Cellular Transformation by Polyomavirus Oncoproteins

by

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Submitted to the Graduate Faculty of the

Kenneth P. Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

Dietrich School of Arts and Sciences

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University of Pittsburgh, 2014

Polyomaviruses have contributed tremendously towards our understanding of molecular biology of the cell and especially in discovering cellular factors and pathways involved in cancer formation and progression. Polyomavirus encoded oncoproteins manipulate specific cellular molecular pathways to create cellular environment conducive for viral replication and persistence. In a non-productive infection, the alteration of such cellular pathways by polyomaviral oncoproteins leads to activation of certain "cancer hallmarks" and results into cell transformation. In one part of this study, I used polyomaviral oncoproteins as a molecular tool to understand and decode cellular pathways involved in cell transformation. In one part of this study, I used a well characterized oncoprotein of polyomavirus Simian Virus 40 (SV40), called the large tumor antigen (TAg), as a molecular and genetic tool to understand the role of RB/E2F pathway in oncogene mediated cell transformation. According to the current paradigm, activator E2Fs are considered essential for cell proliferation and oncogenic transformation. My results, contrary to the current paradigm, suggest that TAg activates an alternative molecular pathway to induce proliferation and transformation in the absence of activator E2Fs.

In another project, I have studied oncoproteins encoded by a less studied polyomavirus, Lymphotropic Papovavirus (LPV). I have discovered previously unknown

splice forms of LPV early region, and their comparative analysis with SV40 oncoproteins suggest distinct roles for the homologous proteins in cellular immortalization and transformation. Importantly, my research shows an essential role of LPV small tumor antigen (sT) in immortalization and transformation of primary murine embryonic fibroblasts.

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PREFACE

A good amount of my work presented in this thesis is a result of collaborations both within the lab (Nick Giacobbi) and with Dr. Jeff Brodsky's (University of Pittsburgh) lab. During my stay in the Pipas lab, I had the pleasure of working with various wonderful people who taught me many things which helped me develop as a better scientist. I would begin my acknowledgements by thanking Dr. James Pipas, who showed faith in my abilities and allowed me to be a part of his lab. My successful graduate studies are a result of his decades of scientific experience and his exceptional leadership abilities.

When I joined the Pipas lab, Dr. Mayte Saenz Robles took me under her wing and taught me various scientific and organizational techniques. Her guidance and continuous scientific discussions have helped me immensely, especially in designing experiments for a particularly challenging project described in Chapter 3 of this thesis. I am highly grateful to Paul Cantalupo for providing me with various constructs and reagents used in my studies. He was the "go to" man in the pipas lab and I would like to thank him for his continuous help and support. Next, I would like to acknowledge Dr. Ping An and Dr. Ashok Srinivasan for their helpful discussions in various aspects of biochemistry and cloning. I am also thankful to Joshua Katz for his time and efforts in helping me understand various aspects of genomics and in performing bioinformatics analysis for my use. I had a great time with Dr. Nicole Seneca and Nick Giacobbi with whom I collaborate in different projects and had various scientific and non-scientific discussions.

Overall, I had a wonderful stay in the Pipas lab from February 2010 to December 2014 and I wish them all the very best for the future.

In addition to the people in the Pipas lab, I had a wonderful committee comprising of Dr. Jeff Brodsky, Dr. Saleem Khan, Dr. Jon Boyle and Dr. Craig Peebles. Their interest in my projects and timely advice helped me in critically thinking about my projects and improve as a researcher.

As per my personal acknowledgements, I would like to thank and dedicate my work to my parents, brother and my grandfather. It is the result of their prayers and sacrifices that I could come this far and obtain the best of education possible. I feel blessed to have a highly educated family to teach me the importance of hard work and good education. Staying in the USA away from my family would not be an easier task without my friends, who stood by my side through thick and thin of the times. In particular, I would like to thank Dr. Aman Kaur, Tushar Koshaley, Vishwas Pathak, Srivatsan Gopalakrishnan and Aditya Goyal for their friendship and motivation.

Just like any other scientific contribution, my studies also follow decades of hard work by several researchers and in the end I am grateful that I got the opportunity to contribute to the wonderful field of science. I wish to continue my journey as a scientist and help mankind to fight with the dreadful disease known as cancer.

1.0 INTRODUCTION

Recent advances in molecular and cell biology coupled with sophisticated high throughput sequencing technology and the advent of computational biology have immensely contributed towards our understanding of perhaps the most complex disease, cancer. Cancer was initially thought a disease caused by viruses and later a disease manifested by interplay of few specific genetic mutations, but is now regarded as a heterogeneous, multistep disease characterized by uncontrolled cell proliferation.

Organs are composed of heterogeneous group of cells organized into tissues specialized in performing different functions. Tumors formed in an organ are composed of multiple different cell types with different developmental origins and proliferation states. The gene expression and epigenetic state of these cells differ widely and so will be the compositions and combinations of the molecular pathways. Despite the heterogeneous nature of tumors, multitudes of studies have recognized nine characteristic phenotypes of malignant solid tumors, regarded as the hallmarks of cancer **(Figure 1)**: 1) sustaining proliferative signaling, 2) evading growth suppression, 3) resisting cell death, 4) replicative immortality, 5) invasion and metastasis, 6) inducing angiogenesis, 7) evading host immune response, 8) reprogramming of energy metabolism and 9) changes in tumor microenvironment (Hanahan and Weinberg 2011). The appearance of the cancer hallmarks may depend upon the stage at which tumors were analyzed, and not all tumors

may possess all the defined hallmarks. Moreover, there may be other cancer phenotypes which are yet to be identified and the current list should not be considered complete and exhaustive.

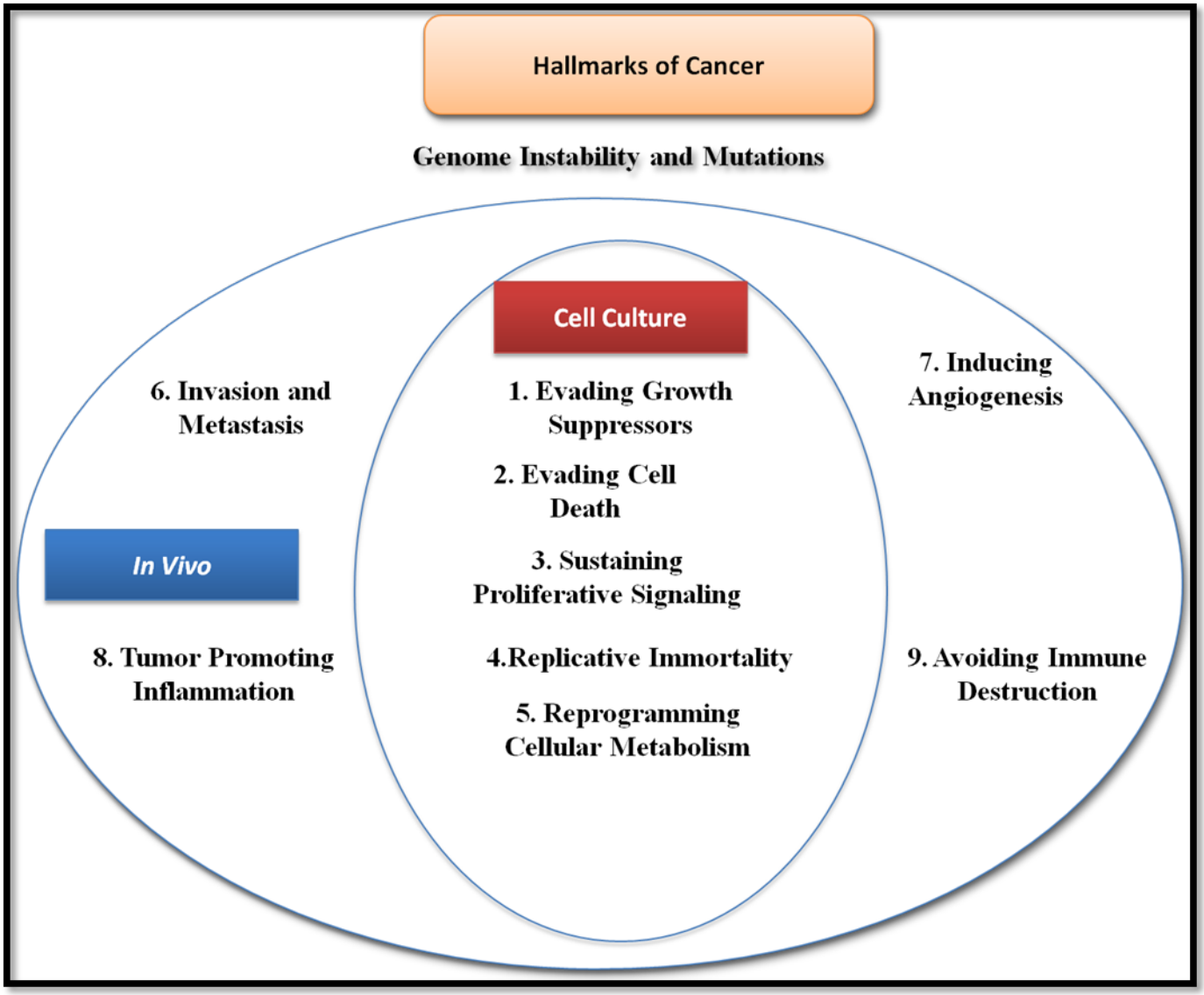


Figure 1: Hallmarks of Cancer

Decades of cancer research has identified nine biological capabilities of solid malignant tumors, generally referred to as "hallmarks" of cancer. Cancer formation is a micro-evolutionary process, and genetic instability forms the basis for acquisition of the genetic diversity. According to the multi-stage cancer model, cancer cells acquire these hallmarks overtime, and formation of malignancy further requires contributions from the surrounding tumor micro-environment, such as hallmark numbers 6, 7, 8 and 9. The ultimate goal of cancer biologists is to relate these observed cancer phenotypes with the cellular regulatory pathways in a tissue specific manner. Cellular pathways regulating some of these cancer hallmarks can be studied *in vitro* using various cell culture systems (Hallmarks 1-5). Not all the tumors may exhibit all nine cancer hallmarks and there may be more hallmarks yet to be discovered.

Each cancer hallmark can be seen as a phenotypic change in cells due to genetic mutations in its regulatory pathway(s). Homeostasis of a normal cell is maintained as a result of complex inter-play between several molecular pathways. Mutations in specific components of a pathway would disrupt its functioning and will activate particular cancer hallmarks. Further accumulation of mutations in the same or different pathways over-time allows the mutated cells to gain more cancer hallmarks and ultimately results into malignancy. Sequencing of spontaneous human tumors and study of tumor cells in cell culture and *in vivo* have identified mutations in several genes linked to activation of different cancer hallmarks (Futreal, Coin et al. 2004, Hanahan and Weinberg 2011). These genes can be broadly divided into tumor suppressors or oncogenes **(Figure 2)**.

Tumor suppressors (growth inhibitory) are genes which encode proteins that inhibit tumor formation, mostly by regulating cell proliferation and death. Mutations of tumor suppressor genes are recessive in nature and for tumor formation, both copies of the gene need to be inactivated. These mutations are called loss-of-function mutations (Futreal, Coin et al. 2004, Hanahan and Weinberg 2011). Oncogenes (growth promoting), encode growth promoting proteins, and gain-of-function mutations in a single copy of these genes results in their dominant activity over their wild type alleles thus contributing to cancer formation. Oncogenes were originally discovered as viral proteins, capable of transforming cells, but later they were identified as mutated, over-expressed cellular genes. Classic experiments, where genes from tumor cells were introduced into rodent cells resulted into their transformation, led to the initial discoveries of various oncogenes (Krontiris and Cooper 1981, Shih, Padhy et al. 1981). Based upon their origin, oncogenes can be divided into cellular or viral oncogenes and the wild type copy of a cellular oncogene is termed as proto-oncogene.

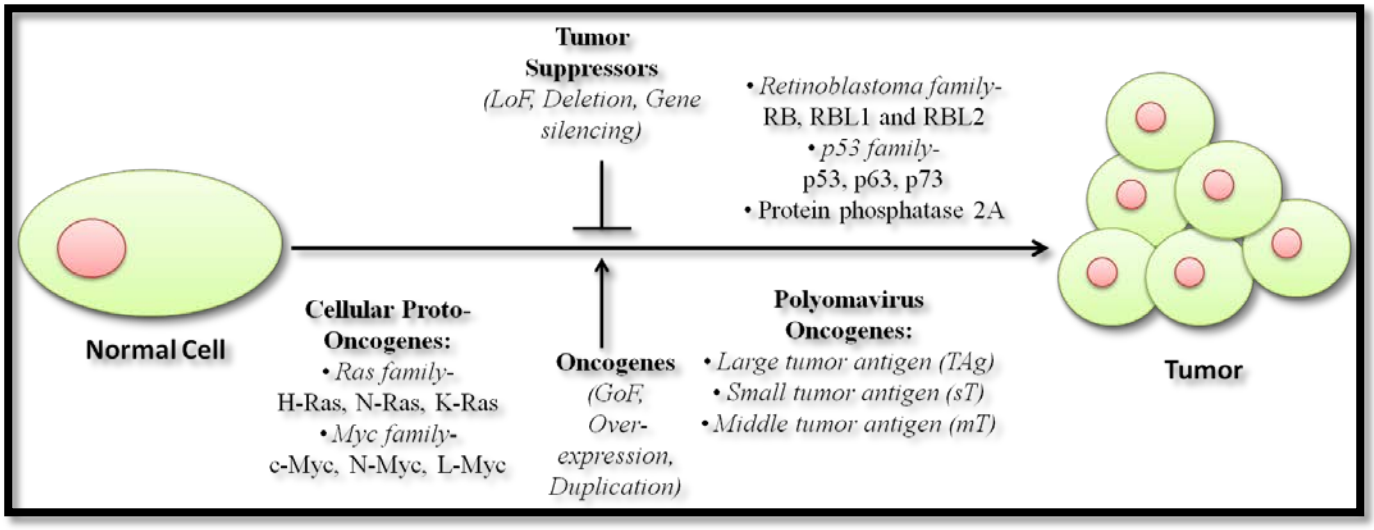


Figure 2: Tumor suppressor and Oncogenes

Based upon the types of mutations the genes involved in tumorigenesis can be divided into tumor suppressors or oncogenes. Oncogenes can be of cellular (proto-oncogenes) or viral origins. Tumor suppressor genes are involved in growth inhibition and therefore their mutations are ressessive in nature. On the other hand, proto-oncogenes encode growth promoting proteins and thus their mutations are dominant in nature. Viral oncogenes induce tumorigenesis when over-expressed experimentally or naturally during viral infections. Few examples of tumor suppressor, proto-oncogenes and viral oncogenes are shown in this figure and are discussed in the sections to follow.

Cancer is a multi-step disease, meaning mutations in a single proto-oncogene or a tumor suppressor gene is insufficient to cause tumorigenesis. For instance, expression of a single cellular oncogene leads to either growth arrest (Ras) or apoptosis (cMyc) of primary cells in vitro, and requires either another co-operating oncogene (cMyc and Ras) or prior immortalization of primary cells. Moreover, *in vivo* studies show that transgenic mice expressing either Myc or Ras oncogenes develop mammary carcinoma at a significantly reduced rate as compared to mice expressing both the oncogenes. Similarly, cellular transformation of primary cells in culture by a single viral oncogene, requires simultaneous alteration of one or more tumor suppressor or oncogenic pathways.

Although we have identified many factors and molecular pathways involved in cancer formation, the list is far from complete. In addition, we still lack information regarding their tissue and cell type specific roles and the cross-talk between them. Therefore, the ultimate goal of cancer biologists is to relate the observable cancer traits to the underlying genetic and epigenetic changes in cancer cells. This would involve a deeper understanding of the molecular

constituents' of the tumorigenic pathways and their interactions with other molecular pathways in a tissue and cell specific manner (Kinzler and Vogelstein 1996, Futreal, Coin et al. 2004).

