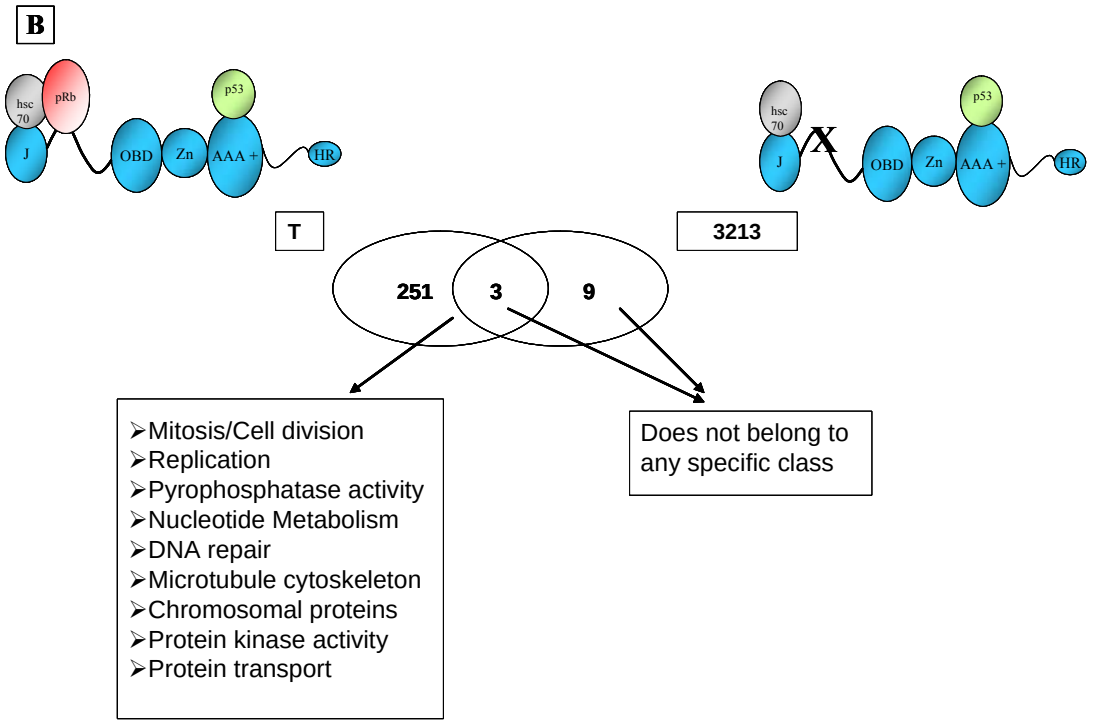
6.2.6 Conclusions from villi microarray

Almost all gene expression regulated by TAg in mouse intestinal enterocytes is dependent on the binding and inactivation of Rb-family proteins. The requirement of the LXCXE motif and the J domain for TAg mediated gene regulation is evident by the fact that TAg and N136 regulate the same set of genes and the regulation is lost by 3213 and D44N. Furthermore, p53 does not play any role in TAg mediated gene regulation in the intestinal epithelium as reflected by the inability of N136 to induce p53-target genes.

Figure 41: Upregulation of genes by TAg and mutants in villi.

In each Venn diagram the intersection represents the number of genes that are commonly upregulated 3-fold or more by TAg and different mutants (N136, 3213, D44N). Left circle represents genes which are uniquely upregulated by TAg while the right circle represent genes which are uniquely upregulated by a mutant. See text for details. Genes were classified into different biological classes using the DAVID 2.0 bioinformatics resource. Biological classes are sorted so that the genes with low p values (high enrichment score) come first in the list.

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HR
X
T
3213
251
3
9
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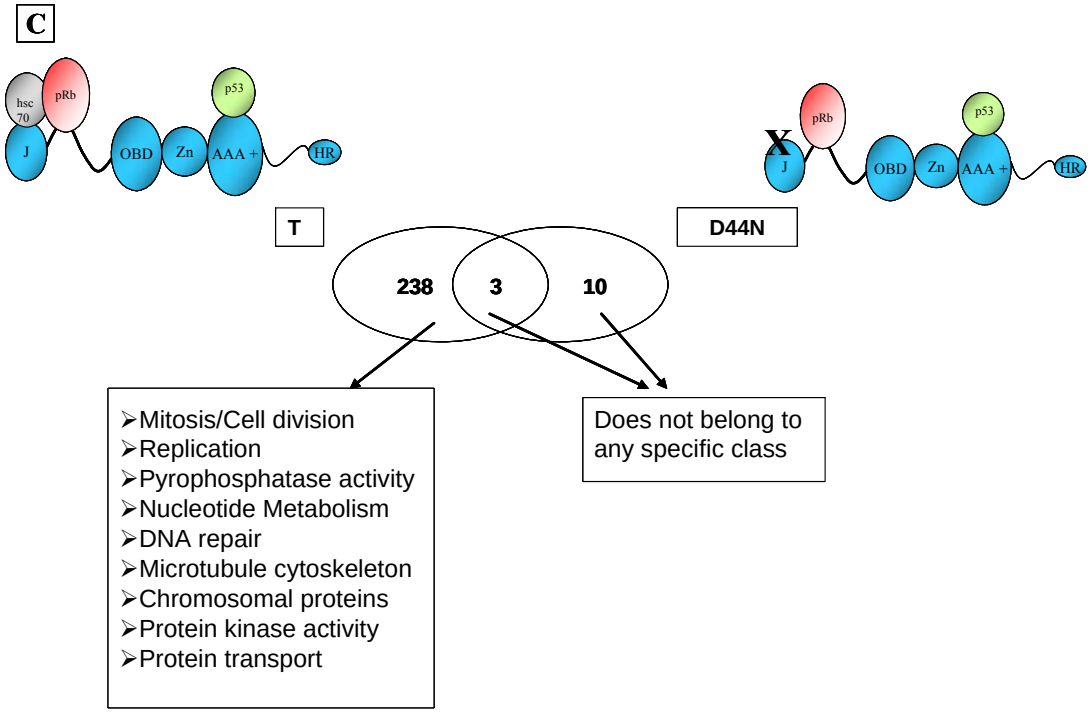
➢Replication
➢Pyrophosphatase activity
➢Nucleotide Metabolism
➢DNA repair
➢Microtubule cytoskeleton
➢Chromosomal proteins
➢Protein kinase activity
➢Protein transport
Does not belong to any specific class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3
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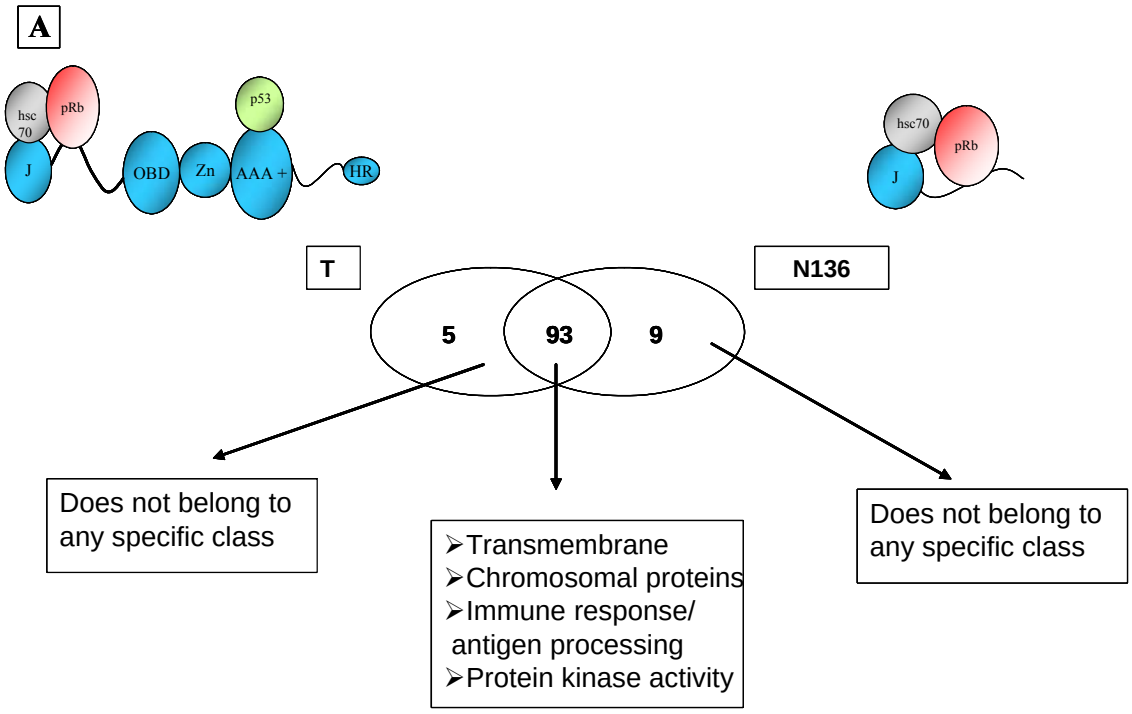
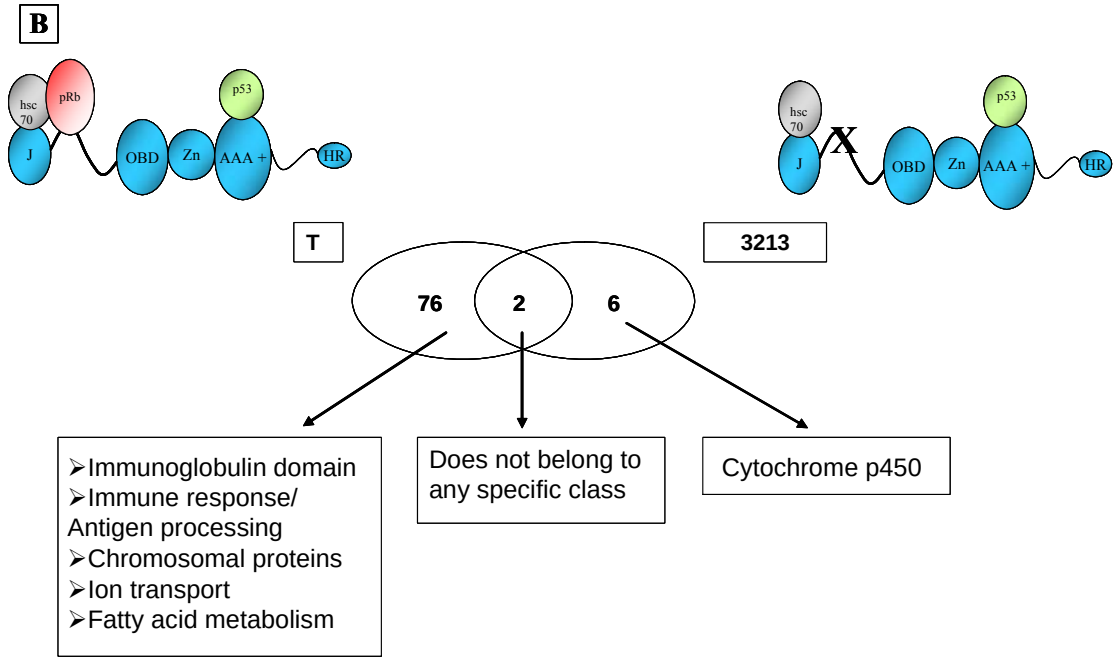
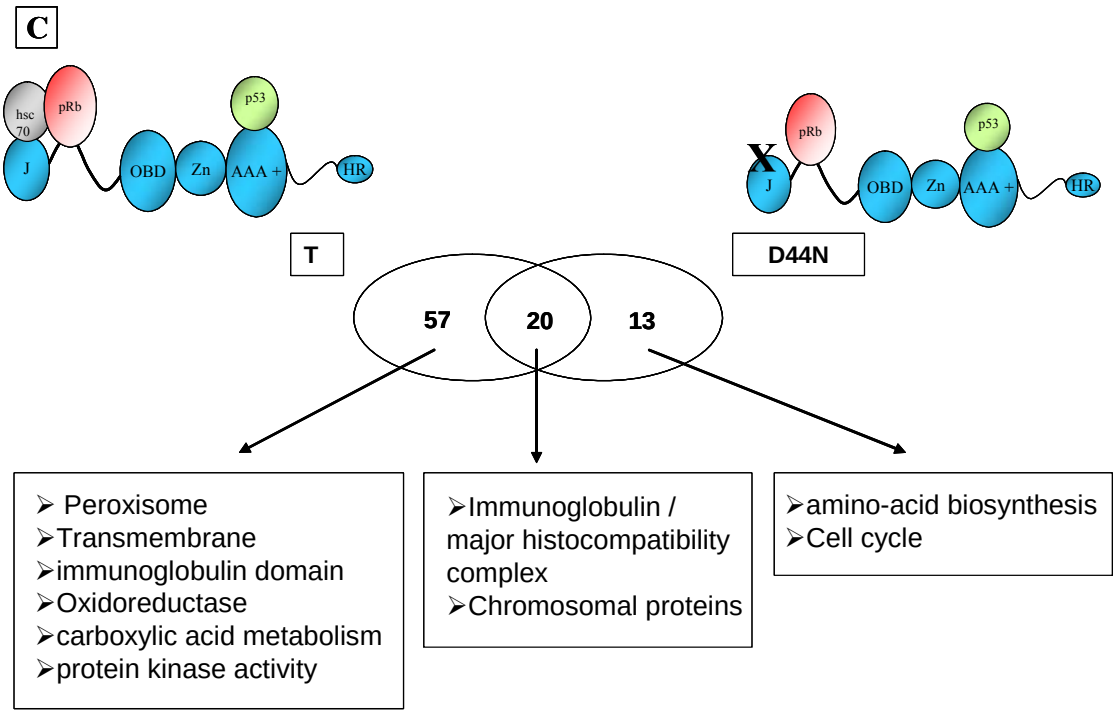
➢Replication
➢Pyrophosphatase activity
➢Nucleotide Metabolism
➢DNA repair
➢Microtubule cytoskeleton
➢Chromosomal proteins
➢Protein kinase activity
➢Protein transport
Does not belong to any specific class