1.1 CANCER BIOLOGY: THE STUDY OF NATURE'S COMPLEXITY

Several labs use various different animal or cell culture model systems to decode cellular pathways and their molecular components involved in tumorigenesis, but no system allows a comprehensive study of all the de-regulated molecular pathways. Although our ability to determine the nature of mutations in various human cancers has been greatly enhanced by sophisticated next generation sequencing technologies, the scientific field is still trying to differentiate between the mutations involved in cancer formation and progression versus those which arise as a result of an unstable system. Similarly, new genetic manipulation techniques for various animal model systems have been developed to study human tumors and tumorigenesis, but high cost, time, complexity and expertise limit their widespread use. Therefore, studying cancer biology using a cell culture system *in vitro* is still a favorable choice.

In fact, most of our current understanding of cancer biology and discoveries of cellular pathways involved in cancer are a result of cell culture studies. Currently, there are several human tumor cell lines used for both basic and therapeutic cancer research, however, they are of limited utility due to their already unstable genome. Alternatively, primary cells from various tissues can be obtained from wild type (normal), genetically modified or transgenic animal models, to study and manipulate *in vitro*. Although, cell culture is a more artificial system than a comparative animal model, the simplicity of performing experiments and genetic modifications

in culture provides a much bigger advantage. In addition, while the study of certain cancer traits like angiogenesis or immune regulation may not be feasible using cell culture systems, other cancer phenotypes like evasion of cell death, sustenance of proliferation and refraction of growth inhibitory signals can still be re-capitulated to an extent **(Figure 1)**.

The scientific term used to re-capitulate cancer phenotypes in cell culture is called transformation. Transformation is defined as the ability of certain cells to survive and proliferate in modified cell culture conditions that are inhibitory to the growth and survival of normal cells. There are various assays used to determine transformation and each assay tests different attributes of cancer cells, and also differs in stringency levels. As primary cells have a limited life span in cell culture, immortality is a pre-requisite to transformation. Immortalization is determined by continuous passaging of cells using a 3T3 or 3T12 protocol (Xu 2005). Immortalization of primary MEFs using similar protocols have been achieved, and studies of such immortalized MEFs have invariably shown mutations in p53/MDM2 pathway **(Discussed Later)** (Xu 2005). Other attributes of transformed cells are growth in low serum conditions (independence of growth factors), focus formation (evading growth suppression), growth in soft agar (anchorage independence) and xenograft in nude mice (malignancy).

Growth in low serum: Normal cells require growth factors in order to activate pro-proliferation signaling pathways. Growth medium supplemented with serum (fetal calf serum or fetal bovine serum), provides these necessary growth factors. However, cancer cells carrying oncogenic mutations in their growth regulatory pathways are self sufficient in maintaining proliferative state even in the absence of serum. This transformation assay therefore tests the activation of "sustaining proliferative signaling" hallmark of cancer.

Focus Formation: Normal cells in cell culture proliferate to a monolayer, and growth arrest upon reaching confluency due to a phenomenon called contact inhibition. However, cancer cells are refractory to contact mediated growth arrest and continue proliferating even after reaching confluency. Such a proliferation results into dense packing of the cells and further growth results into a 3-dimentianal foci formation. This assay tests one aspect of the cancer hallmarks "Evading growth suppression".

Growth in soft agar and Xenografts in animal models: These two assays are considered as the most stringent of all the transformation assays (Bouck, Stoler et al. 1986, Rastinejad, Polverini et al. 1989). Normal cells require solid surface to proliferate in cell culture, while cancer cells have by-passed such a requirement due to yet unidentified mutations. The ability of cancer cells to proliferate in semi-suspension culture conditions is tested by plating cells in semi-solid agar layer. While normal cells would stay as single cells in such conditions, transformed cells proliferate and form quantifiable colonies. This ability of transformed cells to grow in soft agar conditions is co-related with changes in the actin cytoskeleton (Bouck, Stoler et al. 1986). Xenografting of normal cells into nude mice or new born hamsters does not result in tumor formation, however, transformed cells growing in culture form solid tumors. Various characteristics such as incidence of tumor formation, survival time of the animal and size/volume of the tumors formed are determined.

These transformation assays test emergence of different hallmarks of cancer in cell culture and cells growing in culture may be transformed by some assays but not in others. *In vivo* cancer formation is a complex process, while the cell culture transformation assays is an attempt to recreate conditions testing only certain aspects of cancer cells. In order to make biologically relevant conclusions from transformation assay results, study of the pathways involved in

generating the transformation phenotypes need to be characterized *in vivo*, and in spontaneous tumor formation.

1.1.1 Polyomaviridae: A small DNA tumor virus family

Viruses are infectious agents and can be regarded as either simplest microorganisms or complex biomolecules. The genetic makeup of viruses is extremely simple and encodes for just enough genes sufficient for their own replication and long time "fitness". However, they require assistance from the host cell molecular machineries to achieve this goal, and viral genes thus encode the products which have evolved to interact and influence various cellular molecular pathways. The interaction of the viral proteins with the host cell proteins results in various changes in the host cell, including cellular antigenicity, metabolism and cell survival. Viral induced changes in the host and the host response to these changes leads to manifestation of viral diseases. There are two types of viral infections: (1) Productive infections, which allow complete viral replication and production of viral progeny, and (2) Non-productive infections, where viral replication is incomplete and only a part of their genome is expressed. The host cells which "allow" productive infection cycle for a virus are called permissive cells, while others are called non-permissive cells. Although most of the viral diseases are a result of productive viral infections, abortive infections by certain viruses result in more complicated diseases like cancers.

Viruses which lead to cancer formation are grouped under tumor viruses and are further categorized based upon the size and type of their genomes. Polyomaviruses belong to the family of small DNA tumor viruses containing a circular double stranded DNA genome ranging from 5kb to 5.3kb. Their genome is encapsulated in an icosahedral non-enveloped virion (White,

Gordon et al. 2013). The founding member of polyomavirus family is Murine Polyomavirus (MuPyV), discovered and isolated by Ludwik Gross in 1953 from leukemia tissues of AKR mice. Soon after its discovery, a series of studies by Sarah Stewart and Bernice Eddy identified its capabilities of causing multiple type of tumors in rodents, and hence the name polyoma (multiple tumors) (Stewart, Eddy et al. 1958, Javier and Butel 2008, Morgan 2014). Six years after the discovery of MuPyV, another member of this family, Simian Virus 40 (SV40) was discovered as a contaminant in polio vaccines produced using Macacus monkey (*Macaca mulatta*) kidney cells (Sweet and Hilleman 1960). Due to the fact that it was delivered to human population with the polio vaccine, it got immediate attention of virologists and it was soon demonstrated that SV40 causes variety of tumors in experimental animals (Hilleman 1964, Todaro, Green et al. 1966, Diamandopoulos 1972, Diamandopoulos 1973, Garcea and Imperiale 2003). Early discoveries and the tumor causing capabilities led to a series of extensive studies of these two polyomaviruses, making them the model for understanding polyomaviruses infection, life cycle and more importantly the functions of individual gene products.

Sixty years since the discovery of the first polyomavirus, the count has now increased to 91, of which 13 are known to infect humans. With the advent of sequencing technology, astonishingly 10 of the 13 known human polyomaviruses were discovered in the last 7 years. The first human polyomaviruses discovered were BKV and JCV, and serological evidence suggest that around 80% of the human population is infected with these viruses. In normal infected individuals these viruses remain latent in the lymphocytes, brain and urogenital tract of the host (DeCaprio and Garcea 2013, White, Gordon et al. 2013). However, the instances of diseases manifested by these viruses are extremely rare, and mostly occur in immunocompromised patients. The pathologies are extremely debilitating and often fatal. BKV can cause nephropathy

and hemorrhagic cystitis resulting in the graft failure of renal transplant patients, while JCV causes progressive multifocal leucoencephalopathy (PML), a fatal disease of central nervous system (DeCaprio and Garcea 2013). The more recently discovered human viruses, Merkel cell polyomavirus (MCPyV) and trichodysplasia spinulosa associated polyomavirus (TSPyV) are associated with two rare forms of human cancers- Merkel cell carcinoma and trichodysplasia spinulosa piromatrix dysplasia, respectively (Dalianis and Hirsch 2013). Thus far, no diseases have been associated with other human polyoma viruses (Dalianis and Hirsch 2013) **(Table 1)**. The recent technological advances leading to "quick" discoveries of novel human polyomaviruses have outpaced our abilities to characterize them, leaving JCV and BKV as the best characterized human polyomaviruses. Only recently, MCPyV has gained attention due to its cancer causing abilities, and several studies have suggested a considerable difference in mechanism of cellular transformation by MCPyV versus SV40/BKV/JCV (DeCaprio and Garcea 2013).

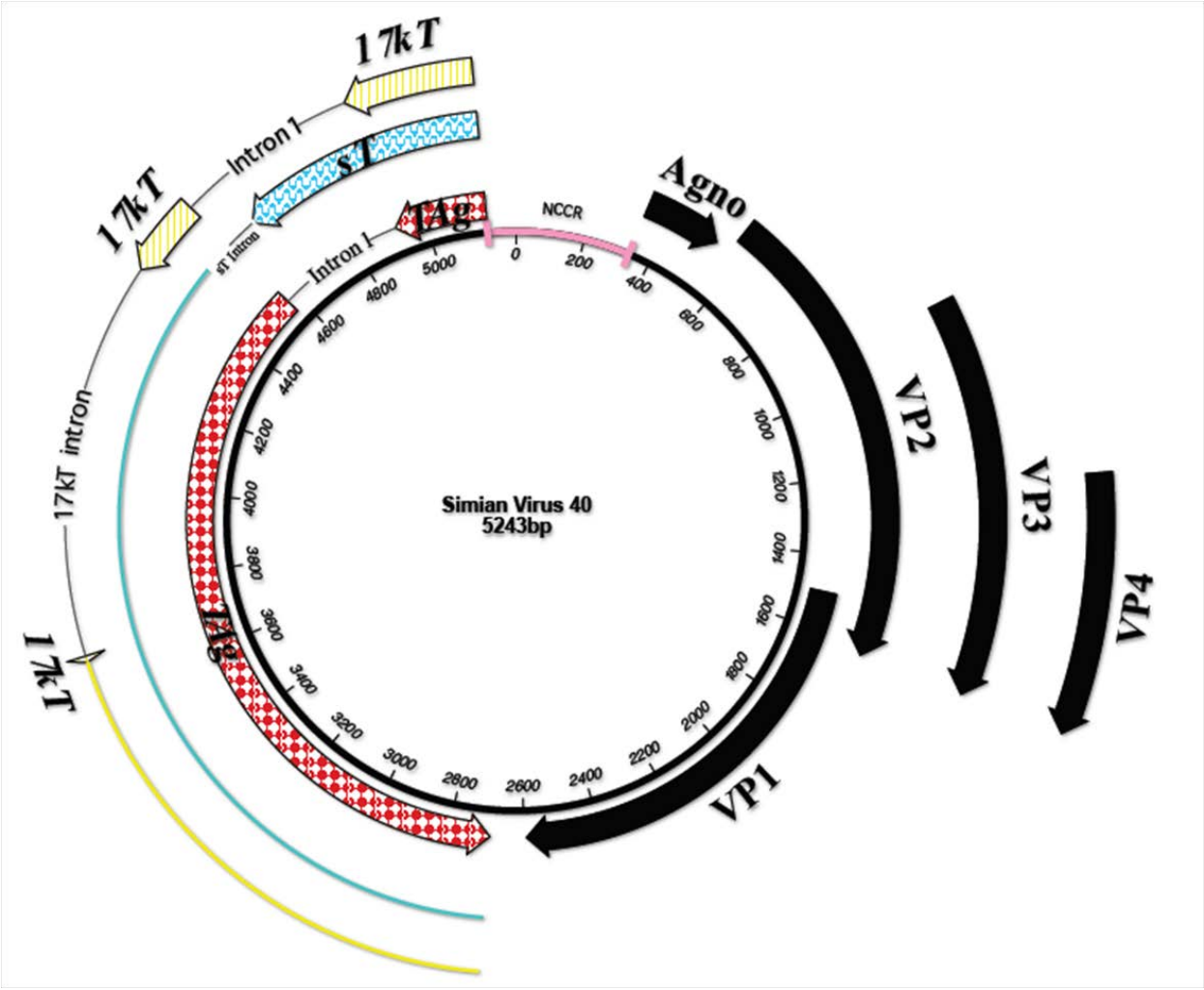
Table 1: List of known human and selective primate and rodent polyomavirus

Virus	Host	Discovery	Human Diseases	Dominating Oncoprotein	Seroprevalence
SA12 (Simian Agent 12)	chacma baboon		NA	TAg	NA
BKV (Patient name)	Human	1971	Polyomavirus associated nephopathy (PVAN), haemorrhagic cyctitis	TAg	80-90%
JCV (Patient name)	Human	1971	Progressive multifocal leukoencephalopathy	TAg	40-55%
SV40 (Simian Virus 40)	African green monkey	1959	NA	TAg	NA
WU (Washington University Polyomavirus)	Human	2007	None	?	70-90%
KI (Karolinska Institute Polyomavirus)	Human	2007	None	?	55-90%
HuPy7 (Human Polyomavirus 7)	Human	2010	None	?	35%
HuPy6 (Human Polyomavirus 6)	Human	2010	None	?	70%
HuPy10 (Malawi Polyomavirus, Human Polyomavirus 10)	Human	2012	None	?	26-68%
STL PyV (Saint Louis Polyomavirus)	Human	2012	None	?	?
LPV (Monkey B lymphotropic papovavirus)	African Green Monkey, Human?	1979	None	?	40%
HuPy9 (Human Polyomavirus 9)	Human	2011	None	?	25-50%
TSPy (Trichodysplasia spinulosa-associated polyomavirus)	Human	2010	Trichodysplasia spinulosa, pilomatrix dysplasia	?	70-80%
NJPy (New Jersey Polyomavirus)	Human	2014	None	?	?
MCPy (Merkel Cell Polyomavirus)	Human	2008	Merkel cell carcinoma	sT	60%
HuPyV12 (Human Polyomavirus 12)	Human	2013	None	?	12-33%
HaPy (Hamster Polyomavirus)	Hamster	1967	NA	mT	NA
MuPyV (Murine Polyomavirus)	Mouse	1960	NA	mT	NA

Figure 3 shows the dsDNA genome organization of a prototypical polyomavirus SV40, which is divided into three structural regions: 1) The Early Region (ER), which encodes for the first transcripts produced upon infection, products of which are essential for viral replication. 2)

The Late Region (LR) which encodes the viral structural proteins and are expressed after viral DNA replication. 3). The NCCR (Non-Coding Control Region) physically separates the early region from the late region and contains promoters for both the ER and the LR. In addition it also contains a bi-directional enhancer and the origin of replication.

The ER of polyomaviruses encodes 3 to 5 differentially spliced products known as tumor antigens which are responsible for the modulation of host cell environment to make it conducive for viral replication and persistence (Berk and Sharp 1978, van Santen and Spritz 1986). ERs of SV40 (van Santen and Spritz 1986) and MuPyV (Heiser and Eckhart 1982)express three spliced products, while JCV ER splicing results into 5 different products (Ishaq and Stoner 1994, Prins and Frisque 2001). All the polyomavirus ERs are known to produce differentially spliced large tumor antigen (TAg) and a small tumor antigen (sT), and other splice product(s) which may differ in number and kind between different polyomaviruses. The TAg mRNA is generated by splicing intron1 and translates into a 708 a.a. protein. sT mRNA reads through the first donor site of TAg and its splicing uses a unique donor site present in intron1 and the accepter site of TAg. While the sT mRNA is longer than the TAg, its translation ends at the stop codon present in intron1 resulting into a 174 a.a. protein product. The other splice products arise due to alternative splicing of TAg transcript and in the case of SV40 give rise to one product called 17k tumor antigen (17kT). The first splicing of 17kT is similar to the TAg splicing, excluding Intron1, while the second splicing takes place within the TAg coding region and results into exclusion of most of the TAg central coding region ultimately including 12 bps (Ahuja, Saenz-Robles et al. 2005).


17kT
Intron 1
ST
17kT
ST intron
17kT intron
17kT
TAg
Intron 1
NCCR
Agno
VP2
VP3
VP4
VP1
TAg
Simian Virus 40
5243bp
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4200
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5000

Figure 3: Genome map of SV40 polyomavirus

The dsDNA genome of prototypical polyomavirus SV40 is divided into three structural regions. The early region (ER) shown in the left, the non coding region marked as NCCR and the late region (LR) shown in the right side of the circular map.

Expression of the ER results into three spliced products; the large T antigen (RED), the small T antigen (BLUE) and the 17k T antigen (YELLOW). The transcripts for each product are color coded with exons are shown as arrows and introns are depicted as black lines. Large T antigen mRNA consists of two exons connected upon splicing of Intron1. 17kT mRNA consists of three exons and originates from differential splicing of the TAg transcript. The second splicing of 17k T results into a change in reading frame which results into an early termination of translation adding only 4 amino acids to its C-terminal end. Small T antigen (sT) mRNA is the largest in size and results from splicing of the sT intron, whose acceptor splice sites are same as that of TAg, but it uses a unique donor site present within Intron1. The translation termination codon is present before the small T intron donor site and therefore the protein product shares exon1 with the TAg and a unique region formed from Intron1 of TAg.

The LR of SV40 results also forms five different products shown as solid black arrows. VP1 and VP2 mRNA are formed due to differential splicing. VP2, VP3 and VP4 share the same reading frame but their protein products are formed using different translation start sites present within the VP2 mRNA. Agnoprotein (Agno), is translated from the LP transcript.

The non coding control region (NCCR) (PINK) physically separates the ER from the LR and contains regulatory elements for the expression of both early and late region transcripts. In addition the NCCR region also contains the origin of replication for the viral genome.

Similar to the ER, the LR of polyomaviruses also encode 3-5 different proteins, whose expression requires an active TAg. Generally all the polyomaviruses LR encode for three capsid proteins called VP1, VP2 and VP3.However, there are a couple of exceptions to the late region encoded proteins; BKV, JCV and many monkey viruses including SV40, also encode

Agnoprotein (Agno) of unknown functions and SV40 late region encodes an additional protein called VP4 which is known to act as a viroporin (Raghava, Giorda et al. 2011). As shown in **Figure 3**, VP1 and VP2 are produced from alternative splicing of the SV40 LR, while VP3 and VP4 are translated from the VP2 transcript using different start codons.

Several studies have shown that the proteins encoded by the early region of polyomaviruses transform cells *in vitro* and cause tumorigenesis in model organisms. Therefore, apart from studying this rapidly expanding family of DNA tumor viruses for their pathological reasons, they also provide a great molecular biology tool to study cellular biology in general and cancer biology in particular. In this chapter, I will focus on characterization of polyomaviral oncoproteins and their mechanism of transformation/tumorigenesis.

1.1.2 Polyomavirus oncoproteins: Molecular tools to study molecular biology of cancer

Polyomavirus ER products are responsible for creating the cellular environment conducive for viral replication and persistence. To achieve this feat, the oncoproteins interact with various cellular factors to alter specific cellular pathways. In a permissive cell, alteration of these pathways results in viral genome replication and in productive infection, however in non-productive infection, where the late genes are not expressed, this process result in activation of certain cancer phenotypes **(Figure 4)**. In cell culture, a non-productive infection or mere expression of these oncoproteins results in cellular immortalization and transformation, while *in vivo* a non-productive infection requires further genetic mutations or immune-suppression to causes tumor formation. Study and characterization of polyomaviral oncoproteins has lead to the discovery and characterization of various cellular pathways and factors involved in spontaneous

human tumorigenesis. Therefore, viral oncoproteins serve as an excellent molecular tool to study specific cellular pathways. Moreover, as cancer is a multi-step process which follows Darwinian selection process to incorporate various co-operating mutations, study of polyomaviral oncoproteins in combination with other viral or cellular oncogenes will allow decoding of complementary tumorigenic pathways.

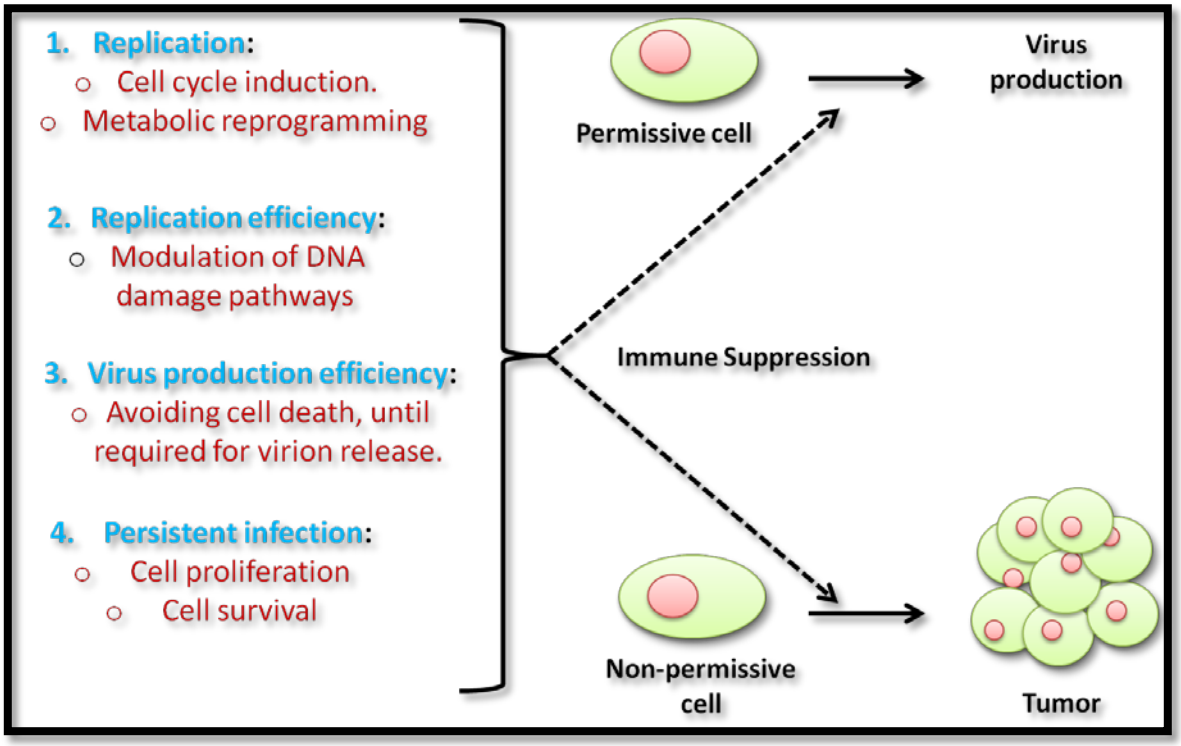


Figure 4: Polyomavirus infection results in productive or non-productive infections resulting in different outcomes.

The early region proteins are responsible for altering specific cellular pathways regulating host cell functions (shown in red color) in order to make cellular environment conducive for different aspects of viral life cycle (shown in blue color). Polyomavirus for most part of its life cycle causes a persistent infection without causing pathology. However, suppression of host immune system results in one of the two outcomes. In a permissive cell, viral replication is complete and more virions are produced leading to cell lysis and virus release. In a non-permissive cell where viral replication is incomplete and only the early proteins are expressed, cell transformation or tumorigenesis takes place. The same cellular pathways altered by the early proteins results into activation of certain cancer hallmarks, and with further genetic alterations, other cancer hallmarks gets activated resulting in tumor formation.

Lastly, virus-host interactions are a continuously evolving processes, which can be seen as a fight for survival. The heterogeneous nature of cancers clearly demands a cell and tissue specific approach to design therapies. Polyomaviruses infect various different cell types, but still require the host cell machinery for their own replication. Cell growth and differentiation is regulated by complex feedback loops involving several molecular pathways acting in a tissues specific manner. Therefore, polyomaviruses infecting distinct cell types or species have evolved mechanisms to alter the same cellular processes but using different strategies. Therefore, studying oncoproteins from distinctly related polyomaviruses thus allows us a way to determine cellular pathways playing dominating roles in different tissues to regulate the same cellular function and eventually would allow us to discover the hubs where different pathways might converge.

Cancer can be a direct consequence of viral infection, or it can be an indirect result of immune suppression by viruses like HIV (Human Immunodeficiency Virus). However, while onco-viral infections are highly prevalent in human populations, only 12-15% of human cancers are linked to viral infections. In addition, only persistent viral infection is known to cause tumorigenesis, which in most cases requires host immune suppression (Mesri, Feitelson et al. 2014). These observations suggest that mere viral infections are not sufficient for cancer formation and would require further spontaneous mutations or cellular pathologies. This observation is also consistent with the current multi-step model of tumorigenesis. In the following sections of this chapter, I will discuss the current understanding of the mechanism of cellular transformation by polyomavirus oncoproteins, the cellular pathways altered in the process and their implications in spontaneous human cancers.

1.1.3 Cell cycle, RB-E2F pathway and Cancer: To cycle or not to cycle?

Cell proliferation is a highly regulated process governed by four sequential phases of cell cycle (G1, S, G2 and M) which are regulated by several inter-connected molecular pathways involving a plethora of proteins. Although all the somatic cells undergo the same sequential phases of cell cycle, there are differences in the initial state of quiescent/differentiated cells versus proliferating/immortalized cells. While a continuously proliferating cell enters G1 phase directly from the M phase, a quiescent cell enters G1 phase from the G0 phase (Takahashi, Rayman et al. 2000) **(Figure 5A and 5B)**.

A fully developed animal contains cells in different proliferative states, ranging from continuously proliferating stem and progenitor cells to non-proliferating quiescent and senescent

cells. Cancer is manifested by uncontrolled cell proliferation, which requires an increase in pro-proliferation signals or mutations which would lower the threshold required for cell cycle commitment (Malumbres and Barbacid 2001). Therefore, understanding of the molecular mechanisms that regulate different phases of cell cycle would immensely help in understanding cancer.

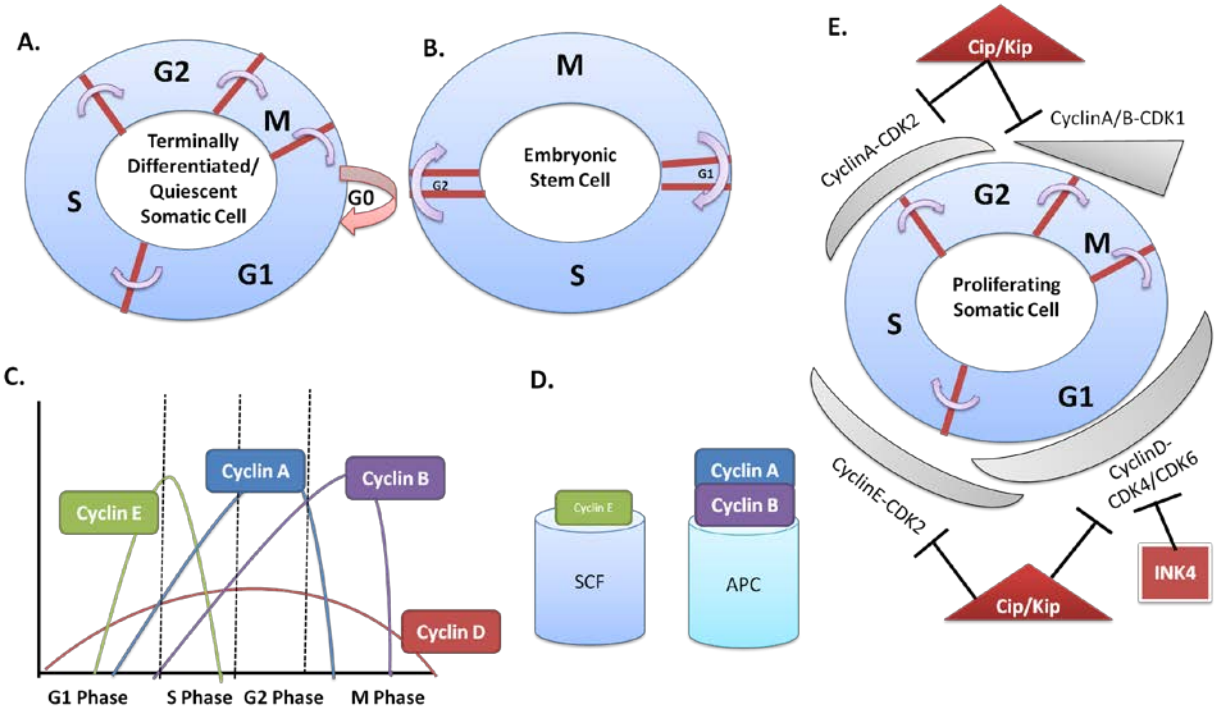


Figure 5: Cell cycle and its regulation

A, B). Different kinetics and variations of cell cycle observed in different types of cells. While a terminally differentiated, quiescent cell (naturally or serum starved) enters into the cell cycle from the growth arrested G0 phase, a continuously dividing somatic cell (stem cells, cancer cells etc) skip the G0 phase and enters into the next round of cell cycle, directly from the M phase. In addition, the normal length of growth phases (G1 and G2) is considerably reduced in stem cells. These changes in the cell cycle between different cell types are manifested by various genetic and epigenetic variations, and may involve different cell cycle regulatory pathways. Therefore, study of cell cycle regulatory molecules or pathways should be performed keeping the proliferative state of cells under consideration. C). Cyclins are the key cell cycle regulatory proteins, governing temporal progression of specific cell phases in response to growth stimuli. The four cell cycle specific cyclins and their cell cycle expression profile is shown. Cyclin D is the first cyclin synthesized upon receiving growth stimuli, and its expression is dependent upon mitogenic signals. The transcriptional activation cyclinE is mitogen independent, and requires cyclinD/CDK complexes enzymatic activity to activate its transcription factors. Similarly transcriptional activation of cyclin A and cyclin B depends upon enzymatic activity of cyclinE/CDK complexes. D). Mitogen signal dependent cyclin D protein is transcriptionally regulated in a cell cycle specific manner, while protein levels of cyclinE, A or B are controlled by proteosomal degradation mediated by two E3 ubiquitin ligase complexes APC or SCF. E). Spatial-temporal regulation of cell cycle progression is mediated by specific cyclin/CDK complexes which phosphorylate various components of cell cycle regulatory pathways and thus maintain normal progression of cell cycle. Activities of specific cyclin/CDK complexes are governed in a temporal-spatial manner by the inhibitory activities of specific CDKI's belonging to two families, INK4 or Kip/Cip. While INK4 family members interact specifically with cyclin/CDK4 complexes, the Kip/Cip family members interact and inhibit all the other cyclin/CDK complexes.

CDK (Cyclin Dependent Kinase), APC (Anaphase Promoting Factors), SCF (Skp, Cullin, F-box), CDKI (CDK Inhibitors), INK4 (Inhibitors of CDK4), Kip (Kinase inhibitory protein), Cip (CDK interacting protein).