Figure 42: Downregulation of genes by TAg and mutants in villi.

In each Venn diagram the intersection represents the number of genes that are commonly downregulated 3-fold or more by TAg and different mutants (N136, 3213, D44N). Left circle represents genes which are uniquely downregulated by TAg while the right circle represent genes which are uniquely downregulated by a mutant. See text for details. Genes were classified into different biological classes using the DAVID 2.0 bioinformatics resource. Biological classes are sorted so that the genes with low p values (high enrichment score) come first in the list.




B
hsc 70
pRb
J
OBD
Zn
p53
AAA +
HR
X
T
3213
76
2
6
➢Immunoglobulin domain
➢Immune response/ Antigen processing
➢Chromosomal proteins
➢Ion transport
➢Fatty acid metabolism
Does not belong to any specific class
Cytochrome p450


C
hsc 70
pRb
J
OBD
Zn
p53
AAA +
HR
X
T
D44N
57
20
13
➢ Peroxisome
➢Transmembrane
➢immunoglobulin domain
➢Oxidoreductase
➢carboxylic acid metabolism
➢protein kinase activity
➢Immunoglobulin / major histocompatibility complex
➢Chromosomal proteins
➢amino-acid biosynthesis
➢Cell cycle

7.0 DISCUSSION

The potent transforming activities of SV40 proteins in multiple cellular environments make this virus a very useful model system for human cancer research. Recently Deeb and coworkers identified an integrated gene signature from multiple T antigen mouse cancer models and found there was an association with the biological behavior and prognosis for several human epithelial tumors (Deeb et al., 2007). The similarity in the gene regulation between T antigen mouse cancer models and human cancers is not surprising due to the fact that TAg targets two major tumor suppressors, pRb and p53, which are found mutated in most human cancers. The goal of this dissertation is to test whether the Rb and p53 pathways are the main players in TAg induced transformation or if additional activities contribute. In the present study, I have used cell culture and animal systems expressing TAg and its mutants to discern the role of different domains/regions of TAg in the regulation of different biological process in a cell-type specific context. To this end, I found that Rb-family protein inactivation by the J domain of TAg is required for induction of intestinal hyperplasia (Chapter 3.0). To explore the role of the C-terminus of TAg, I have screened several transgenic lines expressing an amino-terminal mutant of TAg (N136) and found that these mice develop intestinal hyperplasia and progress to dysplasia after a prolonged latency period (Chapter 4.0). Furthermore, I did a global analysis of gene regulation by TAg and its mutants in MEFs and in mouse intestinal epithelium. I identified sets of genes regulated by TAg that belong to important biological classes, primarily cell cycle

and immune response (Chapter 5 and 6). Additionally, depending on the system used, our studies indicate that different consequences are produced by same oncogenic stimulation. This emphasizes the need to evaluate molecular pathways in a cell-specific context. In the following pages, I summarize the results from each project and discuss the importance of the data and future efforts.

7.1 THE J DOMAIN OF T ANTIGEN IS REQUIRED TO INDUCE ENTEREOCYTE PROLIFERATION AND INTESTINAL HYPERPLASIA

Cell culture studies using various mutants of SV40 TAg suggest that the J domain contributes to transformation. For instance, an in-frame deletion mutant of T antigen missing part of the J domain (*dl1135*) is defective for the induction of dense foci despite being able to bind Rb-proteins and p53 (Pipas et al., 1983; Srinivasan et al., 1997). In contrast, amino-acid substitution mutants of the J domain, such as D44N, are able to induce focus formation and anchorage-independent growth in cultured cells, although at a somewhat reduced frequency compared to wild type TAg (Hahn et al., 2002; Peden and Pipas, 1992; Stubdal et al., 1997). These differences in the observed phenotypes raise the question of why are some J domain alleles defective for focus formation while others are not. One possibility is that the interaction of the J domain with hsc70 is allele specific. For instance, perhaps a particular J domain mutant can stimulate hsc70 enough to achieve partial action on all or some of its substrates, leading to a subset of phenotypes observed with the wild-type TAg (Fewell et al., 2002; Sullivan et al., 2000b). Another possibility is that the SV40 J domain binds cellular proteins in addition to hsc70. In this scenario, some mutant alleles would disrupt T antigen interaction with specific subsets of the unknown cellular targets, thus losing the phenotypes resulting from this interaction while maintaining interaction with hsc70 or *vice versa*. Presumably, alleles resulting from the total loss of the J domain, such as deletions, might result in the loss of both binding functions.