There are three major cell cycle checkpoints: 1) the G1/S checkpoint, which measures the correct size and appropriate proliferation signals, 2) the G2/M checkpoint, which determines the fidelity of DNA synthesis and 3) the metaphase/anaphase checkpoint which determines appropriate alignment and adhesion of daughter chromosomes to the spindle fibers. The extracellular environment determines the fate of cellular proliferation, with nutrient availability and mitogenic stimuli being the limiting factors. Cell surface receptors sense the nutrient availability or mitogenic signals from outside the cell, and relay them within, to start a series of signaling events (Masckauchan, Shawber et al. 2005). Various pro-and anti-proliferation signals are received by the cell, and it is the final summation of these signals which determines the fate of cell cycle progression. Tumors proliferate in the absence of pro-proliferation signals or in the presence of anti-proliferation signals. Over-expression or oncogenic mutations of various growth factor receptors (receptor tyrisine kinases, epidermal growth factor receptor family) are found in human cancers, which constitutively activate pro-proliferation down-stream signaling pathways (Malumbres and Barbacid 2001, Malumbres and Barbacid 2005). The signaling cascade events eventually results in inhibition of tumor suppressor retinoblastoma family members (RBs), and this leads to the release and activation of E2F family of transcription factors and induces cell cycle genes expression **(Figure 6)**.

Cyclins and CDKs (cyclin dependent kinases) are regulators of cell cycle, and are themselves regulated by multiple feedback loops. After receiving mitogenic signals, D-type cyclins (D1, D2 and D3) are synthesized, and they bind to CDK4 and CDK6 to form cyclin/cdk active enzyme complexes that phosphorylate RBs early in the G1 phase. Subsequently, mitogen-independent E-type cyclins are synthesized, which associate with CDK2, and further phosphorylate RBs in the late G1-S phase (Harbour, Luo et al. 1999). Various types of mutations

are observed in the cyclins and CDKs of the G1-S phase of the cell cycle in human cancers. Particularly, D1 and E1 cyclins and CDK2 and CDK4 are over-expressed, mutated or their genomic loci are amplified (Chen, Tsai et al. 2009, Malumbres and Barbacid 2009) **(Figure 6)**.

Cyclin levels are regulated in a cell cycle stage manner, both by transcriptional control and proteosomal degradation via various ubiquitin ligases such as APC (Anaphase Promoting Complex) and SCF (Skp, Cullin, F-box) E3 ligase **(Figure 5D)**. Mutations in APC and members of SFC complex, which would prevent degradation of cyclins are found in human cancers. While cyclins are expressed in a cell cycle specific manner **(Figure 5 C),** CDKs are ubiquitously present during the complete cell cycle and are regulated by post-translational modifications and by cyclin dependent kinase inhibitors (CDKI's). CDKI's are broadly divided into two families, INK4 (INhibitor of CDK4) and cip/kip family, depending upon their mode of action (Moore 2013). Various studies of human cancers have shown decreased expression of CDKI's, which has been attributed to both genetic and epigenetic changes (Malumbres and Barbacid 2009) **(Figure 6)**.

The RB-E2F pathway is considered the central regulator of cell proliferation, and therefore most human cancers bear mutations in this pathway. As shown in **Figure 6**, studies of human tumors have identified an array of mutations in different components of this pathway, ultimately leading to RB (retinoblastoma proteins) inhibition and activation of E2Fs. Most of our current understanding of the RB-E2F pathway comes from a series of cell culture experiments involving serum starved cell cycle synchronized cells. Depletion of serum from the growth medium in cell culture results into growth arrest of primary cells, which can be induced to proliferate synchronously upon re-addition of serum. This strategy has allowed researchers to discover and characterize various cell cycle regulatory and effector molecules, their temporal

expression patterns and their mechanism of action. **Figure 6,** delineates the general mechanism of cell cycle activation and regulation at different steps.

In summary, the regulators of cell cycle G1/S phase are found mutated in several human cancers, ultimately leading to de-regulated cell proliferation. In particular, the negative regulators of G1/S phase, like the CDKI's, APC and RBs are found to bear *lof* and deletion mutations, while the positive regulators (Cyclins, CDKs and E2Fs) are found to be over-expressed, or bear *gof* mutations. These results emphasize on the importance of G1/S cell cycle checkpoint in regulating normal cell cycle progression and the importance of RBs and E2Fs in controlling cell proliferation.

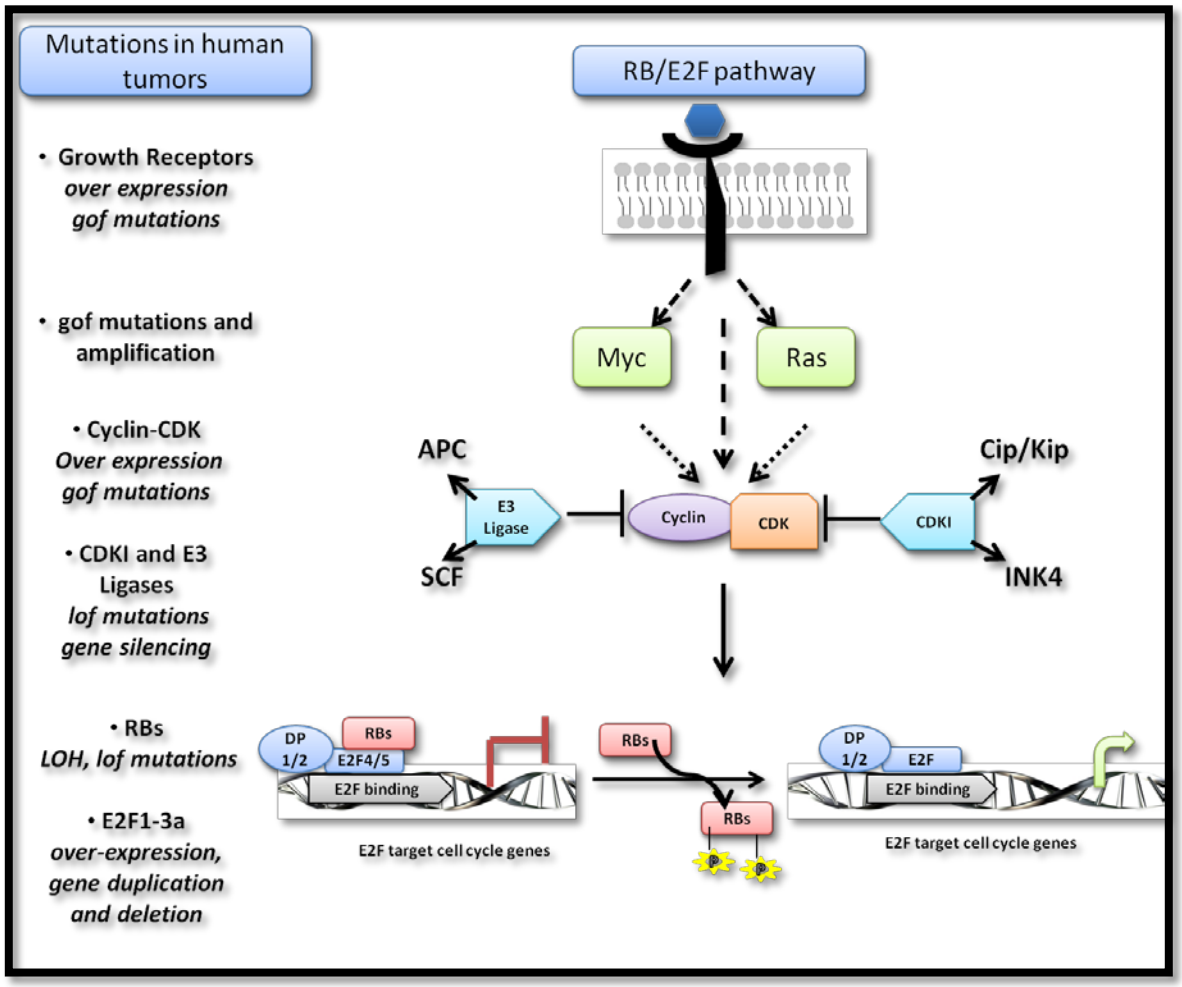


Figure 6: Mutations found in various components of RB/E2F pathway in spontaneous human tumors.

The RB/E2F pathway is the central regulator of cell proliferation controlling the G1/S phase cell cycle progression. The types of mutations found in various components of this pathway ultimately results into RBs inactivation and the release of E2F family of transcription factors and ultimately cell cycle genes activation. Growth factor receptors like RTKs and EGF or cellular oncogenes like Myc and Ras are found with mutations like gof or gene amplification. Similarly, cyclins and CDKs are found to be over-expressed or containing gof mutations, leading to their enhanced activity. On the contrary, CDKIs and components of E3 ubiquitin ligase complexes APC or SCF are found to contain genetic or epigenetic changes leading to decreased expression or lof. Finally, RBs are found to contain lof or gene silencing mutations, most of which are known to occur in RB1 gene coding for pRb protein.

lof (loss of function), LOH (loss of heterozygosity), gof (gain of function).

1.1.4 Retinoblastoma family of tumor suppressors

Retinoblastoma gene (pRb, RB1) is the first tumor suppressor to be cloned, and since its discovery, two more family members p107 (RB like 1, RBL1) and p130 (RB like 2, RBL2) have been identified. Together, the three proteins constitute the retinoblastoma family (RB) and are implicated in various cellular processes, such as, cell proliferation, differentiation, DNA repair and apoptosis (Classon and Harlow 2002). pRb was discovered as a mutation in a human eye malignancy called retinoblastoma (Friend, Bernards et al. 1986, Lee, Bookstein et al. 1987). It later gained importance due to the discovery that the independently evolved oncoproteins of small DNA tumor viruses, interact and inhibit the members of RB family (Whyte, Buchkovich et al. 1988, Chellappan, Kraus et al. 1992, Zalvide and DeCaprio 1995).

RBs in general, interact with various cellular and viral proteins containing the LXCXE motif via the pocket domain B (Classon and Dyson 2001, Classon and Harlow 2002). Based upon the sequence homology, p107 and p130 are more closely related to each other than pRb. The two pocket domains, A and B are the regions of maximum homology among the three RB proteins, and are involved in interactions with several cellular proteins including transcription factors and chromatin modifying enzymes (Lee, Russo et al. 1998, Brehm and Kouzarides 1999, Harbour and Dean 2000). The "spacer" between the two pocket domains in p107 and p130 is required for binding to cyclin/CDK complexes, which in turn phosphorylate the RBs and regulate their activity and expression. pRb also differs from p130 and p107, as its C-terminus region uniquely contains docking sites for E2F1, protein phosphatase 1 (PP1) or cyclin/CDK complexes **(Figure 7A).**

RBs activity and protein levels are governed at different stages of the cell cycle. During each phase of cell cycle, RBs are regulated by cyclin/CDK mediated phosphorylation, RNA transcription or protein stability. As shown in **Figure 7B**, while pRb levels remain constant during the cell cycle, p130 and p107 protein levels change dynamically. In growth arrested cells, p130 levels are maximum, which start decreasing due to proteosomal degradation as cell cycle progresses. In contrast, p107 protein levels are lowest in growth arrested cells, but increase progressively with cell cycle progression. p107 is a E2F target gene and the increase in p107 protein levels is directly linked to the increase in its transcript levels due to E2F transcriptional activity. RBs activity and interactions with E2Fs and with various other cellular proteins is regulated by their sequential phosphorylation mediated by cyclin/CDK complexes (Classon and Harlow 2002). During growth arrested phase, pRb and p130 are in a hypophosphorylated state, and interact with E2Fs and other LXCXE motif containing histone modifier proteins (Sadasivam and DeCaprio 2013).

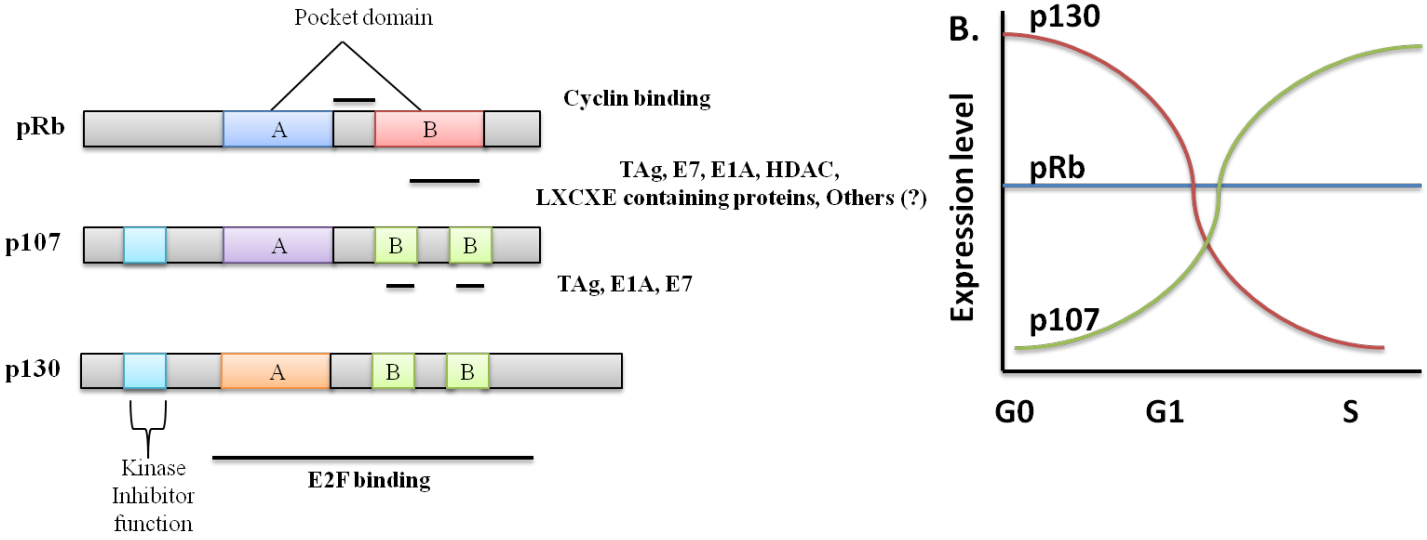


Figure 7: Retinoblastoma family of tumor suppressor proteins (Classon and Harlow 2002).

The RB family consists of three members, pRb, p107 and p130. The common region of sequence homology lies in the pocket domains A and B, with which they interact with various cellular or viral proteins containing LXCXE motifs and E2F family of transcription factors. The pocket domain B in p107 and p130 contains an insertion. The spacer region between the two pocket domains is involved in interactions with cyclins. p107 and p130 both contain a N-terminus kinase inhibitor domain, while pRb alone interacts with E2F1 and protein phosphatase 1 (PP1) from its C-terminus region. B). The RB family members are differentially expressed throughout the cell cycle. While pRb protein levels are maintained through the cell cycle, p107 and p130 protein levels dynamically change during the cell cycle progression. p107 is transcriptionally regulated and its expression begins during G1/S phase and peaks during late S phase. In contrast, p130 protein is post translationally regulated, and its levels peak during the G0 phase while gradually decreasing as the cell cycle progresses and becomes un-detectable during the S phase.