Different J domain mutants show different phenotypes in cell culture and thus present a confusing picture about the role of J domain in transformation. Therefore, we decided to test the requirement of the J domain in a native tissue, namely the small intestine. The TAg J domain

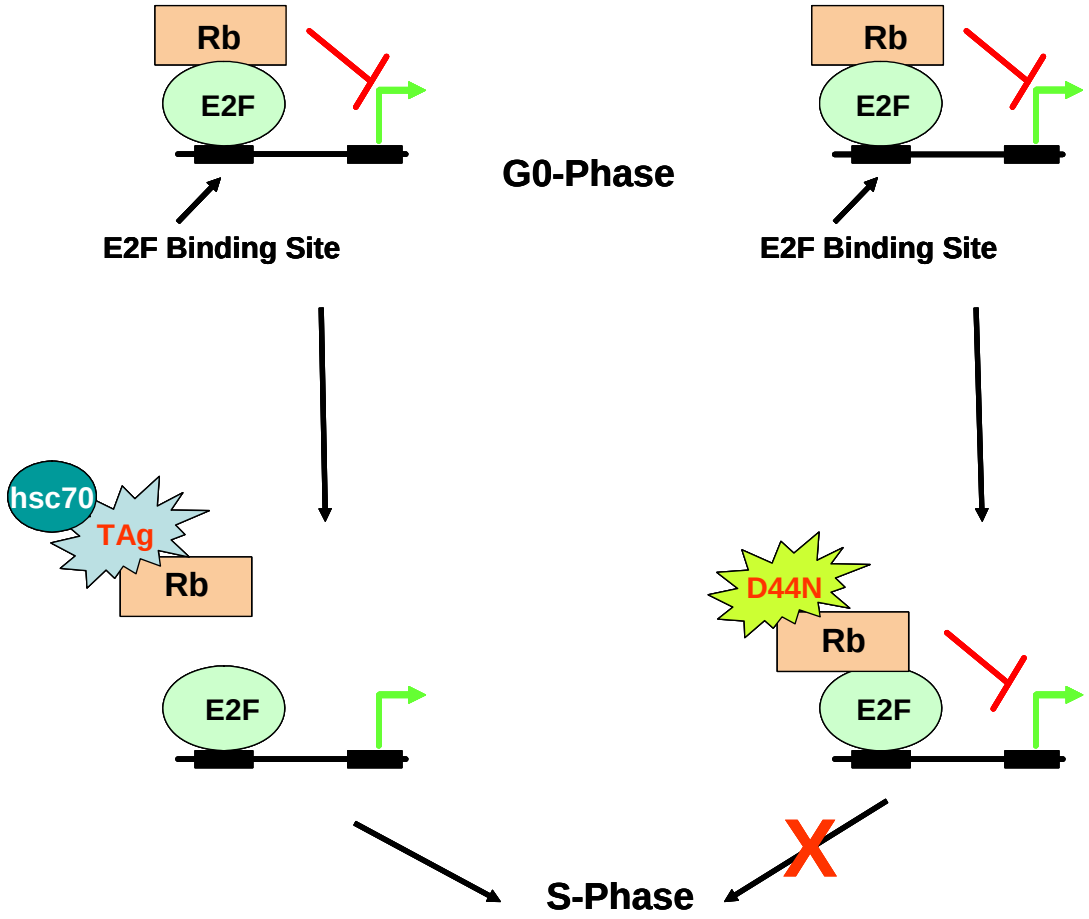
mutant D44N is capable of inducing foci and anchorage-independent growth in cell culture but, in contrast, we have found that its expression in villus enterocytes does not induce ectopic cell proliferation. The intestines of TAg^{D44N} mice retained normal morphological features in spite of the high levels of transgene expression in several transgenic lines tested. Based on these observations we conclude that TAg requires a functional J domain to induce enterocyte proliferation and intestinal hyperplasia.

7.1.1 The J domain of T antigen is required to inactivate p130.

Several cell culture studies have shown that the J domain of TAg cooperates with the Rb binding motif (LXCXE) to inactivate the growth suppressive functions of p130, p107 and pRb. Unlike TAg^{wt}, J-domain mutants do not alter the phosphorylation of p130 and p107 and are unable to degrade p130 (Stubdal et al., 1997). The TAg J-domain confers a growth advantage to normal mouse embryo fibroblasts (MEFs), but it is dispensable in the case of MEFs lacking both p130 and p107. This indicates that p107 and p130 have overlapping growth-suppressing activities whose inactivation is mediated by the J domain of TAg. *In vivo* and *in vitro* studies have shown that the TAg^{D44N} protein is unable to bind hsc70 and does not disrupt p130-E2F4 complexes (Sullivan et al., 2004) (Fig.43). All these observations strongly suggest that the J domain of TAg is required to overcome p130 and/or p107-mediated repression of E2F-dependent transcription, and thus contributes to transformation induced by T antigen. In agreement with the cell culture studies, we found that an intact J-domain is required to reduce the p130 protein levels in villus enterocytes (Fig.21). Our gel shift analysis indicated the presence of intact p130-E2F4 complexes in TAg^{D44N} mice which were completely absent in TAg^{wt} mice (Fig.23). These observations strongly suggest that TAg requires a functional J domain to downregulate p130.

Figure 43: J domain mutant D44N is not able to disrupt Rb-E2F complexes.

TAg^{D44N} protein is unable to bind hsc70 and does not disrupt p130-E2F4 complexes and therefore unable to induce E2F dependent genes required for S-phase.



In contrast, TAg^{wt} is able to increase both the RNA and protein levels of p107 while TAg^{D44N} upregulates only protein levels, suggesting that a functional J domain is required for the upregulation of the RNA levels. However, the increased p107 protein levels in the TAg^{D44N} intestines might be due to decreased turnover or degradation of the protein, resulting in protein stabilization. On the other hand, TAg^{wt} does not affect either the RNA or protein levels of pRb, though expression of TAg^{D44N} results in the accumulation of pRb protein, perhaps through alterations in the turnover rate. At this point we do not know the details of either mechanisms or if TAg requires interaction with other cellular factors to modify the pRb proteins.

7.1.2 A functional J domain is required for the induction of activator E2Fs.

Transgenic mice expressing TAg^{wt} in villus enterocytes upregulate protein and mRNA levels of all activator E2Fs (E2F1, E2F2 and E2F3a) (Saenz-Robles et al., 2007). This activation in turn induces cell proliferation and results in intestinal hyperplasia. Consistent with the normal morphology observed in TAg^{D44N} mice, we did not find upregulation of E2F1, and only a modest increase in E2F2 and E2F3a protein levels were found. The protein levels of E2F2 and E2F3a in TAg^{D44N} were higher than the nontransgenic but significantly less than the TAg^{wt} and therefore they might be insufficient to induce enterocyte proliferation. Furthermore, we were unable to detect Rb-free E2F2 or E2F3a activity by gel shift analysis. Additionally, the transcripts of several E2F targeted genes were unaffected, indicating no activation of the pathway (Fig.24). These results indicate that the E2F2 and E2F3a protein found in TAg^{D44N} enterocytes is not active transcriptionally.

7.1.3 Loss of p130 alone is not sufficient to induce intestinal hyperplasia.

Mice with null mutations in either *p107* or *p130* are viable and normal in certain genetic backgrounds, implying that loss of p130 or p107 alone is not sufficient to induce hyperplasia (Cobrinik et al., 1996; Lee et al., 1996). However, mice lacking both p107 and p130 die at birth (LeCouter et al., 1998a; LeCouter et al., 1998b), suggesting that p107 and p130 perform overlapping functions. It has been shown that the inactivation of pRb in conjunction with either p130 or p107 in villus enterocytes is sufficient to cause intestinal hyperplasia (Haigis et al., 2006). In agreement with these observations our results show that loss of p130 is insufficient to induce intestinal hyperplasia. At present, we can not assess the combined effects of a J domain and pRb in induction of hyperplasia because Rb-/- mice are embryonic lethal by day 13.5 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Future studies will require a conditional Rb knock out in a TAg^{D44N} background. Similarly, elucidation of the role of p107 will require analysis of TAg^{D44N} mice in a p130-/-; p107+/- background. These goals represent future efforts in the lab.

Animal studies using various J domain mutants suggest that the requirement for the J domain in transformation is cell-type and context specific. Although defective in cell culture, the J domain mutant *dl1135* (lacking small t expression) specifically induced the transformation of T lymphocytes in mouse. However, the same mutant was not able to transform B lymphocytes, suggesting a cell-type specific requirement of J domain for the transformation (Symonds et al., 1993). Similarly, our study also suggests the requirement of J domain in the induction of intestinal hyperplasia. At this point we can not exclude the role of small t antigen J domain in the transformation process. To our knowledge only one other study has used a J domain mutant of

TAg in another transgenic system (Ratineau et al., 2000). However, the requirement of the J domain alone in the development of tumors was not addressed.

Several possible explanations could contribute to the differences between the phenotype observed upon expression of TAg^{D44N} in cell culture and in our transgenic system. On one hand, the cell culture does not represent real *in vivo* conditions. Additionally, the mouse embryo fibroblasts, used in most of the cell culture studies, are derived from a tissue different than intestine. The differences observed between both experimental systems support the notion of tissue specific requirements for the J domain. Since TAg encodes multiple transformation-related activities, the subset of TAg functions needed and the corresponding target proteins could differ among tissue/cell types.