Transcription factor E2Fs (discussed later), bind to the promoters of cell cycle genes via their DNA Binding Domain (DBD), and recruit RBs to these promoters. RBs, on the other hand, interact with LXCXE motif containing histone modifiers, like histone deacetylase1 (HDAC1), histone methyltransferase Suv39h1 and heterochromatin protein1 (HP1). Therefore, by recruiting chromatin modifiers, RBs induce growth suppressive effects on the promoters of cell cycle genes, thus keeping the cells in a growth arrested state. Various studies have found predominant p130/E2F4-5 and few pRb/E2F3b complexes occupying the promoters of cell cycle genes in G_0 or early G1 phases of the cell cycle. In early to late G1 phase, p130 and pRb containing repressor complexes are disrupted due to the cyclin/CDK mediated inhibition of RBs, and E2F mediated cell cycle genes expression proceeds. p107 is an E2F target gene, and hence its transcription block is also removed, resulting into an increase in its protein levels during S-G2-M phase. This marks the exit from cell cycle. p107 in turn binds to E2F4/5, again forming a repressor complex on the promoters of cell cycle genes, and thus switching off its own transcription. To add to the complexity, cyclins are E2F target genes as well, and p107/E2F mediated repression induces their transcription suppression, resulting into a decrease in their protein levels, and thus stabilization of p130 proteins. p130 then replaces p107 from the E2F containing repressor complexes, thus completing the sequential array of events (Vairo, Livingston et al. 1995, Mulligan and Jacks 1998, Classon and Harlow 2002).

Genetic studies in mouse models suggest redundant and tissue specific roles of RBs

Although pRb was discovered as a mutation in human retinoblastoma, most of our understanding of its functions and regulations has come from studying transgenic and knockout mouse models. Individual and combinatorial deletion of RB family members in mouse models have suggested an overlapping as well as specific roles in development and cell proliferation.

Depletion of functional pRb from mice causes' embryonic lethality with various apparent defects as summarized in **Table 2** (Classon and Harlow 2002). While pRb deletion causes embryonic lethality in mice, loss of either p107 or p130 does not lead to any detectable developmental disorder (Cobrinik, Lee et al. 1996, Lee, Williams et al. 1996). However, combinatorial deletion of p107 and p130 results into neonatal lethality (Cobrinik, Lee et al. 1996). Moreover, combinatorial deletion of pRb/p107 and pRb/p130 from mice embryos resulted into an early embryonic death in comparison to pRb null embryos (Lipinski and Jacks 1999). These results suggest specific as well as redundant roles of the three RB family members in mice development.

Table 2: Role of retinoblastoma family of proteins in development and tumorigenesis

Genotype	Phenotypes	Remarks	References
$pRb^{+/-}$	• Tumors in Pituitary and Thymus. • Apoptosis in retina	Tumors associated with LoH. Early death @8.5 months.	(Jacks, Fazeli et al. 1992)
$pRb^{-/-}$	• Hyper proliferation accompanied with apoptosis in lens and CNS, Anemia. • Skeletal muscles defects. • Tumors in Pituitary and Thymus	• Tumors in Chimeras. • Embryonic Lethal @ 13.5-15 days.	(Jacks, Fazeli et al. 1992, Zacksenhaus, Jiang et al. 1996)
$pRb^{loxP/loxP}$; Mox2-Cre	• Lens Developmental defects	• Lethal @ P0.5 • Unlike $pRb^{-/-}$, these embryos	(de Bruin, Wu et al. 2003)

	• Skeletal muscles dysplasia	developed from wt placenta.	
$p130^{-/-}$	No observable defects	• Viable and fertile. • Strain specific defects.	(Cobrinik, Lee et al. 1996, LeCouter, Kablar et al. 1998)
$p107^{-/-}$	No observable defects	• Viable and fertile. • Strain specific defects	(LeCouter, Kablar et al. 1998)
$p130^{-/-}$;$p107^{-/-}$	• Several developmental and growth defects. • Enhanced cellular differentiation rate.	• Lethal @ P0.5	(Cobrinik, Lee et al. 1996)
$pRb^{-/-}$; $p130^{-/-}$	Pituitary and Thyroid tumors	• Embryonic lethal @ 11.5	(Lipinski and Jacks 1999)
$pRb^{-/-}$; $p107^{-/-}$	• Higher rates of apoptosis in liver and CNS when compared to $pRb^{-/-.}$ • Retinoblastoma, pituitary and thyroid tumors	• Embryonic lethal @ 11.5	(Lipinski and Jacks 1999)
$pRb^{+/-}$; $p53^{-/-}$	Retinal Dysplasia, pituitary and thyroid tumors	• Tumors associated with LoH	(Jacks 1996)

Based upon their inhibitory roles in cell proliferation, RB proteins are expected to behave as tumor suppressors. In fact, over-expression of each RB family member in rodent cells in cell culture resulted in cell growth (Classon and Harlow 2002). Similarly, *in vivo* and cell culture studies suggest that viral oncoproteins need to inhibit all three RB family members to induce proliferation and transformation in cell culture and transgenic expression of SV40 large T antigen. Consistent with this, SV40 T antigen, which inhibits all the members of RB family members, induces retinoblastoma in mice (Classon and Harlow 2002). Interestingly, while pRb haploinsufficiency causes retinoblastoma in humans, mainly due to spontaneous loss of the second copy, pRb conditional knockout in mice retina results in normal retinal development. Loss of pRb requires simultaneous loss of p107 gene in order to cause retinal dysplasia in mice, suggesting a compensatory role of p107 in mouse retina (Robanus-Maandag, Dekker et al. 1998, Vooijs, te Riele et al. 2002).

However, similar to their specific role in mice development, there are discrepancies observed in their tumor suppressive behavior in studies of mice and human tumors. Whereas deletion of pRb alone in mice results in pituitary and thyroid tumorigenesis, depletion of 107 and/or p130 does not result in tumorigenesis (Mulligan and Jacks 1998, Vooijs and Berns 1999). Similarly, while *lof* mutations in pRb gene have been identified by sequencing human tumors mutations are very rarely found in the p107 or p130 genes, arguing against their roles as tumor suppressors (Classon and Harlow 2002). Moreover, pituitary tumors observed in $pRb^{-/+}$ chimeric mice were abrogated upon deletion of p107 from these mice, suggesting an oncogenic role of p107 in the pRb knockout context (Vooijs and Berns 1999).

Based upon these studies, pRb is an established tumor suppressor while the role of p130 and p107 as tumor suppressors is still debatable. Finally, mouse studies showing tumorigenesis

in specific tissues upon depletion of pRb, suggests a strong tissue specific role. Similarly, the action of p107 as a tumor suppressor occurs only in the retina and only in pRb knockout context suggesting a tissue specific co-operation between the two RB proteins. Apart from playing key regulatory role in cell proliferation RBs are also implicated in apoptosis and DNA damage response (Hooper 1994, Lipinski and Jacks 1999), however the exact pathways affected are still unclear. In summary, the exact roles in tumorigenesis and tissue specificity of RB family require a comprehensive knowledge of their expression pattern and interacting partners. As mentioned above, RBs interact with several cellular proteins, but most of their growth suppressive effects are attributed to their regulation of members of E2F transcription family.

1.1.5 E2F family of transcription factors

RBs interact with various transcription factors implicated in cell cycle progression and cell differentiation. In this section, I will focus on the E2F family of transcription factors which regulate cell cycle progression downstream of RBs. Currently there are ten known E2F family members (E2F1-8) in mammalian cells, encoded by eight different chromosomal loci. E2F3 and E2F7 have two isoforms, which in the case of E2F3 (E2F3a and 3b) arise from a separate promoter while in E2F7 (E2F7a and 7b) arise from alternative splicing. E2F 1-5 can act as both activators and repressors of cell cycle genes, depending upon their interactions with other cellular proteins. However, various over-expression, depletion, co-immunoprecipitation (Co-IP) and chromatin immunoprecipitation (ChIP) studies coupled with gene expression analysis in unsynchronized and synchronized cells have divided the E2F family into two broad groups:

Activators of gene expression (E2F1-2-3a) and Repressors of gene expression (E2F3b-4-8) (Chen, Tsai et al. 2009).

As shown in **Figure 8**, activator E2Fs possess three major domains- (i). DNA binding domain (DBD), (ii). Dimerization domain (DD) and (iii). Transactivation domain. In addition, activator E2Fs also possess a nuclear localization signal (NLS) and cyclinA binding motif at the amino-terminus. Repressor E2F4-5 possess the same three domains as activator E2Fs but lack the NLS and cyclinA binding motifs. Instead they possess a bipartite nuclear export signal (NES). E2F6 contains the DNA binding and dimerization domains but lacks the transactivaton domain and localization signals. E2F7-8 only possess the DBD and lack the other canonical E2F domains and motifs. The repressor E2F6-8 lack transactivaton domain, and it is presumed that they inhibit cell cycle genes independent of RBs (Di Stefano, Jensen et al. 2003) (Ramirez-Parra, Lopez-Matas et al. 2004, Chen, Tsai et al. 2009).

While E2F1-5 contain RB binding motifs in their transactivation domain, they do not interact with all the members of RB family similarly (pRb, p130, and p107). pRb mainly interacts with E2F1-4, while p107 and p130 specifically binds to E2F4-5. The interaction and inhibition of E2Fs by RBs is tightly and sequentially regulated in a cell cycle specific manner (Chen, Tsai et al. 2009). E2Fs, when bound to RBs form repressor complexes with chromatin remodeling proteins like BRG-1, histone methyl-transferases like SUV39H1 and histone deacetylases (HDACs), and thus act as transrepressors (Sadasivam and DeCaprio 2013). When they are released from RBs, E2Fs form complexes with histone acetyltransferases like CBP, p300, and Tip60 via their transactivation domain, and thus activates cell cycle genes (Chen, Tsai et al. 2009). Although, "free" E2F1-5 all have the potential to transactivate cell cycle genes,

E2F4-5 are exported out of the nucleus when released from RBs (Takahashi, Rayman et al. 2000, Attwooll, Lazzerini Denchi et al. 2004, Frolov and Dyson 2004).

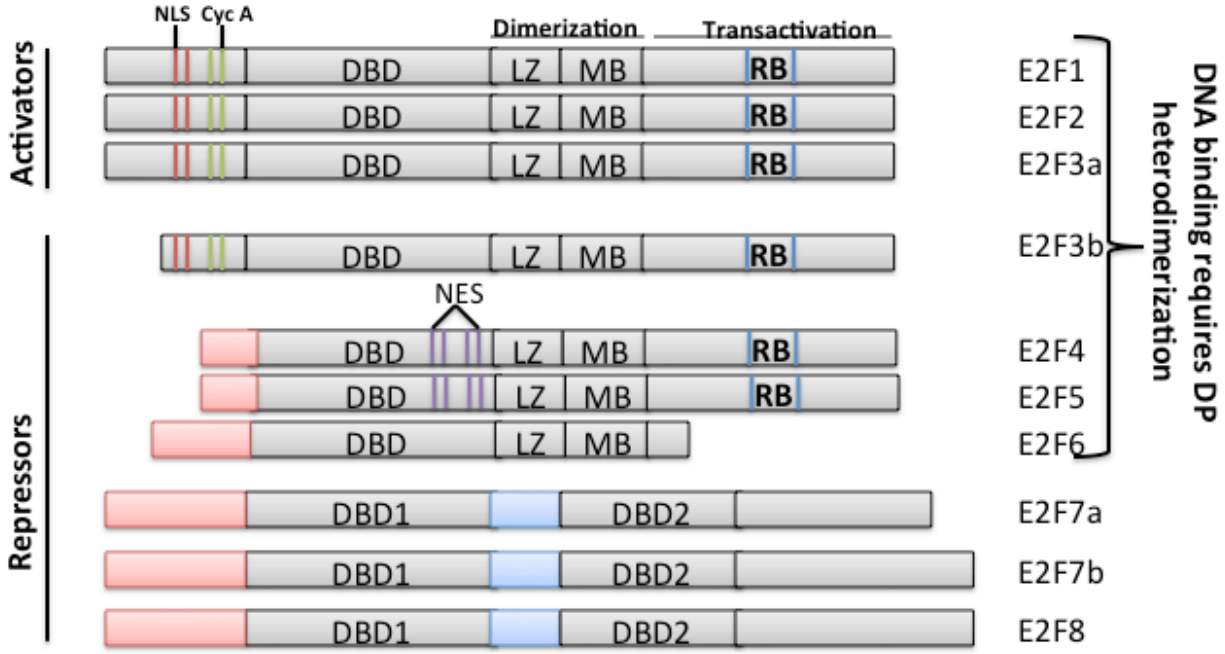


Figure 8: E2F family of transcription factors (Chen, Tsai et al. 2009).

E2F family of transcription factors consists of 10 members which are broadly divided into activators (E2F1-2-3a) or repressors (E2F3b-8). All the 10 members share sequence homology in the DBD domain. E2F1-6 also contain dimerization domain which is divided into regions containing leucine heptad repeats (LZ) to form heterodimers with DP proteins (DP1-3) and marked box motif (MB) of yet unknown activity. E2F1-5 contain C-terminus tranactivation domain which contain sites for interactions with specific RB family members (RB) and specific chromatin modifiers. E2F1-2-3a-3b also contain N-terminus NLS and Cyclin A (cycA) binding motifs. All other E2Fs lack these two N-terminus regions, and E2F4-5 contain a bipartite NES within their DBD. E2F7 and E2F8 are most distant from all other E2F family members, as they lack the transactivation and dimerization domains. Instead they possess two DBD (DBD1 and DBD2) and are believed to repress E2F target genes by forming homodimers. E2F3a and E2Fb are formed due to the use of different promoters in E2F3 locus. Isoforms E2F7a and E2F7b are formed by differential splicing of E2F7 primary transcript.

E2F (E2 binding Factors), LZ (Leucine Zipper), DP (Dimerization Partners), MB (Marked Box), DBD (DNA Binding Domain), NLS (Nuclear Localization Signal), NES (Nuclear Export Signal).

E2Fs were discovered as the factors required for Adenovirus E1A protein mediated activation of the viral E2 promoter, and hence the name E2F. Since then, several studies using genome wide approaches have identified E2F target genes involved in G1-S and G2-M cell cycle progression, DNA repair, apoptosis and development (Takahashi, Rayman et al. 2000, Wells, Boyd et al. 2000, Wells, Graveel et al. 2002, Cam, Balciunaite et al. 2004, Jin, Rabinovich et al. 2006). *In silico* analysis of the E2F binding promoters and reporter assays have shown that all the activator E2F family members bind to the same consensus DNA sequences and *in vivo* knockout mouse studies suggest a high degree of functional redundancy among activator E2Fs in regulating cell cycle genes (Jin, Rabinovich et al. 2006). Examples of genes whose expression is critical for cell cycle progression and which are regulated by E2Fs binding to their promoters

include DNA polymarase alpha (Pol α), dihydrofolate reductase (DHFR), minichromosome maintenance (MCM), thymidine kinase (TK) and Cyclin A and E. Apart from these genes, E2Fs also regulate expression of their own family members as well as of p107 (Bieda, Xu et al. 2006, Chen, Tsai et al. 2009).

Complications associated with the functional characterization of E2Fs

As mentioned above, E2F family consists of 10 members with high sequence homology. They all carry DBDs and apart from binding to the same consensus DNA sequences, they also bind to other non-consensus DNA sequences. The DNA binding promiscuity and high sequence homology leads to high functional redundancy among E2Fs (Chen, Tsai et al. 2009).

Another difficulty associated with the study of E2Fs is functional antagonism. This is evident from the studies performed in D. melanogaster, which, in contrast to mammalian cells has only two E2Fs-dE2F1 and dE2F2. Knockout studies show that depletion of dE2F1 causes repression of E2F target genes and cell growth arrest. These phenotypes are rescued upon depletion of dE2F2, suggesting an antagonistic role played by the two E2Fs in regulating cell cycle genes and cell proliferation (Asano, Nevins et al. 1996, Du, Xie et al. 1996, Royzman, Austin et al. 1999, Frolov, Huen et al. 2001).

The third difficulty arises from the complex feedback loop among E2Fs in regulating expression of their own family members. Knockout studies have shown that E2F1 gene is transcriptionally regulated by E2F7 and E2F8, while E2F7 and E2F8 promoters are themselves regulated by other E2F family members (Di Stefano, Jensen et al. 2003, Christensen, Cloos et al. 2005). These characteristics make the functional study of E2Fs extremely challenging and have resulted into various contrasting models regarding the roles of E2Fs in regulating cell proliferation.

Classical Model: Divides E2Fs into two functional classes

Experiments with the synchronized growth arrested cells in cell culture or differentiated quiescent cells *in vivo*, showed enrichment of p130/E2F4-5 containing DREAM (DP, RBL, E2F, and MuvB) complexes on the promoters of E2F target genes **(Figure 9)** (Sadasivam and DeCaprio 2013). This result correlates with chromatin condensation, histone methylation and cell cycle genes repression during G0 state of cell cycle. Upon serum stimulation of these cells, in the late G1 phase to early S phase, the p130/E2F4 repressor complexes were replaced with activator E2Fs. This exchange of promoter occupancy correlates with chromatin relaxation, histone acetylation and cell cycle genes expression (Takahashi, Rayman et al. 2000). Consistent to these results, co-immunoprecipitation studies have identified E2F4 association via p130 with DNA methyltransferase (DNMT1) and histone deacetylase (HDAC1), while activator E2Fs direct association with histone acetylases (p300, CBP) (Blais and Dynlacht 2007). In early to late S-phase, activators E2Fs are further replaced with p107/E2F4-5 complexes with a decrease in expression of cell cycle genes **(Figure 9)**.

Consistent with these studies, over-expression of activator E2Fs in quiescent mammalian cells results in induction of G1-S phase transition and circumventing $p16^{INK4}$ mediated growth arrest (Lukas, Petersen et al. 1996). Moreover, deletion of the activator E2F from Drosophila embryo (dE2F1) results in incomplete larval development, and failure in G1/S transition, over-expression of dE2F1 or dDP in growth arrested cells of Drosophila imaginal disc (Asano, Nevins et al. 1996) and eye (Du, Xie et al. 1996) leads to G1/S progression and DNA synthesis. Moreover, combinatorial deletion of the three activator E2Fs result into embryonic lethality in mice and cause growth arrest and cell death of mouse embryonic fibroblasts (MEFs) in cell culture. The same study also showed a significant down-regulation of cell cycle genes in MEFs

depleted of the three activator E2Fs (Wu, Timmers et al. 2001). These studies suggested cell cycle genes transactivation and cell proliferation requires "free" E2F1-2-3a.

Theoretically, based upon the current paradigm, activator E2Fs should act as oncogenes while repressor E2Fs should act as tumor suppressors in spontaneous or oncogene induced tumorigenesis. In fact, several human tumors show gene duplication or over-expression of activator E2Fs (Nevins 1998, Chen, Tsai et al. 2009). *In vivo* studies performed in $pRb^{-/-}$ mouse models also support their oncogenic role, suggesting them as downstream effectors of pRb. As mentioned above, pRb deletion in mice leads to embryonic lethality, accompanied by excessive cell proliferation, apoptosis and development of pituitary tumors, but deletion of activator E2Fs, especially E2F1 and E2F3a, rescues the $pRb^{-/-}$ phenotypes to a large extent, suggesting them as downstream effectors of pRb. In contrast, E2F4 or E2F5 deletions in pRb-/- background fail to revert the pRb-/- phenotypes (Chen, Tsai et al. 2009, Chong, Tsai et al. 2009). Lastly, c-Myc and HRas oncogenes are well known to co-operate in transforming rodent cells. Consistent with this, they transform wt and E2F1-2 double knockout (DKO) MEFs, but do not induce transformation in E2F1-2-3 triple knockout (TKO) MEFs (Sharma, Timmers et al. 2006). This suggests a requirement for at least one activator E2F in oncogene mediated cell transformation. In summary, these *in vivo* and cell culture studies have categorized E2F family members as activators and repressors of cell cycle genes. Therefore, based upon these studies, the current paradigm suggests that activator E2Fs are essential for cell cycle genes activation, and normal and oncogenic cell proliferation **(Figure 9)**.

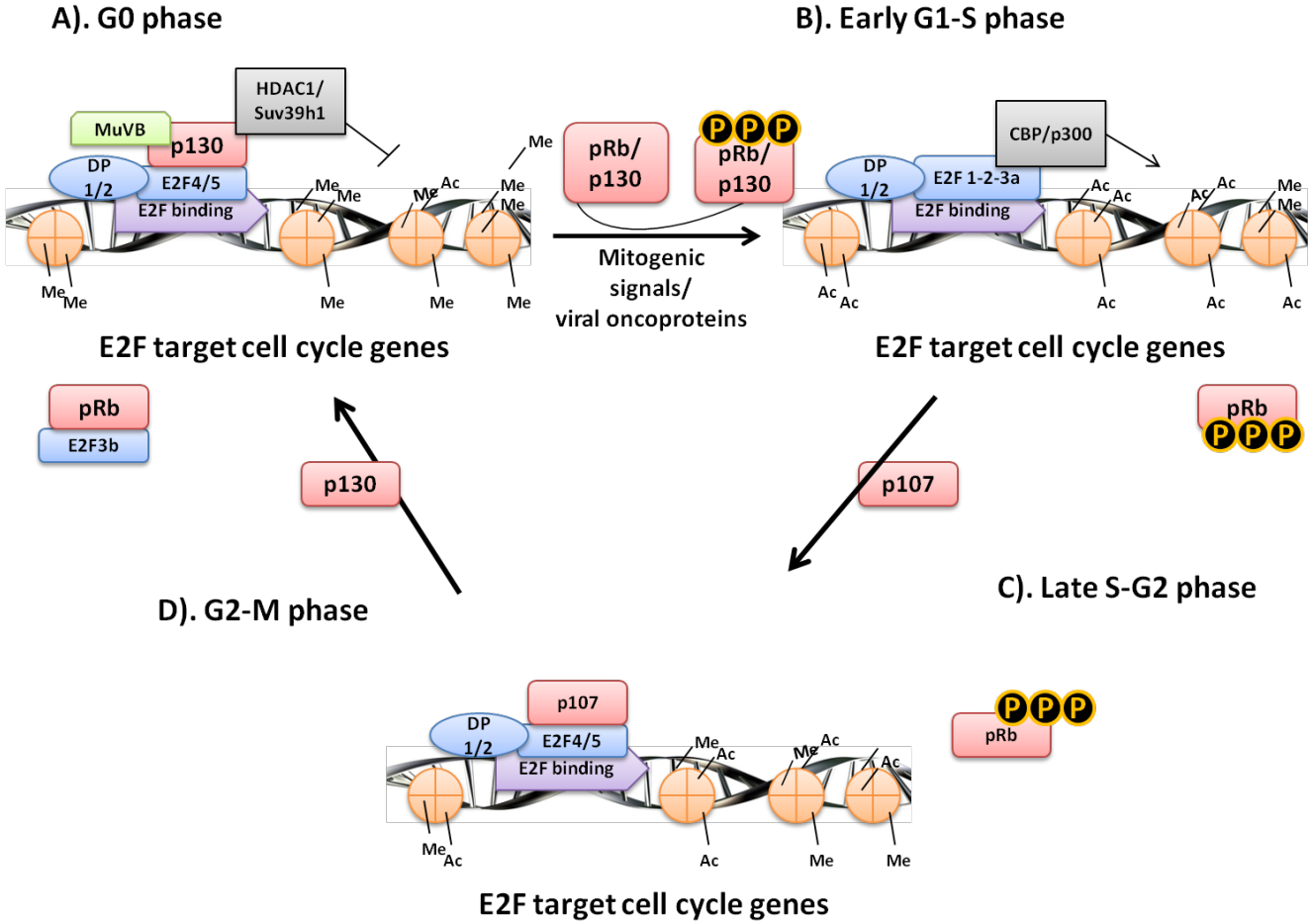

A). G0 phase
B). Early G1-S phase
HDAC1/
Suv39h1
MuVB
p130
DP
1/2
E2F4/5
E2F binding
pRb/
p130
pRb/
p130
CBP/p300
E2F 1-2-3a
Mitogenic
signals/
viral oncoproteins
E2F target cell cycle genes
E2F target cell cycle genes
pRb
E2F3b
p130
p107
pRb
C). Late S-G2 phase
D). G2-M phase
p107
DP
1/2
E2F4/5
E2F binding
pRb
E2F target cell cycle genes

Figure 9: Classical model of E2F target genes activation

According to the model E2Fs are grouped as activators (E2F1-2-3a) or repressors (E2F3b-8) of cell cycle genes. The studies leading to this model show activator E2Fs to be essential for cell survival and proliferation. **A)**. In the growth arrested G0 state of the cell cycle, DREAM complexes consisting of p130 and E2F4/5 occupies the promoters of E2F target genes and prevent them from expressing. This activity is dependent upon two features of the DREAM complex: 1) occupation of promoters by repressor E2Fs, thus making it inaccessible for transcription activators, and 2). histone modifications (de-acetylation and methylation) mediated by p130 associated histone modifiers. **B)**. Upon receiving pro-proliferation signals during the G1-S phase, pRb and p130 gets phosphorylated, resulting into the release of E2Fs. While repressor E2Fs are exported out of the nucleus, activator E2Fs now occupy the promoters of E2F target genes, and activate their expression. **C, D)** Gene expression activity of activator E2Fs results in transcription activation of p107, which accumulates during the late S phase to G2 phase of cell cycle. p107 binds to repressor E2Fs and forms the repressor complex on the promoters of cell cycle genes, replacing activator E2Fs and thus switches off cell cycle genes expression and induces cell cycle exit. Due to the suppression of E2F target genes, cyclins levels decrease, causing inhibition of CDK activity and preventing phosphorylation of p130 and pRb. This sequence activates p130 and pRb, which binds to E2Fs. While pRb binds and inhibits activator E2Fs, p130 replaces p107 and again forms the DREAM complex.

Cell context dependent role of activator E2Fs in activating cell cycle genes- Paradigm shift?

Studies categorizing E2Fs as activators or repressors were performed in either $RB^{-/-}$ background or in synchronized cells. Mammalian cells carry multiple isoforms of E2Fs, and mouse embryos depleted of a single activator E2F although show tissue specific defects, but still develop to adulthood and are fertile **(Table 3)**. Moreover, MEFs obtained from these mice proliferate similar to the wt MEFs in cell culture, with normal expression profile of the cell cycle genes. While, these results suggest a tissue specific role of activator E2Fs, they also show a functionally redundant role in cell cycle genes regulation and mouse development. Due to the functional redundancy of activator E2Fs in mammalian cells, *in vivo* and cell culture studies with combinatorial deletion of activator E2Fs were carried out. These *in vivo* or in cell culture studies suggest more complex roles of E2Fs in regulating cell cycle genes and cell proliferation, and have resulted into alternative model.

Table 3: Developmental defects in E2F knockout mice.

Genotypes	Phenotypes	Tumor Incidence	References
$E2F1^{-/-}$	Lymphocyte differentiation, Testicular atrophy	Increase tumorigenesis in several tissues	Yamasaki, L et al. 1996, Field et al. 1996
$E2F2^{-/-}$	Autoimmune disorders, Hematopoietic defects	None	Murga M et al. 2001
$E2F3^{-/-}$	Embryonic lethal @12.5 days, strain specific survival defects	None	Humbert, P.O. et al. 2000, Chen Hui-Zi et al. 2009
$E2F3a^{-/-}$	Reduced WAT deposits	None	Tsai, S et al. 2008
$E2F3b^{-/-}$	None	None	Tsai, S et al. 2008
$E2F4^{-/-}$	Neonatal lithality, craniofacial defects, hematopoietic defects, and gut developmental defects	None	Sherr and Roberts et al. 2004, Rempel et al. 2000
$E2F5^{-/-}$	Short life span, Hydrocephalus	None	Lindeman et al. 1998
$E2F6^{-/-}$	Normal lifespan except axial skeleton developmental defects	None	Pohlers, M et al. 2005
$E2F7^{-/-}$	None	None	Li, J et al. 2008
$E2F8^{-/-}$	None	None	Li, J et al. 2008
$E2F1^{-/-};E2F2^{-/-}$	Pancreatic defects and Diabetes	None	Li, F et al. 2003
$E2F1^{-/-};E2F3a^{-/-}$	Death @ 1 month, reduced white adipose tissue deposits, under-developed sexual organs and pancreatic disorders	None	Tsai, S et al. 2008
$E2F1^{-/-};E2F3b^{-/-}$	Viable and normal	None	Tsai, S et al. 2008
$E2F7^{-/-};E2F8^{-/-}$	Embryonic lethal @12.5 days, widespread apoptosis, vascular dialation and	None	Li, J et al. 2008
$E2F1^{-/-};E2F2^{-/-};E2F3a^{-/-}$	Neonatal lithality @19.5 days	None	Tsai, S et al. 2008, Danielian et al. 2008
$E2F1^{-/-};E2F2^{-/-};E2F3b^{-/-}$	Under-developed sexual organs	None	Tsai, S et al. 2008, Danielian et al. 2008
$E2F1^{-/-};E2F2^{-/-};E2F3^{-/-}$	Embryonic lethal @10.5 days, and **no** defects in cell proliferation	None	Chen, D et al. 2009 , Chong, J-L et al. 2009

As mentioned above, deletion of E2F1 and E2F2 allowed normal mouse development, deletion of the entire class of activator E2Fs resulted into embryonic lethality at 10.5 days (Wu, Timmers et al. 2001). However, the same study also showed normal cell proliferation in E2F1-2-3 null (TKO) embryos until they die at 10.5 days. In addition to these results, other *in vivo* and cell culture studies have also shown proliferation of cells in the absence of E2F1-2-3. Mouse embryonic stem cells in cell culture, and progenitor cells of mouse retina, lens and intestinal crypts *in vivo* proliferate in the absence of activator E2Fs (Chen, Pacal et al. 2009, Chong, Wenzel et al. 2009, Wenzel, Chong et al. 2011). Notably, the E2F target genes were up-regulated in TKO retina and lens cells while remaining unchanged in TKO crypts. Wild type progenitor crypt cells differentiate into quiescent cells of villi with a down-regulation of E2F target genes. While the differentiation of TKO crypts into villi cells was unaffected, E2F target genes were found up-regulated in TKO villi (Chong, Wenzel et al. 2009). These results suggest a switch in the roles of activator E2Fs from activators to repressors of cell cycle genes during the differentiation process (Chong, Wenzel et al. 2009). Therefore, while the earlier studies suggested a requirement of activator E2Fs in serum induced proliferation of growth arrested cells, these results suggest that certain cell types can proliferate even in the absence of activator E2Fs.

Similar to the contrasting studies regarding the role of activator E2Fs in cell proliferation, their role in tumorigenesis is also unclear. *In vivo* deletion of E2F1 or E2F2 leads to lymphoma and c-Myc induced T-cell lymphomagenesis respectively (Opavsky, Tsai et al. 2007, Parisi, Yuan et al. 2007). Similarly, E2F3 deletion in $pRB^{-/+}$ background induces development of

medullary thyroid tumors (Parisi, Yuan et al. 2007). These studies suggest a tissue specific tumor-suppressive role of specific activator E2Fs. Similar contrasting results were obtained for repressor E2Fs as well. Consistent with their repressive effects on cell cycle genes expression, repressor E2Fs are expected to act as tumor suppressors. However, they have been found to act as oncogenes in a few instances (Lee, Cam et al. 2002) (Chen, Tsai et al. 2009). Similarly, in spontaneous human cancers there are instances, for example in thyroid and pancreatic cancers, where huge deletions and suppressed expression of activator E2Fs are observed. Interestingly, a few instances have also shown a deletion or repressed expression of repressor E2Fs in human cancers, suggesting their role as a tumor suppressor. In fact, more instances have been observed where repressor E2F genes are amplified or are over-expressed (Chen, Tsai et al. 2009). These results in contrast to the current model, suggest a that generalization of E2F1-2-3a as activators and E2F3b-8 as repressors may not be the correct functional characterization. They also point towards more specialized roles for specific E2Fs in regulating cell cycle progression in a tissue specific manner. Lastly, these results suggest a dispensable role of activator E2Fs in cell cycle progression and cell survival depending upon the cell types (stem cells versus differentiated cells) or the initial proliferative state of the cells (continuously proliferating versus quiescent cells).