7.2 AN AMINO-TERMINAL TRUNCATION MUTANT OF T ANTIGEN INDUCES INTESTINAL HYPERPLASIA AND PROGRESSION TO DYSPLASIA AFTER A PROLONGED LATENCY PERIOD

The results presented here indicate that the transgenic mice expressing an amino-terminal region of TAg containing amino acids 1-136 under the control of the I-FABP promoter display ectopic enterocyte proliferation and present abnormally long intestines. However, the appearance of signs of dysplasia was significantly delayed (10-12 months) in comparison to similar morphological changes observed in TAg^{wt} mice (4 to 6 months). The late phenotype in TAg^{N136} mice may be due to lower percentage of proliferating cells in comparison with the TAg^{wt} mice. Consistent with the gradual severity in the phenotype, the fraction of S-phase enterocytes present in villi increased with the age of TAg^{N136} mice (Fig.27C-E).

7.2.1 Role of inactivation of Rb-family proteins in induction of hyperplasia

To determine the role of Rb-proteins in the induction of tumors in different cell types many studies have used a TAg mutant N121, which is very similar to N136 except for the absence of an nuclear localization signal. Both N121 and N136 proteins possess an intact LXCXE motif and a J domain, thus are able to bind and inactivate all Rb–family members similar to wild-type TAg, resulting in transactivation of E2F-target genes (Saenz-Robles et al., 2007). These amino-terminal mutants induce hyperplasia, although not as early as wild-type TAg does, suggesting that the inactivation of Rb family members is sufficient to induce the phenotype (Kim et al.,

1994). Our previous studies already demonstrated that the expression of TAg with a mutation in the LXCXE motif (3213) or the J domain (D44N) in villus enterocytes does not induce hyperplasia (Chandrasekaran et al., 1996; Rathi et al., 2007). Consistent with our studies it has been shown that the inactivation of individual Rb family members in the small intestinal and colonic epithelium had little or no effect, while loss of pRb combined with p107 or p130 produced chronic hyperplasia and dysplasia of the tissues (Haigis et al., 2006). We can conclude that the C-terminus of TAg is not required for the induction of hyperplasia.

7.2.2 Role of the C-terminus of TAg in progression to dysplasia

One major activity mapping to the C-terminus of TAg is interaction with p53. The capacity of large T antigen to bind the tumor suppressor protein p53 influences both the type of tumors developed and the kinetics of their appearance. Full-length large T antigen expressed under the lymphotropic papovavirus (LPV) transcriptional control region induces tumors in the choroid plexus epithelium (CPE), T lymphocytes, and B lymphocytes in transgenic mice (Chen et al., 1992). In contrast, under the same conditions, N121 induces only CPE tumors (Chen et al., 1992; Saenz Robles et al., 1994). These CPE tumors grow more slowly than those found in mice expressing full-length T antigen (Saenz Robles et al., 1994; Symonds et al., 1993). When N121 transgenic mice are bred with p53-null mice, rapidly growing CPE tumors develop (Symonds et al., 1993). In addition, N121/p53-null mice develop T- and B-cell lymphomas. Similarly, inactivation of p53 in N121 mice by coexpression of a p53 dominant-negative mutant protein results in aggressive CPE tumors (Bowman et al., 1996). These results indicate that the spectrum of tumors induced by mutant T antigens depends on specific activities that they retain and

emphasize the importance of inactivating p53 in the development of aggressive CPE tumors and lymphomas.

On the other hand, many studies show that the inactivation of p53 by TAg is not always required for transformation or tumorigenesis. Cell culture studies show that coexpression of the dominant-negative p53 molecule along with N136 is unable to induce focus formation or saturation density. This suggests that inactivation of p53 is not sufficient for transformation and may required a novel transforming activity mapping to the C-termiuns of TAg (Sachsenmeier and Pipas, 2001; Srinivasan et al., 1997). Several studies have been performed with transgenic mice expressing TAg or amino-terminal truncation mutants in various tissues, and indicate no role for p53 in tumor induction. For instance, the expression of an amino-terminal fragment of T antigen (N147) in the acinar cells of the pancreas induces tumors that are indistinguishable from those induced by wild-type T antigen. The tumors induced by N147 still express a wild-type p53 (Tevethia et al., 1997). On the contrary, the expression of the N121 truncated T antigen in astrocytes results in their re-entry into the cell cycle, accompanied by extensive apoptosis. However, this apoptosis is not alleviated by the elimination of p53, rather it is dependent on a functional PTEN protein (Xiao et al., 2002).

Both wild-type T antigen and N121 induce S-phase and hyperplasia when expressed in intestinal enterocytes (Hauft et al., 1992; Kim et al., 1994). Nevertheless, inactivation of Rb-proteins by N121 does not result in increased apoptosis and N121 expresing mice do not show any signs of dysplasia even at very old age. Molecular studies indicate that enterocytes do not express significant amounts of p53 and that there are no detectable T antigen–p53 complexes present in the intestines of these transgenic mice (Markovics et al., 2005). This indicates that the delayed progression to dysplasia observed in this study by N136 can not be associated with p53.

7.2.3 Levels of protein / concentration dependent phenotype

There have been reports correlating the levels (amount) of TAg expression with the tumor phenotype. In the study by Kim et al , two lines of TAg mice shows very different phenotypes depending on the amount of protein expressed (Kim et al., 1993). Another study showed that liver tumors were induced earlier in N121 homozygous mice than in heterozygotes, leading to much more aggressive tumors and the rapid death of the mice (Bennoun et al., 1998). We did not find such a concentration dependent phenotype. Two independent N136 transgenic lines expressing similar levels of protein, and comparable to wild-type TAg, were unable to induce dysplasia as early as TAg.

7.2.4 Patterns of TAg staining

We found some significant differences in the patterns of TAg expression in villus enterocytes of TAg and N136 mice by immunostaining. TAg mice show a dense nuclear staining throughout the villi, villi/crypt differentiation zone and occasionally (not in every crypt) extended to the top of the crypts (Fig.30C). On the other hand, N136 mice show staining only in the top 3/4th of villi and the staining never goes down to the villi/crypt differentiation zone (Fig.30B). These observations raise a question if the location of TAg expression is responsible for the observed phenotypes in TAg and N136 mice. In general, we can ask does initiation and progression of tumors depend on the specific cell population crypts (cycling) or villi (quiescent) challenged by oncogenic signals? More experiments are needed to prove if this is the major factor affecting the rate of tumor progression. One such experiment would be making transgenic mice which express TAg or N136 specifically in the crypts.

Additionally, nuclear vs. cytoplsmic distribution of an oncogene can be very crucial for cell fate/phenotype. Even though N136 has an NLS, we still see staining in the cytoplasm. Due to the very small size of this protein (136 amino acids, ~21kDa), free entry and exit from the nucleus makes it more accessible for interaction with cytoplasmic proteins. Similarly, N121 which does not have an NLS was found in both the nuclei and cytoplasm of CPE tumors (Saenz Robles et al., 1994).

In summary, the first 136 amino acids of TAg are sufficient to induce hyperplasia in mouse intestinal epithelium whereas the p53-binding function is dispensable. The inactivation of Rb-proteins appears to be the main requirement for the induction of hyperplasia. However, at this point we can not eliminate the possibility of N136 having activity/ies, in addition to Rb inactivation that playing role in the induction of hyperplasia. In this report we suggest that delayed onset of proliferation and prolonged latency period in TAg^{N136} mice can be caused by (1) lack of concentrated nuclear expression of the protein, (2) protein expression only in the top $3/4^{th}$ of the villi, and 3) absence of some unknown transforming activity of TAg residing in the C-terminus.

7.3 ROLE OF MULTIPLE DOMAINS OF T ANTIGEN IN GENE REGULATION AND TRANSFORMATION IN MOUSE EMBRYO FIBROBLASTS AND IN MOUSE INTESTINAL EPITHELIUM

Large TAg contains several intrinsic biochemically active domains with distinct functions. The expression of TAg induces cell proliferation, inhibition of apoptosis which ultimately leads to immortalization of primary cells, induces cell transformation and induction of tumors in mice. The present study is aimed to determine if large TAg exerts many of these activities through influencing cellular gene expression by (a) inactivating RB-family proteins and inducing E2F dependent transcription (b) inactivating p53 and blocking p53 dependent transcription (c) regulating gene expression by interacting with other targets (such as transcription factors TBP, TEF-1, TAFs, TFIIB, and RNAPolII) (Table 8).

We have performed microarray analysis to observe the change in global gene expression induced by TAg or its mutants presumably via one of the mechanisms described above and to link changes in gene expression with the known transforming functions or novel activity of TAg. We have examined effect on gene expression in MEFs and transgenic mice expressing wild-type TAg or its mutants N136, 3213 or D44N.

Table 8: Interaction of SV40 large T antigen with cellular proteins to elicit transformation.