In summary, the exact role of E2F family members in cell proliferation and tumorigenesis is debatable, and contrasting results have challenged the current paradigm which categorizes them into activators or repressors. These studies have conclusively shown that activator E2Fs are dispensable for cell proliferation or tumorigenesis at least in certain cell types, and further studies are required to distinguish between the possible models and alternative pathways.

1.1.6 p53-MDM2 tumor suppressor pathway cross-talks with the RB-E2F pathway

p53 was discovered in 1979 in Co-Immunoprecipitation (Co-IP) assays as a protein bounded to SV40 TAg (Lane and Crawford 1979) (Linzer and Levine 1979). Since its discovery, it has been implicated as an effecter protein in several cellular processes involved in sensing various types of cellular stresses. p53 acts as a transcription factor, downstream of these stress sensing pathways and regulates cell growth arrest, senescence or apoptosis (Bond, Hu et al. 2005). In normal conditions, p53 protein levels are kept low due to a negative feedback loop involving a RING finger E3 ubiquitin ligase MDM2. p53 binds to MDM2 promoter and induces its expression, which in turn binds to p53 protein and induces its proteosomal mediated degradation, and thus regulating its levels (Momand, Zambetti et al. 1992). Upon activation of stress pathways, post translational modifications like phosphorylation and acetylation prevents p53 interactions with MDM2, causing an increase in its cellular levels and facilitating nuclear localization. Activated p53 forms homotetrameric complexes, which bind to specific DNA sites and act as a transcription in activating or repressing its target genes. Another protein $p19^{Arf}$ is a positive regulator of p53, which upon activation prevents MDM2 mediated degradation of p53, leading to its stabilization and thus allows p53 mediated growth inhibitory activities (Bond, Hu et al. 2005). Due to its growth inhibitory activity in response to various stresses, discovery of p53 mutations in human cancers is not surprising. It is found mutated in cancers of all tissue types and almost 50% of all human cancers are believed to bear mutations in p53 gene itself (Hooper 1994, Hickman, Moroni et al. 2002). As shown in **Figure 10**, p53 protein is divided into five major domains, each of which have been shown to be mutated in human cancers, with almost 90% of cancers bearing mutations in the DNA binding domain alone. Apart from mutations in the p53

gene, cancers bearing mutations in MDM2 or other upstream proteins negatively regulating p53 are also prevalent. In addition, viral oncoproteins are known to by-pass p53 growth suppressive effect either by directly interacting with it and inhibiting it, or by preventing its activation by acting on its up-stream regulators (Kinzler and Vogelstein 1996).

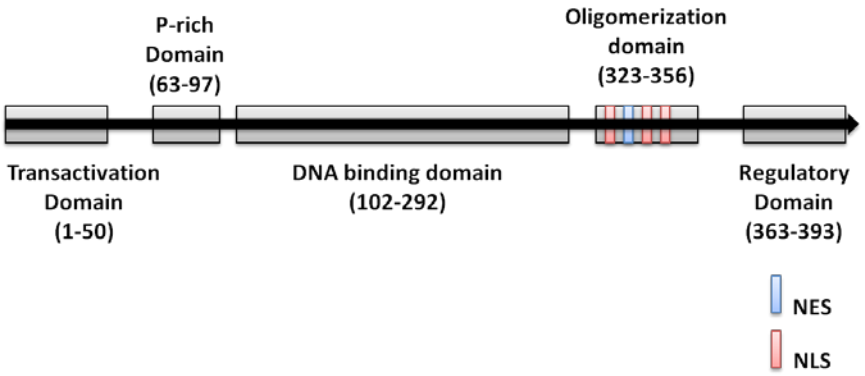


Figure 10: Domain structure of human p53

p53 is a multi-domain protein, consisting of five domains. The N-terminus transactivation domain is rich in acidic amino acids and is required for interactions with specific chromatin modifiers like CBP/ p300 and MDM2 protein. The second domain is rich in proline residues and also acts as transactivation domain. The central region (DBD) is required for interactions with specific DNA sequences in the promoters of p53 target genes. The oligomerization domain is required for forming p53 tetramers, and consists of a beta-strand followed by alpha helix. There are three NLS and one NES present in the oligomerization domain. Finally, the C-terminal domain is a non-specific DNA binding region and acts as a regulatory region by preventing specific DNA binding via DBD.

DBD (DNA Binding Domain), NLS (Nuclear Localization Signal), NES (Nuclear Export Signal).

p53-MDM2 pathway cross-talks with the RB-E2F pathway

Several lines of evidence suggest an anti-apoptotic activity of pRb (Hooper 1994, Lipinski and Jacks 1999, Hickman, Moroni et al. 2002). While pRb depleted mouse retina develops normally, a simultaneous depletion of p53 gene results into retinal dysplasia. Moreover, where expression of HPV oncoprotein E7 results into apoptosis of retinal cells, a simultaneous expression of HPV E6 (which inhibits p53), results into retinoblastoma formation (Howes, Ransom et al. 1994). Similarly, pRb deletion in lens cells of mice have been shown to cause hyper-proliferation, accompanied by p53 mediated apoptosis (Morgenbesser, Williams et al. 1994, Williams, Remington et al. 1994).

Similar results were obtained during over-expression studies of E2F1 (Shan and Lee 1994, Wu and Levine 1994), which was shown to induce hyper-proliferation accompanied by p53 induced cell death. Deletion of either E2F1 or E2F3 also prevents a significant level of apoptosis reported in pRb null embryos (Tsai, Hu et al. 1998, Ziebold, Reza et al. 2001). These results suggest that pRb deletion results into a de-regulated activity of activator E2Fs, which induces p53 mediated apoptosis. In fact, at least E2F1 is shown to transcriptionally activate p19ARF, and thus stabilizing p53 protein levels (Bates, Phillips et al. 1998). Interestingly, deletion of all three activator E2Fs from MEFs in cell culture or from progenitor cells of retina or lens also induces p53 activity without changing its protein levels by an unknown mechanism, which results into either growth arrest or apoptosis (Sharma, Timmers et al. 2006). Therefore, p53 response is tightly regulated by the RB/E2F pathway with activator E2Fs acting as both positive and negative regulators of p53 activity. Similarly, p53 downstream signaling also regulates the RB/E2F pathway. p53 activation in response to various stress stimuli results into its

transcriptional activity and CDKI $p21^{cip}$ is a p53 response gene. p53 mediated activation of $p21^{cip}$ results into inhibition of cyclinD/CDK4/6 complexes and induces growth arrest or senescence due to inhibition of E2Fs by RB proteins **(Figure 11)**.

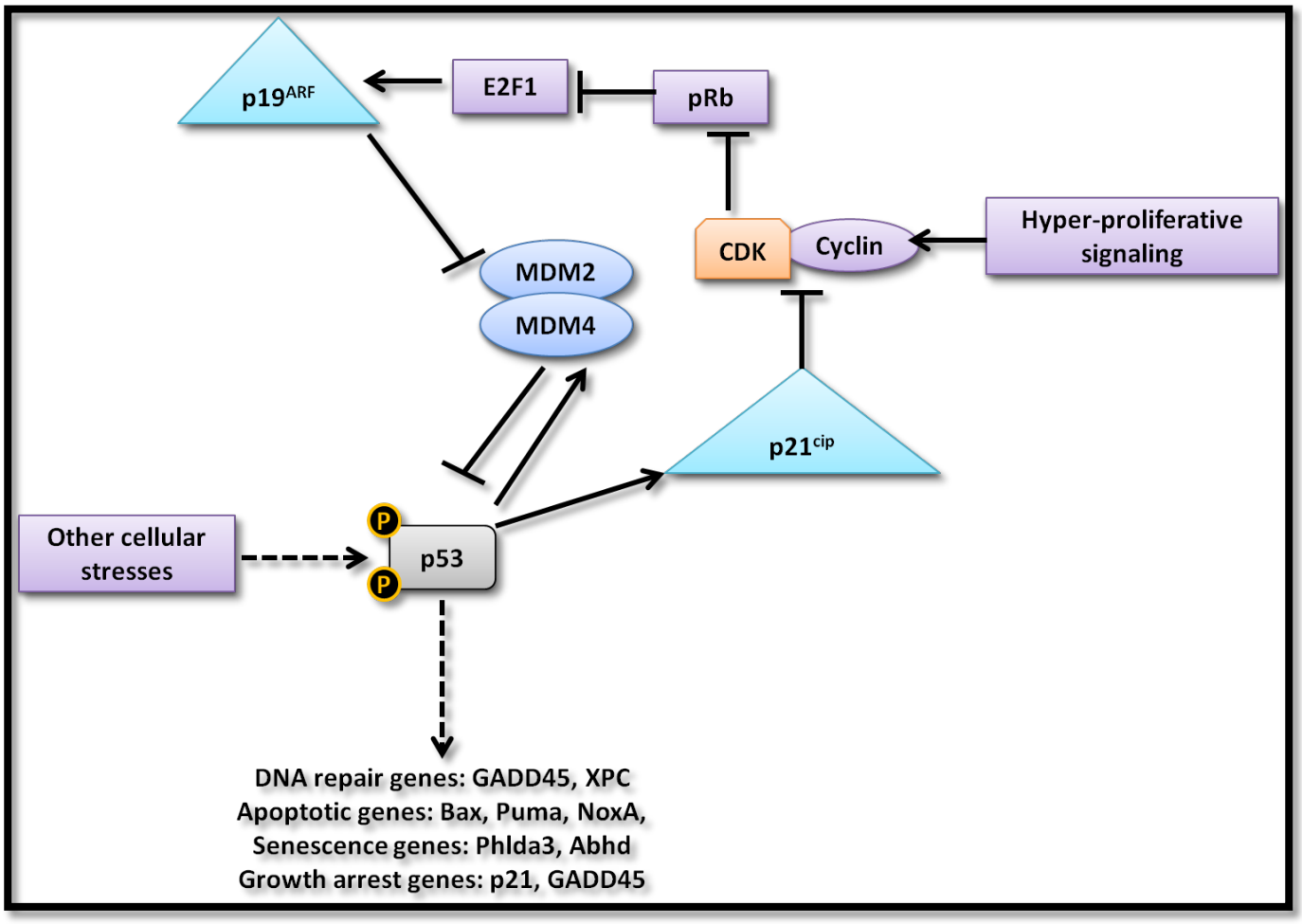


Figure 11: p53/MDM2 pathway cross-talk with RB/E2Fpathway: Various cellular stresses like acute

DNA damage, replicative stress, hypoxia, spindle damage and oncogenes lies upstream of p53/MDM2 pathway. The stress signals activate p53 response by either increasing its protein stabilization or by inducing specific post-translational modifications of p53 protein or both. Activated p53 induces its downstream signaling by transactivating its target genes promoter and resulting into one of the four cellular responses- DNA repair, Apoptosis, Senescence or Growth arrest. p53/MDM2 pathway is in tight cross-talk with the RB/E2F pathway. Hyper-proliferative signaling induces inhibition of RBs and results into hyper-activity of activator E2Fs. p19ARF is a E2F target gene, and a negative regulator of MDM2. Specifically, E2F1 transcriptional activation results into increased p19ARF levels which inhibits MDM2 activity by directly interacting with it, and thus prevents p53 protein degradation. This results into an increase in p53 protein levels and its transcriptional activity. p21cip is a p53 response gene which acts as a CDKI and inhibits cyclin/CDK4/6 complexes and thus switching off the pro-proliferation signaling and inducing either growth arrest or senescence.

1.1.7 Protein phosphatase 2A tumor suppressor

Protein phosphatase 2A (PP2A) is a major cellular serine/threonine phosphatase, implicated in various human cancers. PP2A is a heterotrimeirc protein complex, composed of a catalytic subunit C, a regulatory subunit B and a scaffolding subunit A. Each subunit has various isoforms and splice variants which potentially can form around 100 different holoenzyme complexes. The A subunit has two isoforms Aα and Aβ, which share 87% amino acid identity, but Aα isoform is several fold more abundant and have different physiological roles in the cell than Aβ. Similar to the A subunit, the catalytic C subunit also has two extremely identical isoforms, Cα and Cβ, while the B subunit has 20 different isoforms. While the A and C isoforms are ubiquitously expressed, B isoforms expression is tissue and developmental state specific. The distinct ABC holoenzymes complexes regulate more than 50 protein kinases and affect various cellular pathways like PI3K/Akt, and Wnt/β-catenin and thus regulate various cellular processes like cell growth, apoptosis, cell adhesion and tumorigenesis (Millward, Zolnierowicz et al. 1999). Several studies have found PP2A mutations in human cancers, and various labs have shown that inhibition of PP2A by different methods induce cell transformation both *in vivo* and in cell culture. Most of the PP2A mutations observed in human tumors are in the structural subunit isoforms Aα and Aβ, and these mutations render defects in holoenzyme formation and activity, suggesting tumor suppressive properties of the protein. These observations were verified by over-expression of the isoform, which suppressed transformation phenotypes and reducing the protein levels induced cell transformation (Millward, Zolnierowicz et al. 1999).

Different combinations of PP2A subunit isoforms would result into alterations in different subsets of molecular pathways, influencing various cellular processes. Our understanding of different PP2A isoforms arrangements and their biological consequences is extremely limited, and requires combination of genetics and comprehensive genome wide expression analysis. Polyomavirus sT and mT specifically binds and disrupts PP2A holoenzyme activity and therefore polyomavirus oncoproteins can serve as a great molecular tool to perform genetics and in determining biological implications of different holoenzyme combinations.

1.1.8 The Large Tumor Antigen (TAg)

The large tumor antigen (TAg) is a product of polyomavirus early region, and is the first gene expressed upon viral infection. Its activities are required for polyomavirus late genes expression, viral DNA replication, and virion assembly (Basilico and Zouzias 1976, DeCaprio and Garcea 2013). Polyomavirus requires host cell machinery for its own DNA replication, and thus requires the infected cells to be in a proliferative state (Basilico and Zouzias 1976). TAg is a multi-domain protein which interacts with specific cellular factors and modulates growth regulatory cellular pathways to induce host cell proliferation. Although each TAg domain has its own functional importance in viral genome replication, induction of cell proliferation and transformation are attributed to three different regions of TAg (DeCaprio and Garcea 2013). Biochemical and structural studies have shown that TAg binds and inhibits activities of two cellular tumor suppressor proteins-RB and p53, and thus influences the major growth regulating pathways involved in cell proliferation and death (Ahuja, Saenz-Robles et al. 2005). In addition to these two major tumor suppressors, TAg also interacts with other cellular proteins, directly or

indirectly, and affects cellular processes like histone modifications, protein degradation and cell cycle checkpoint regulation (Ahuja, Saenz-Robles et al. 2005).

TAg expression alone results into hyper-proliferation and transformation of various primary cell types in cell culture, and also induces tumors in various tissues of transgenic model organisms. Therefore, TAg is regarded as a potent viral oncoprotein, widely studied, and extensively used to decipher cellular pathways and molecular components involved in tumorigenesis. In addition, cell biologists also use TAg as a tool to extend lifespan of primary cells, which otherwise have limited growth capacity in cell culture, this allows their use for studying various cellular phenomenon or to be used as packaging cell lines for retro- or lenti-viruses production. Lastly, due to extensive characterization of SV40 ER promoter and polyadenylation signals, they are widely used in expression vectors.

In this chapter, I will focus on the current knowledge of different regions of SV40 TAg and their role in viral replication and cellular transformation.