Cellular protein	Biological consequences	References
pRb/p130/p107	promotes G1/S transition	(DeCaprio et al., 1988)
p53	promotes G1/S transition, block apoptosis	(Pipas and Levine, 2001)
Hsc70	Disruption of pRb-E2F complex	(Sullivan et al., 2000a; Sullivan and Pipas, 2002)
p300/CBP	Unknown	(Poulin et al., 2004)
Cul7	Unknown	(Ali et al., 2004; Kohrman and Imperiale, 1992)
Bub1	Override spindle checkpoint	(Cotsiki et al., 2004)
Nbs1	Genomic instability	(Digweed et al., 2002; Wu et al., 2004)
Fbw7	Unknown	(Welcker and Clurman, 2005)
IRS-1	Signal transduction	(DeAngelis et al., 2006)
TEF-1	Change in gene expression	(Dickmanns et al., 1994)
AP-2	Change in gene expression	(Mitchell et al., 1987)
Sp1	Change in gene expression	Reviewed in (Moens et al., 1997)
TBP	Change in gene expression	(Moens et al., 1997)
TAF1 ($TAF_{II}250$)	Change in gene expression	(Moens et al., 1997)
TAF4 ($TAF_{II}130$)	Change in gene expression	(Moens et al., 1997)
TAF5 ($TAF_{II}100$)	Change in gene expression	(Moens et al., 1997)
RNA polII (140kDa subunit	unknown	(Moens et al., 1997)
AP-1 (c-Jun and c-Fos)	Change in gene expression	(Moens et al., 1997)

7.3.1 TAg interaction with RB-family proteins is required for regulation of E2F-target genes

TAg-induced cell cycle deregulation is mainly achieved by the direct targeting of key regulators of the cell cycle. According to the current model, TAg binds and inactivates Rb proteins which results in the release of E2F from Rb-complexes and subsequently the induction of E2F dependent transcription of S-phase genes. If this is true then N136 should be able to regulate the same set of E2F target genes as full-length TAg. N136 has an intact LXCXE motif and J domain and therefore it should be capable of inactivating Rb proteins and inducing E2F dependent transcription. Indeed, we found that N136 upregulates E2F-target genes as efficiently as wild-type TAg in both MEFs and enterocytes. E2F-target genes are involved in key regulatory process such as DNA replication, mitosis/cytokinesis, DNA repair and transcriptional control. For example, all mini-chromosome maintenance proteins (MCM 2-7) are profoundly upregulated by both TAg and N136. MCMs are components of the prereplicative complex (pre-RC) that are essential for DNA replication (Tye, 1999). Other E2F target genes upregulated by both TAg and N136 are involved in nucleotide metabolism (such as dihydrofolate reductase, thymidine kinase, ribonucleotide reductase) and in DNA replication (such as DNA polymerase α, DNA topoisomerase alpha, PCNA, replication protein A2, DNA ligase 1 etc). Furthermore, cell cycle (mitosis/cytokinesis) genes such as Cyclins E1, A2, and B, Cdc2a, Cdc6, Cdc20 and DNA repair genes such as BRCA1 and RAD51 show profound upregulation by both TAg and N136. Some of the E2F target genes such as histones H2afz and H2afx, p107, Ezh2, Mybl2, Smc2l1 and Hmgb2 are directly involved in transcriptional control of downstream genes.

According to the model, the LXCXE motif and J domain are required for the complete inactivation of Rb-family members and thus for the induction of the above genes. Transgenic

mice support the model since 3213 and D44N lose regulation of almost all genes upregulated by TAg and N136 and are therefore unable to induce intestinal hyperplasia. This suggests that almost all TAg mediated gene regulation in enterocytes of transgenic mice is mediated by Rb-E2F pathway.

Consistent with the model MEFs expressing TAg and N136 also show upregulation of E2F-target genes. However, 3213 and D44N do not completely loose the regulation of these genes. One of the possible explanations for the intermediate upregulation of E2F-target genes by 3213 and D44N is the presence of some other factors/activity (independent of LXCXE and J domain) at the C-terminus of TAg which may contributes to Rb-family inactivation. It has been reported that MEFs expressing either TAg N-terminal fragment (1-147) or C-terminal fragment (251-708) have an extended life span, but are not immortal. The cooperation between these two fragments is required for immortalization (Tevethia et al., 1998). Furthermore, the C-terminal fragment (251-708) of TAg cooperates with oncogenic ras to transform primary REFs independent of p53, suggesting the presence of an activity in the C-terminus of TAg that bypasses Ras-induced senescence (Beachy et al., 2002).

Alternatively, it is also possible that 3213 and D44N have leaky phenotype and that they do not completely defective in inactivating Rb-fmaily members. However, this possibility is unlikely since 3213 and D44N both are unable to downregulate p130 protein levels. Third possibility is that the small t is relieving E2Fs from Rb repression via upregulation of Cyclin D1.

7.3.2 Inactivation of p53 by TAg is required for the inhibition of p53-target genes

As an immediate response to virus infection, the host cell activates a cascade of genes with the aim of inhibiting cell proliferation or inducing apoptosis. One such gene is p53 which is a potent transcriptional activator. As p53 steady-state levels rise it upregulates a number of genes, some of which mediate cell cycle arrest or apoptosis. Genes with a p53 binding motif in their regulatory regions that are activated by p53 include p21, MDM2, Cyclin G1, Apaf1, Fas, PERP and NOXA (Table 5). TAg interacts with the DNA-binding surface of p53, blocking its ability to bind promoters and thus to regulate gene expression. Consistent with this fact, we do not see regulation of p53-target genes in MEFs expressing TAg. On the other hand, N136 lacks p53 binding region and therefore can not block p53-dependent transcription of target genes. Upregulation of p53-target genes in N136 MEFs can cause cell cycle arrest and apoptosis and may contribute to transformation negative phenotype. Large TAg may also affect p53 mediated transcription by competing with the TATA-binding protein (TBP). TAg and p53 were shown to bind the same region of TBP (Martin et al., 1993).

Neither wild-type TAg nor N136 regulate p53-dependent genes in enterocytes of transgenic mice. Thus it appears that in enterocytes, TAg regulates cellular gene expression mainly through Rb-E2F pathway and there is no evidence for p53 response. This finding is consistent with the previous study from our lab which reported that villus enterocytes do not express p53 (Markovics et al., 2005). The comparison between the regulation of p53-dependent genes by N136 in MEFs and enterocytes is shown in Table 5.

We found that N136, but not wild-typeTAg upregulaties several growth factors such as amphiregulin, PDGF-α, LIF, BMP8a, proliferin and GD-15 in MEFs. Upregulation of some of

these growth factors (such as amphiregulin, proliferin, PDGF-α and GD-15) may be dependent on p53. One intriguing possibility is that these factors may explain the growth of N136 expressing MEFs in cell culture even in the presence of active p53. These growth factors might be involved in proliferation and antiapoptotic responses to counteract p53 mediated cell cycle arrest and apoptosis. One of such example is amphiregulin which is a member of epidermal growth factor (EGF) family. Amphiregulin acts as an autocrine, antiapoptotic growth factor as well as a mitogen for a broad range of target cells (Castillo et al., 2006). Additionally, platelet-derived growth factor (PDGF-alpha) is a potent mitogen for cells of mesenchymal origin. Moreover, Proliferin provides a growth stimulus to target cells in maternal and fetal tissues during the development of the embryo at mid-gestation (Adamson et al., 2002). All these examples suggest a role for growth factors in nullifying the effects of p53 induced cell cycle arrest or apoptosis in MEFs expressing N136. On the other hand, GD-15 which belongs to the TGF-beta family, recently was shown to serve as a secreted biomarker for activation of the p53 pathway and induces growth arrest and apoptosis by paracrine mechanism (Yang et al., 2003).

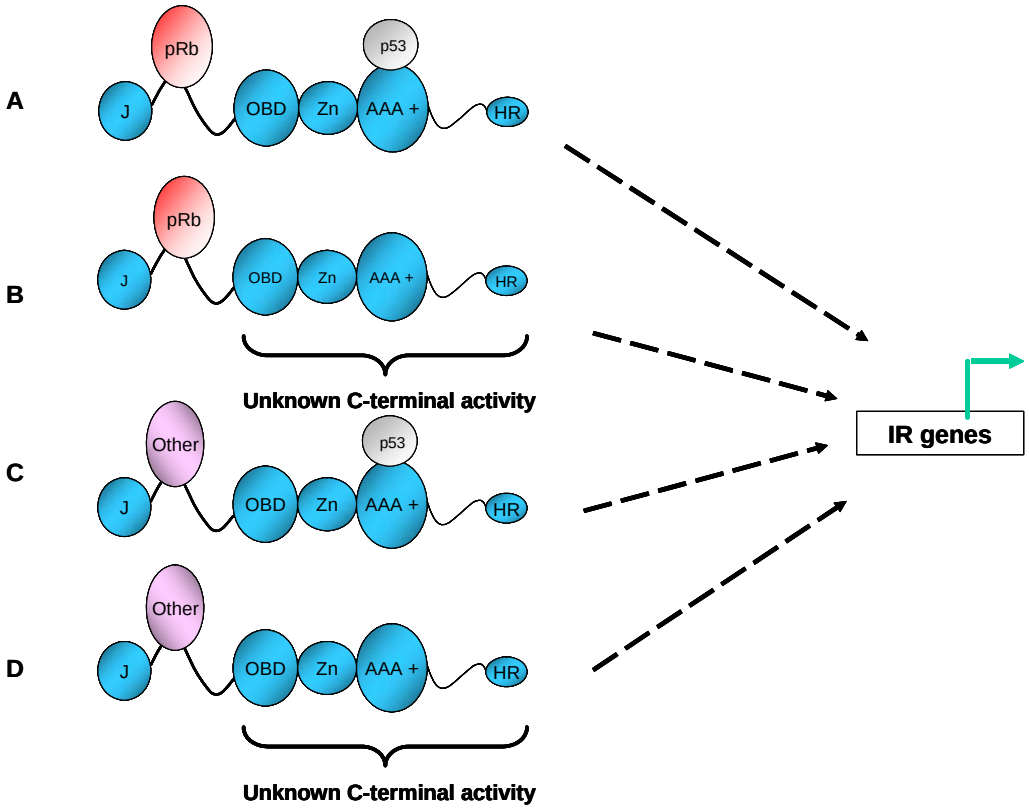
7.3.3 TAg regulate expression of immune response genes

One of the major findings of this study is the upregulation of immune response genes (such as STAT1, Ifi44, Ifi27, Oas2, GTPase2, Rsad2, Mx1, Oasl2, IRF7 and PKR) by MEFs expressing TAg and D44N but not by N136 and 3213. This suggests a requirement for the C-terminus of TAg and LXCXE motif in their regulation. Based on these findings we hypothesize that regulation of these genes require TAg interaction with (1) Rb-family members (Fig44 A, B). (2) p53 (Fig.44 A, C) (3) unknown target (that interacts with LXCXE motif) and an activity reside on the C-terminus of TAg (Fig.44 D). To test these hypotheses, future experiments will be

required: (1) Expressing N136 in p53-/- MEFs. If we find upregulation of IR genes then we conclude that inactivation of p53 and some LXCXE binding protein (Rb or other) is required for the induction of IR genes (Fig. 44 A and C). (2) Expressing 3213 in MEFs devoid of pRb, p107 and p130 (TKO MEFs). If we see upregulation of IR genes then we conclude that inactivation of Rb-family proteins and p53 or some other C-terminal activity are required for the induction of IR genes (Fig. 44 A and B). (3) Determine the basal levels of IR genes in Patch1 (p53-binding defective mutant). If we see upregulation of IR genes then we conclude that inactivation of Rb or other LXCXE binding protein and some unknown activity of C-terminal is required for the induction (Fig 44 B and D)

Figure 44: Four possible mechanisms for regulation of immune response genes by Large T antigen.

"Other" represents any LXCXE binding protein except RB-family proteins. IR = Immune Response



Surprisingly, we did not find upregulation of immune response genes (such as STAT1, Ifi44, Ifi27, Oas2, GTPase2, Rsad2, Mx1, Oasl2, and IRF7) in enterocytes of mice expressing TAg. We hypothesized that in enterocytes TAg does not upregulate these genes. However, one of the possibilities is that in enterocytes the levels of these genes are already high and therefore we do not see activation of these genes in our microarray analysis. In fact, comparison between the raw signal values for enterocytes of nontransgenic mice and normal MEFs reveal that enterocytes of nontransgenic mice have very high signal values for immune response genes in comparison to normal MEFs.

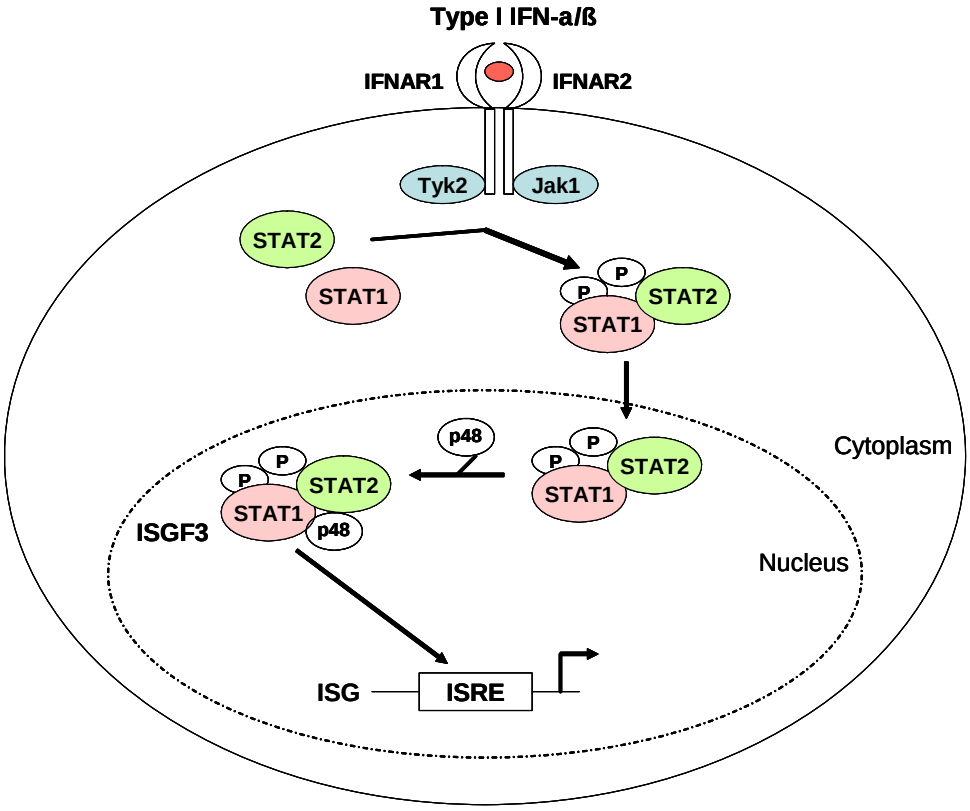
Furthermore, we found that some of the immune response genes such as STAT1, Ifi44, GTPase2, Mx1 and Oasl2 are downregulated 2 to 3 fold by TAg, N136 and D44N mice but not by 3213 mice. This finding suggests that TAg binding to Rb-proteins is required to downregulate these immune response genes in villus enterocytes. Alternatively, TAg can bind to LXCXE binding protein(s) other than Rb-family proteins and thus affect the expression of immune response genes in enterocytes.

The majority of immune response genes upregulated by TAg in MEFs are belong to the class of interferon-stimulated genes (ISG). Therefore, I would like to provide a brief description about the interferon (IFN) pathway. IFNs are multifunctional secreted proteins that involved in antiviral defense, cell growth regulation and immune activation. IFNs are classified into two distinct types namely Type I and Type II. Type I IFNs are produced in direct response to virus infection and consists of two main members INF-α and INF-β. Type II INFs consists of IFN-γ and produced in response to the recognition of infected cells by activated T lymphocytes and natural killer (NK) cells (reviewed in (Samuel, 2001)). Further details are focused on Type I IFN-α/ β.

IFN-α/ β binds to their multisubunit receptor (IFNAR1 and IFNAR2) and initiates the tyrosine phosphorylation of Janus kinase Tyk2 and JAK1 and the receptor. Subsequently, two cytoplasmic signal transducing activators of transcription (STAT) proteins, STAT1 and STAT2, are phosphorylated at specific tyrosine residues. The phosphorylated STAT1/STAT2 heterodimers then dissociates from the receptor and translocate to the nucleus. In the nucleus they associate with the DNA-binding protein p48 to form a heterotrimeric complex called ISGF3, which binds the IFN-stimulated response element (ISRE) of IFN-α/ β stimulated genes (ISG)(reviewed in (Darnell et al., 1994; Goodbourn et al., 2000) (Fig.45).

Figure 45: Signaling pathway activated by IFN-α/β.

Interferons bind to their cognate receptors (IFNAR1 and IFNAR2) and initiate a signaling cascade that involves the STAT family of transcription factors. Upon activation by phosphorylation, STAT1 forms heterodimer with STAT2 and then translocates to the nucleus. In the nucleus STAT1/STA2 heterodimer bind to p48 and form a complex ISGF3. This complex then binds and activates interferon-stimulated response elements (ISRE) of target genes (ISGs).



The best characterized IFN-inducible components of the antiviral response are protein kinase PKR, 2'-5' oligoadenylate synthetase (OAS), protein MxA GTPase and RNA-specific adenosine deaminase ADAR1. All these genes are highly upregulated by SV40 infection and also in MEF-T. The purpose for infecting MEFs with SV40 virions was to ensure that alterations to the expression of ISGs were not artifacts of the transfection of cells with SV40 DNA. Induction of all of the above genes by SV40 in MEFs following infection confirms that this response is specifically mediated by SV40.

PKR is a cytoplasmic serine/threonine kinase induced by both type I and II IFN in the presence of dsRNA (Ben-Asouli et al., 2002). Activated (phosphorylated) PKR can further phosphorylate several substrates, including eIF-2α. Phosphorylated eIF-2α sequesters eIF-2B, which is crucial for the recycling of eIF-2 between successive rounds of initiation, leading to inhibition of protein synthesis (Hinnebusch, 2005).

The 2'5' OAS proteins catalyze the polymerization of ATP to 2'-5'-linked oligoadenylates (2'-5'-A). 2'-5'-A binds with RNase L and induce the formation of active RNAase L. Activation of RNase L results in the degradation of viral mRNAs, blocking protein synthesis, and mediates the induction of apoptosis (Dong and Silverman, 1995; Rusch et al., 2000).

MxA protein, a dynamin superfamily GTPase, interferes with viral replication probably by blocking the activity of viral polymerases (Frese et al., 1996; Kochs and Haller, 1999a; Kochs and Haller, 1999b).

The IFN-inducible dsRNA specific adenosine deaminase (ADAR) catalyzes C6 deamination of adenosine to inosine in viral and cellular RNA (Liu and Samuel, 1996; Patterson and Samuel, 1995). Consequently, target RNA coding capacities are changed which causes

amino acid substitution which in turn results in synthesis of proteins with altered functions (Liu and Samuel, 1996).

There are several other less studied ISGs such as ISG56, ISG15, Rsad2, P200 gene family were significantly upregulated in SV40 infected and transfected MEFs. A member of the p56 family of proteins, ISG56, is one of the strongly induced genes in response to IFN, dsRNA and many viruses. P56 family members bind to eIF-3 via multiple tetratricopeptides motifs and cause impairment of eIF-3 which results in inhibition of viral protein synthesis (Hui et al., 2003; Hui et al., 2005; Terenzi et al., 2005).

As with ISG56, ISG15 is strongly induced by IFN, dsRNA, and virus infection. ISG-15 show strong upregulation (10 fold) in MEF-T cells. ISG15 encodes a small, ubiquitin-like protein that gets conjugated to many cellular proteins. ISG15 may have a major role in IFN's action against certain viruses, because the NS1B protein of influenza B virus binds ISG15 and blocks its conjugation to cellular proteins (Yuan and Krug, 2001).

We also found induction of another IFN-inducible gene, cig5 or Rsad2. This antiviral protein is expressed in response to human cytomegalovirus (HCMV) infection, and shown to inhibit viral replication by its envelope protein glycoprotein B (Chin and Cresswell, 2001).

We found upregulation of two members of the P200 gene family, p202 and p204. The p202 protein inhibits cell proliferation by binding to and inhibiting the functions of several transcription factors such as NF-kB, E2F, p53, c-fos, c-jun, and Rb. The possible role for the interaction between p202 and Rb in the growth inhibitory activity of interferons is not clear (Choubey et al., 1996). On the other hand, p204 protein inhibits ribosomal RNA (rRNA) transcription by binding to the UBF1 transcription factor that is required for rRNA synthesis (Liu et al., 1999). It has been shown that TAg expression activates a cellular kinase resulting in

increased phosphorylation of the transcription factor UBF which promotes the formation of a stable UBF-SL1 (TBP-TAF_I) complex and the induction of rRNA synthesis (Zhai and Comai, 1999). These observations present a clear scenario of competition between host and virus for binding to same cellular transcription factor for their own benefit.

Cellular IFN regulatory factors, IRF3 and IRF7, are a family of transcriptional regulators and are important for viral induction of IFN genes and ISG. IRF3, a key transcriptional activator, is a subunit of dsRNA-activated transcription factor complex (DRAF) (Lin et al., 1999). Activation of IRF3 by virus infection or by dsRNA results in its translocation to the nucleus where it binds to the transcriptional co-activator p300/CBP and contributes to the activation of IFN α/β responsive genes (Bandyopadhyay et al., 1995; Daly and Reich, 1993; Hummer et al., 2001). Though our data do not show direct induction of IRF3, many of the genes induced by IRF3 such as ISG56, ISG49, OAS and cig5 (Grandvaux et al., 2002) are up-regulated in the MEFs expressing TAg. On the other hand, IRF7 was upregulated 4-fold in our experimental system. IRF7 is a critical determinant for induction of the IFN genes and functions in part by a positive feedback mechanism (Marie et al., 1998).

Interestingly, TAg is capable of inducing downstream interferon pathway without affecting the levels of IFN-α and IFN-β itself. One explanation for an IFN-independent signaling cascade in SV40 infected MEFs could be the activation of ISG by virus specific dsRNA. During the replication cycle of SV40 there are several stages when dsRNA can form as an intermediate product of transcription. However this can not explain IFN-independent regulation of ISG in MEFs transfected with DNA expressing early region of SV40. One of the possibility is that TAg itself mimics IFN-α and IFN-β and triggers the downstream pathway. Alternatively, TAg can directly (independent of receptor activation) activate gene (s) of intereferon pathway. Future

experiment will be required to determine (1) the phosphorylation status of STAT1 and STAT2 (2) nuclear localization of STAT1/STAT2 heterodimer.

Several viruses target the IFN signaling system and elicit immune responses during infection. The induction is more pronounced in RNA viruses, like Hantaan virus, human parainfluenza virus and Rous sarcoma virus (Garcia-Sastre et al., 1998; Kong et al., 2003; Nam et al., 2003). Most DNA viruses, such as adenoviruses and human papillomavirus, use multiple mechanisms to weaken the STAT-mediated antiviral responses that are presumed to help evade host immune surveillance. Nevertheless, the SV40-induced response described in this report is very similar to that induced by several herpesviruses, such as Epstein–Barr virus, herpes simplex virus, Kaposi's sarcoma-associated herpesvirus and HCMV. All of these viruses activate IFN-responsive genes, such as MxA and OAS (Browne et al., 2001; Mossman et al., 2001; Poole et al., 2002; Ruvolo et al., 2003; Zhu et al., 1998).

One of the important questions that arise is how MEFs expressing TAg transform in the presence of high levels of ISGs. It is possible that somewhere downstream TAg (by inducing other pathways) counteracts the growth inhibitory and apoptotic activities of ISGs.

7.3.4 Gene regulation by C-terminus of TAg

About 20 genes were found to be uniquely downregulated in N136 expressing MEFs. Perhaps some activity mapping to the C-terminus of TAg is required to relieve the repression of these genes. Alternatively, it is also possible that some new interactions of N136 itself may playing a role in regulation of these genes. Genes downregulated by N136 belong to the class of cell adhesion molecules (such as cadherins, VCAM1), lipoprotein lipase, secreted frizzeled-related sequence protein 2 (Sfrp2), GLI-Kruppel family member GLI2. VCAM1 is important in cell-cell

recognition and appears to function in leukocyte-endothelial cell adhesion. Sfrp2 is a Wnt inhibitor and function as a modulator of Wnt signaling through direct interaction with Wnts. They have a role in regulating cell growth and differentiation in specific cell types (Oshima et al., 2005). SGLI2 is a transcription factor involved in transducing Sonic hedgehog (Shh) signals in vertebrates. It has been suggested that Shh-dependent Gli2 activation plays a critical role in epithelial homeostasis by promoting proliferation through the transcriptional control of cell cycle regulators (Mill et al., 2003).

I recognize that microarry data show the regulation of gene expression at the mRNA level only. We have no evidence that the protein levels of these genes change similarly or that their regulation has any significance. However, based on the literature we can make some predictions about the role of other interactions of TAg in regulation of above genes. TAg has been reported to interact with the transcription factors TEF-1 (Transcription enhancing factor-1) and TBP (TATA binding protein). These interactions are missing in N136. It has been shown that TAg OBD binds to the DNA-binding domain of TEF-1 and inhibits the binding of TEF-1 to target DNA sequences. A TEF-1 binding deficient mutant of TAg (S189N) is seven-to eight fold less efficient than wild-type TAg in focus formation assay (Dickmanns et al., 1994) suggesting that TEF-1 might also regulate genes involved in growth control. The inability of N136 to bind TEF-1 may also contribute to the transformation defective phenotype of N136. Furthermore, TBP is a subunit of TFIID, a major component of the transcription initiation complex. Several groups have reported *in vitro* and *in vivo* interaction of amino terminal of TAg (amino acids 5 to 172) with the conserved C-terminal domain of TBP (Damania and Alwine, 1996; Gruda et al., 1993; Johnston et al., 1996; Martin et al., 1993). The effect of this interaction on transcription is yet to be determined, although TBP is required for all three RNA polymerases and thus TAg

could potentially affect transcription by all these. It will be interesting to check if Patch 1 is able to downregulate these genes as it is expected to bind to both TEF-1 and TBP. Another study suggests that TAg might function as an anti-repressor by disrupting the binding of transcriptional repressor Dr1 with TBP (Lai et al., 1992). Based on this report we can assume that N136 may not able to disrupt the binding of Dr1 with TBP due to inability to bind with TBP. This may result in the transcriptional repression of above genes.

7.3.5 Gene regulation by unknown activity of TAg

Large number of genes are downregulated by TAg, N136, 3213 and D44N. This may suggests a new activity of TAg independent of the C-terminus, LXCXE motif and J domain. Alternatively, based on the fact that TAg and mutants are capable of making small t, give rise to possibility of regulation of these genes by small t. A total of 127 genes were downregulated at least 3 fold in all transfected cell lines. However, this number drops significantly to 30 when including the SV40 infection microarrays. This difference in gene regulation could be an artifact of creating the MEF cell lines or that these genes require a longer exposure to TAg (SV40 infection last only 3-4 days) for their regulation. Genes downregulated by SV40 infection and transfected MEFs belongs to biological classes such as chromatin (Histone H2B), regulation of apoptosis (IGF-BP), EGF-like domain (Dlk1, Itgbl1, Nell2, Dner), cellular protein metabolism (elastin, Cpa6, Mgll). Again, I want to emphasize that these results show that the regulation of gene expression is at the mRNA level only. We have no evidence that the protein levels of these genes change similarly or that their regulation has any significance. On the other hand, involvement of these genes in so many different regulatory processes (described below) in the cell raises the possibility of their contribution in TAg-mediated transformation.

The insulin-like growth factor (IGF) signaling pathway plays a crucial role in the regulation of cell growth, differentiation, apoptosis and aging. IGF binding proteins (IGFBPs) are important members of the IGF axis. Interestingly IGFBP-3, which is a target of p53, is downregulated by MEFs expressing N136, suggesting p53-independent regulation of this gene. Recently it has been reported that the oncogenic human papillomavirus type 16 encoded protein E7 can bind and inactivate IGFBP-3 thereby causing inhibition of IGFBP-3-induced apoptotic cell death (Santer et al., 2007).

Elastin is downregulated more than 5 fold in MEFs expressing TAg and mutants. In vascular smooth muscle cells (SMCs), elastin induces actin stress fiber organization, inhibits proliferation, regulates migration and signals via a non-integrin, heterotrimeric G-protein-coupled pathway (Karnik et al., 2003). It has been reported that b-myb represses SMC elastin gene expression (Hofmann et al., 2005; Marhamati and Sonenshein, 1996). This report raises the possibility that the repression of elastin in our experimental system may be a secondary event of b-myb activation by E2F.

7.3.6 Cooperation between different domains/regions of TAg is required for cyclin D1 regulation

There is now a large body of evidence that links the deregulation of cyclin Dl protein expression to a variety of neoplastic cell types. The observed reduction of cyclin Dl protein expression in viral oncoprotein-transformed cells has been proposed to be a direct result of Rb inactivation (Spitkovsky et al., 1995). This is also supported by our data where MEFs expressing Rb-binding defective mutant 3213 or J domain mutant D44N are unable to downregulate cyclin D1 levels. Also, MEFs expressing N136, which is capable of inactivating Rb, was not able to downregulate

cyclin D1. This suggests that the association of SV40 large T antigen with the Rb protein by itself is not sufficient to alter cyclin Dl protein expression. Only MEFs expressing full-length of TAg showed a reduction in cyclin D1 protein expression. This suggests a cooperative role of the C-terminus and N-terminus (LXCXE motif + J domain) of TAg. It will be interesting to test whether inactivation of both Rb and p53 are required for downregulation of cyclin D1. We can test this by expressing N136 in p53-/- MEFs.

7.3.7 Summary of MEFs and Villi microarray

Our study presents a global picture of differential gene expression patterns in MEFs and in enterocytes of transgenic mice expressing wild-type TAg and mutants. The majority of genes upregulated by MEFs and enterocytes expressing TAg and N136 belong to the cellular pathways of cell cycle, nucleotide metabolism, DNA repair and chromatin modifiers. 3213 and D44N do not regulate these genes in enterocytes and only slightly elevate their expression in MEFs. This suggests an important role of the J domain and LXCXE motif in inactivation of Rb-family members. Furthermore, we found that p53-target genes are upregulated in MEFs expressing N136 but not in MEFs expressing TAg, 3213 or D44N. This suggests that TAg blocks the p53 dependent response by binding to p53. We did not find upregulation of p53-target genes in enterocytes of mice expressing N136. This finding is consistent with the previous report in our lab that villus enterocytes do not express p53.

One of the major findings of this work is the regulation of immune response genes by TAg and D44N, but not by N136 and 3213 in MEFs. This suggests a requirement for the LXCXE motif and C-terminus of TAg in regulation of these genes. Furthermore, we found upregulation of a class of growth factors by N136 in MEFs. Our microarray experiments also

revealed a set of genes that are downregulated by TAg and mutants. Further investigation of these genes may lead to the discovery of some activity of TAg that is independent of the J domain, LXCXE motif and p53 binding domain activities. An interesting finding of this work is the downregulation of Cyclin D1 by TAg in MEFs. Regulation of Cyclin D1 is unique in the sense that it required cooperation between different domains/regions of TAg.

7.4 CONCLUSIONS

In last four decades SV40 T antigen has continuously provided insight into various cellular processes such as DNA replication, gene expression, posttranscriptional processing, tumor suppressor functions and cell cycle regulation. The ability of TAg to affect so many diverse biological functions is partly explained by the fact that it is a multifunctional protein consisting of six structural domains/regions, each of which interacts with both viral and cellular targets. TAg is unique in its ability to induce transformation in multiple cell lines and therefore serves as an important tool to understand the mechanisms of cellular transformation in a cell type specific context. Moreover, a series of TAg mutants have been made which are defective in one or more of its transforming activities and thus provide tools to decipher the role of different cellular pathways in transformation. The goal of this thesis is to determine whether the Rb and p53 pathways are the only important targets in TAg mediated transformation or if additional activities contribute.

Through this work I have made some new discoveries that will contribute to a better understanding of how TAg induces transformation. However, my studies also raised many new questions that remain unresolved. Several studies have suggested a role for the J domain of TAg in altering the function of Rb- family members in cells. These studies support a model in which the J domain recruits and stimulates the ATPase activity of the cellular chaperone Hsc70, and the resulting energy is used to release E2F from Rb. However, the effects of J domain mutations in

transformation in cell culture systems are modest. For example, J domain mutants induce dense foci and anchorage independent growth of primary and established cell lines, but are compromised for the induction of growth in low serum. To address the contribution of the TAg J domain to cellular proliferation and transformation in a normal tissue model, we have generated transgenic mice that express a J domain mutant (D44N) in villus enterocytes. I found that D44N was defective for inducing enterocyte proliferation and hyperplasia. Furthermore, unlike wild-type TAg, D44N neither induces p130 degradation nor disrupts p130-E2F complexes. Additionally D44N is unable to induce E2F dependent transcription. I found that loss of p130 alone is not sufficient to induce intestinal hyperplasia. The combined effect of a J domain and loss of pRb or p107 in induction of hyperplasia is yet to be determined. To resolve these questions future studies will require a conditional Rb knockout in a D44N background and analysis of D44N mice in a p130+/-; p107-/- background.

The intestinal hyperplasia requires TAg action on the Rb family of tumor suppressors since 3213 mice (defective in Rb binding) and D44N mice (defective in Hsc70 binding) are unable to induce hyperplasia. Additionally an amino-terminal truncation mutant N121, capable of inactivating Rb proteins but defective for p53 binding, develops hyperplasia but does not progress to dysplasia. Based on these findings we hypothesized that inactivation of Rb-proteins by TAg is sufficient to induce enterocyte proliferation, but that an activity mapping to the C-terminus of TAg is required for progression to dysplasia. To test this we have generated several lines of transgenic mice expressing the amino-truncation mutant, N136. I found that N136 induces hyperplasia without any indication of increased apoptosis consistent with the previous finding in the lab that enterocytes lack p53. Unlike N121 mice, several N136 expressing mice from different transgenic lines showed clear signs of dysplasia and presented abnormally long

intestines. However, I found that the appearance of signs of dysplasia was significantly delayed (10-12 months) in comparison to wild-type TAg mice (4-6 months). At this stage I am unable to determine the requirement of the C-terminus activity of TAg (independent of p53) in progression to dysplasia. I found some significant differences in the patterns of TAg expression in villus enterocytes of mice expressing TAg or N136, which may contribute to the delayed phenotype in N136 mice. I observed that TAg mice show a dense nuclear staining throughout the villi, villi/crypt differentiation zone and occasionally extended to the top of the crypts. On the other hand N136 mice show nuclear and cytoplasmic staining only in the top $3/4^{th}$ of the villi and expression never goes down to the villi/crypt differentiation zone. To test whether the expression patterns of TAg can affect the phenotype, future studies will require a generation of transgenic mice expressing N136 in crypts. Additionally, a genetic cross between N136 and 3213 or D44N mice will be required to elucidate the role of C-terminus of TAg in progression to dysplasia.

I have performed microarray experiments with MEFs and enterocytes expressing TAg and mutants (N136, 3213 and D44N) to analyze the effect of TAg or mutant expression on cellular gene expression. The goals of these experiments were to determine if: (a) the Rb-binding motif and J domain are required to stimulate E2F-dependent transcription; (b) TAg blocks p53 dependent transcription; and (c) TAg regulates genes through targets other than the Rb-E2F and p53 pathways. I found that nearly all TAg-mediated gene regulation in enterocytes requires both a functional J domain and Rb binding motif. Furthermore, N136 regulated nearly the same set of cellular genes as wild-type TAg. My analysis suggests that almost all TAg-mediated gene regulation in enterocytes is mediated by the Rb-E2F pathway. Accordingly, these genes are associated with DNA replication and repair, nucleotide metabolism, cell cycle progression and chromatin assembly and modification. MEFs expressing TAg or N136 also show upregulation of

the same set of genes, however I found that 3213 and D44N shows an intermediate upregulation of these genes. One of the possible explanations for this finding is the presence of some other factor/ activity (independent of the LXCXE motif and J domain) at the C-terminus of TAg which may contribute to Rb-family inactivation in MEFs. Alternatively, it is also possible that small t is releveing E2Fs from Rb repression by induction of Cyclin D1 and thus inducing E2F-traget genes. Another possibility is that 3213 and D44N have leaky phenotype.

To achieve the second goal of microarray experiments I have compared the regulation of p53-dependent genes by TAg and N136 in MEFs and enterocytes. Consistent with the hypothesis TAg blocks the activation of p53-target genes in MEFs. Expression of N136, which is unable to bind and inactivate p53, resulted in the upregulation of p53-target genes. On the contrary, I found that there is no upregulation of p53-target genes in enterocytes expressing N136. Thus, my experiments suggest that in enterocytes all TAg regulation of cellular gene expression is mediated through Rb-family inactivation and there is no evidence for a p53 response.

One of the major findings of the MEFs microarray experiments is the differential regulation of immune response genes by TAg and its mutants. I found profound upregulation of immune response genes by MEFs expressing TAg or D44N, but not by N136 or 3213. This finding suggests a requirement for the C-terminus and LXCXE motif in their regulation. Furthermore, I found upregulation of growth factors by MEFs expressing N136. This may provide an explanation for their survival when faced with the growth inhibitory environment imposed by p53. I also found a set of genes which are downregulated by TAg and mutants. This suggests the role of J domain, LXCXE motif and C-terminus independent activity of TAg in their regulation. Alternatively, these genes are regulated by small t. At this point I am unable to distinguish whether regulation of immune response genes and growth factors are dependent on

the Rb and/or p53 pathways or some other pathway/ factor is involved in their regulation. However, my findings will direct the future experiments in the lab to resolve these unanswered questions. This is the first microarray study which uses mutants of SV40 TAg to extend the knowledge about how different regions/domains of virus encoded proteins affect host gene expression.

Finally, I hope through this work I have not only advanced our understanding about how TAg mediates gene regulation and transformation, but also provided a platform for the future efforts in the lab to resolve the mysteries of one of the universe's most amazing molecules.

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