J-domain and the linker region: The first N-terminal 82 amino acids of the large TAg share sequence similarity with the DnaJ class of molecular chaperone. Similar to the cellular DnaJ, J-domain of TAg, interacts with DnaK class of chaperone Hsc70, via its highly conserved HPDKGG motif, and induces its ATPase activity to target cellular substrates. Hsc70 is a major DnaK chaperone, which targets several cellular substrates; one of the substrate identified for the J-domain-Hsc70 complex is RB-E2F complex bound to the LXCXE motif of TAg (Sullivan, Cantalupo et al. 2000, Sullivan, Gilbert et al. 2001, Sullivan and Pipas 2002). Both *in vivo* and *in vitro* studies have shown that TAg mediated disruption of RB-E2F complexes requires ATPase activity of Hsc70 bounded to J-domain.

The D44N mutant, which only abolishes Hsc70 binding to TAg, allows foci formation and soft agar growth, but shows defects in proliferation under low serum conditions (Peden and Pipas 1992, Stubdal, Zalvide et al. 1997, Hahn, Dessain et al. 2002). The inability of D44N mutants to induce growth in low serum can be attributed to its activity in disrupting p130/E2F4 repressor complexes which accumulate under low serum conditions (Sadasivam and DeCaprio 2013). In fact, the J-domain is required to provide proliferative advantage to $pRb^{-/-}$ cells (Zalvide and DeCaprio 1995), but is dispensable for conferring growth advantage in p130;p107 double knockout cells (Stubdal, Zalvide et al. 1997). These results suggest a requirement of J-domain to inhibit p107/p130-E2F4-5 repressor complexes, but not for pRb-E2F complexes.

Importantly, TAg mutants lacking the complete J-domain can immortalized primary cells in cell culture (Hahn, Dessain et al. 2002), but it is defective in foci formation assays (Srinivasan, McClellan et al. 1997). The lack of transformation capabilities of J-domain deletion mutants in foci formation assays, suggests an additional activity of J-domain other than interactions with Hsc70, is required for "complete" transformation of primary cells.

The region of TAg between amino acids 71 to 98 is involved in interactions with two cellular substrates- Cullin7 (CUL7), a core component of cullin-Ring E3 ubiquitin ligase 7 (CRL7) and Bub1, a spindle checkpoint protein. Two point mutants F74A and F98A are defective in binding CUL7 while W94A and W95A point mutants are defective in binding to Bub1. While, Bub1 binding mutants are defective in focus formation assays, Cul7 binding mutants are defective in anchorage independence and low serum assays. TAg interaction with Cul7 is suggested to stabilize Insulin Receptor Substrate 1 (IRS1) both *in vivo* and *in vitro* assays. IRS1 stabilization is linked to activation of PI-3K/Akt and Erk mitogen-activated kinase pathways (Hartmann, Xu et al. 2014).

Linker region: The J-domain and OBD (Origin Binding Domain) of TAg are linked via unstructured peptide called the linker region. It is present in all the known polyomaviruses but varies in length, and contains two conserved and highly characterized motifs-LXCXE and a mono-partite nuclear localization signal (NLS). The role of LXCXE motif in cell transformation is discussed in detail, later in this review.

Origin Binding Domain: The OBD of TAg binds specifically to a pentanucleotide DNA sequence GAGGC present at the ori of viral genome. Apart from binding to the ori, OBD domain also interacts with the cellular replication protein A (RPA), and both these activities are essential for viral DNA replication but are dispensable for TAg induced transformation. TAg, via its OBD interacts with a DNA repair component of MRN complex, Nbs1. This interaction is thought to contribute in genome instability, but no role in cellular transformation has been reported.

Zn binding domain and ATPase domain: The Zn binding and ATPase domains carry out active DNA unwinding during the replication process. They form the core of TAg's helicase activity, and are essential for DNA replication. The Zn binding domain allows TAg hexamer formation, a structural state required for the enzymatic activity of the ATPase domain. While the Zn binding domain is dispensable for TAg induced cellular transformation, at least one activity in the ATPase domain is required for TAg induced transformation **(discussed later)**.

Variable and host range region (VHR): The C-terminal end of TAg consists of unstructured, flexible chain of amino acids called the VHR region. This region contributes in enhancing viral host range and allows productive infection in different cells. It is not shown to contribute in TAg mediated transformation, but it interacts with cellular protein component of SCF ubiquitin ligase complex. Moreover, few studies have reported phosphorylation and acetylation of residues T701 (Welcker and Clurman 2005) and K697 (Poulin, Kung et al. 2004)

respectively, and their possible roles in regulating TAg protein stability. F-box/WD repeat containing protein 7 (Fbxw7), is a tumor suppressor protein which acts as a substrate recognition protein in Cul7 mediated proteosomal degradation of CyclinE. Importantly, T701 acts as a phosphodegeron decoy in recruiting Fbxw7 protein and thus enhancing stability of Cyclin E (Welcker and Clurman 2005).

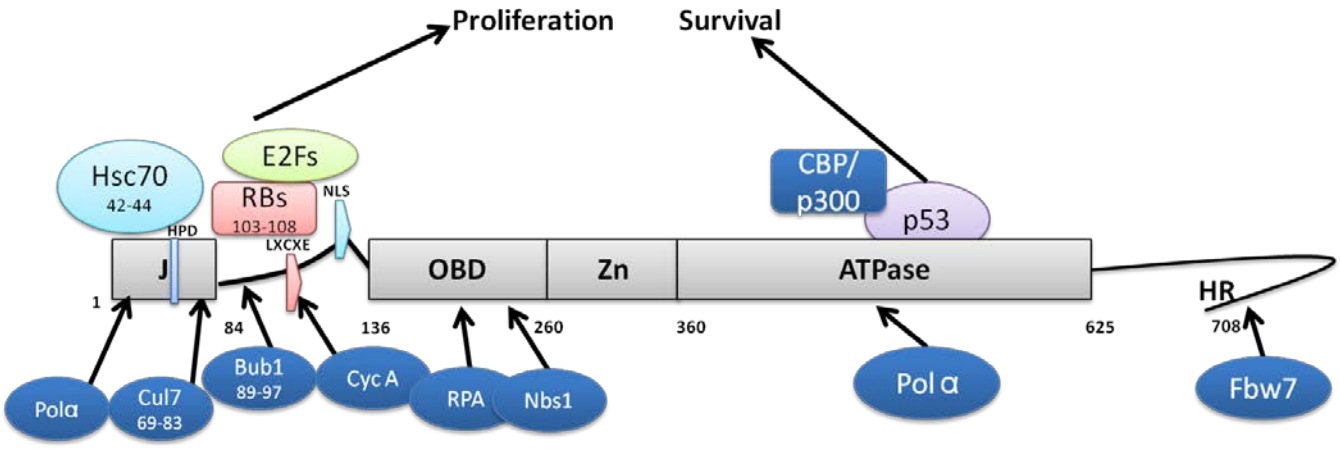


Figure 12: SV40 large tumor antigen (TAg), a multi-domain protein and its interactions with various cellular factors

The TAg of SV40 consists of four domains (grey boxes) and two unstructured regions (black loops), one present at the C-terminus and the other connects J-domain to the OBD domain. It interacts with various cellular factors via specific regions along its length, and alters their normal cellular activities. Its transformation activity is dependent upon at least three specific interactions: 1. It recruits Hsc70 via its HPD motif present in the N-terminus J-domain, 2. It interacts with RB/E2F complexes via its LXCXE motif present in the unstructured linker region, and 3. It binds to tumor suppressor p53 via its ATPase domain. It induces ATPase activity of Hsc70 to disrupt RB/E2F complexes and releases activator E2Fs to induce cellular proliferation, while its interactions with p53 result into suppression of p53 growth suppressor activity, which allows cell survival.

Polα (DNA polymerase alpha), Cul7 (component of E3 ubiquitin ligase complex), CycA (CyclinA), RPA (Replication Protein A), Bub1 (Mitotic checkpoint S/T kinase, Budding uninhibited by benzimidazoles 1), Nbs1 (Nijmegen breakage syndrome 1, component of MRN complex), Fbw1 (F-box 1, component of E3 ubiquitin ligase complex), CBP/p300 (Histone acetylases).

1.1.9 TAg inhibits the RB-E2F tumor suppressor pathway

The transformation activity of SV40 TAg in primary human and rodent cells is attributed to its interactions and inhibition of two cellular growth suppressive, RB-E2F and p53-MDM2 pathways (Ahuja, Saenz-Robles et al. 2005). In order to induce cell proliferation, TAg requires control of the RB-E2F pathway. TAg binds to RB-E2F complexes via its LXCXE (residues 103 to 107) motif present in the linker region (DeCaprio, Ludlow et al. 1988), and actively disrupts these complexes using ATPase activity of Hsc70 bound to the HPD (residues 42 to 44) motif of N-terminus J-domain **(Figure 12)** (Stubdal, Zalvide et al. 1997, Sullivan, Cantalupo et al. 2000, Sullivan, Gilbert et al. 2001). The LXCXE and the HPD containing motifs are conserved in all polyomavirus TAgs, suggesting a conserved mechanism of action on the growth suppressor RB-E2F pathway. In fact, TAg-RB interaction has been demonstrated for at least seven different polyomaviruses (Dyson, Bernards et al. 1990). While TAg interacts with all three members of the RB family, it affects the RB proteins differently. Whereas TAg expression causes a decrease in p130 protein levels, pRb protein levels remain unchanged, and p107 protein levels increase (DeCaprio, Ludlow et al. 1988). The decrease in p130 levels has been shown to be mediated by proteosomal degradation while p107 increase is linked to its transcriptional activation. Interestingly, while TAg interacts with the three RBs only in their un(der)-phosphorylated forms (active), it induces phosphorylation of p107 and p130, but not pRb (DeCaprio, Ludlow et al.

1988, Zalvide and DeCaprio 1995). The altered phosphorylation of p107 and p130 are dependent upon an intact J-domain and LXCXE motif (Stubdal, Zalvide et al. 1997). These results suggest a different biochemical mechanism for TAg-pRb interaction vs TAg-p107/p130 interaction. Interestingly, the effects of TAg expression on the levels and phosphorylation states of RBs are strikingly similar to that in normal, proliferating cells. Therefore, whether TAg induced proliferation results in the observed changes in RBs or the TAg induced changes in RBs result in proliferation, is still an open question.

RBs bind to E2Fs and forms repressor complexes on the promoters of cell cycle genes, and TAg interaction and inhibition of RBs disrupts these complexes, thus releasing E2Fs from RB repression. According to the current dogma TAg mediated RB repression is required to release activator E2Fs so that they can unleash their transcriptional activity to induce cell cycle genes expression. In fact, microarray studies performed in SV40 TAg expressing MEFs and mouse intestinal villi cells, shows an up-regulation of E2F target genes, which was dependent upon intact HPDK and LXCXE residues (Rathi, Saenz Robles et al. 2009).

Although, these studies do support the notion that activator E2Fs act as oncogenic effectors down-stream of TAg, only two studies have directly tested the role of activator E2Fs in TAg induced proliferation or transformation. *In vivo* studies performed using transgenic mice expressing TAg or its N-truncation mutant (N-terminal 121 amino acids, T^{N121}), in two different mouse tissues showed a requirement of at least one activator E2F in TAg mediated hyperplasia. The villi cells of mouse intestine are terminally differentiated cells and do not proliferate. Expression of TAg in these cells induces S phase entry and results in hyperplasia, that is significantly reduced in E2F2 alone or E2F2;E2F3a double knockout mice, suggesting an oncogenic role of at least E2F2 in TAg induced hyperplasia in enterocytes (Saenz-Robles,

Markovics et al. 2007). In another study, transgenic mice were prepared by expressing T^{N121}, which expresses the first 121 a.a. and retains the capability to bind and inhibit RBs. Expression of T^{N121} caused epithelial brain tumors in mice by inducing ectopic proliferation in normally growth arrested cells of choroid plexus. Depletion of E2F1 in T^{N121} transgenic mice showed considerable reduction in the number of S-phase and M-phase cells and also increased tumor latency period (Pan, Yin et al. 1998). This study suggested an oncogenic role of E2F1 in bringing TAg induced cell proliferation and tumorigenesis.

As described in the earlier sections, redundancy, antagonism, and feedback regulations among members of the E2F family make their study complicated and different E2Fs can act as tumor suppressors or oncogenes, under different circumstances. Although, the above mentioned studies suggest an oncogenic role of activator E2Fs, both these studies were performed in differentiated quiescent cells. As mentioned in the previous sections the requirement of activator E2Fs in cell proliferation or transformation can change depending upon the proliferative state of the cells studied. Thus, the role of activator E2Fs in TAg induced proliferation and transformation requires further investigation, perhaps in different cellular context.

1.1.10 TAg inhibits p53 tumor suppressor

The truncation mutants of TAg (N121 or N136), lack p53 interacting regions and their expression enhance the expression of p53 target genes (Rathi, Saenz Robles et al. 2009), suggesting that some N-terminus activity of TAg activates p53 pathway. TAg mediated inhibition of pRb releases activator E2Fs and induces uncontrolled cell proliferation. As mentioned in the previous section, p53/MDM2 pathway cross-talks with the RB/E2F pathway in

positive and negative feedback loops and uncontrolled cell proliferation can also result into activation of DNA damage pathways. Thus, TAg expression induces several types of oncogenic stresses which may lead to p53 activation and thus an induction of p53 growth suppressive responses. SV40 TAg, binds to DNA binding region of p53 and inhibits it from activating its target genes. The TAg-p53 interaction stabilizes p53 and increases its protein levels, but as shown by microarray analysis, p53 target genes remain repressed (Rathi, Saenz Robles et al. 2009) **(Figure 12)**.

TAg-p53 interaction is essential for TAg induced transformation. TAg point mutants, defective in binding p53 are also defective in foci formation and soft agar transformation assays (Ahuja, Rathi et al. 2009). Moreover, depletion of p53 in transgenic mice expressing T^{N121} leads to a significant increase in neoplastic growth of mice choroid plexus with a significant decrease in survival time (Pan, Yin et al. 1998).

1.1.11 The Small Tumor Antigen (sT)

All polyomaviruses express a differentially spliced product of ER called the small T antigen (sT). The sT shares N-terminus J-domain with the TAg, but contains a unique C-terminus region containing two zinc-binding motifs and PP2A interaction sites. Similar to TAg, sT interacts with Hsc70 through its N-terminus J-domain (Rundell and Parakati 2001) but most of its biological activities arise from its interaction with protein phosphatase 2A (PP2A) via its unique region (Mungre, Enderle et al. 1994, Arroyo and Hahn 2005). The unique region of sT contains two cysteine rich clusters (CXCXXC) which are involved in zinc ion binding and are essential for sT structural stability. Structural and biochemical analyses have identified specific

residues R7, R21, P132 and W147 in SV40 sT which are involved in PP2A interaction (Cho, Morrone et al. 2007). sT interacts with the N-terminus of the structural A subunit of PP2A, via its motif containing second Zn binding cluster and the J-domain, preventing its interactions with the regulatory B subunit, and thus preventing holoenzyme formation (Mungre, Enderle et al. 1994, Skoczylas, Fahrbach et al. 2004). The catalytic C subunit of PP2A interacts with the C-terminal region of the A subunit. Structural superimposition of sT-PP2A structure on PP2A holoenzyme structure suggests a possible interaction between PP2A C-subunit and sT motif containing the first Zn binding cluster.

Displacement of the PP2A B subunit by sT inhibits PP2A activity towards some substrates. Interestingly, sT-PP2A interaction also results in an increase in phosphatase activity of PP2A towards histone H1. Therefore, sT-PP2A interactions may result into alteration in PP2A substrate specificity, and sT may itself act as the PP2A B-subunit.

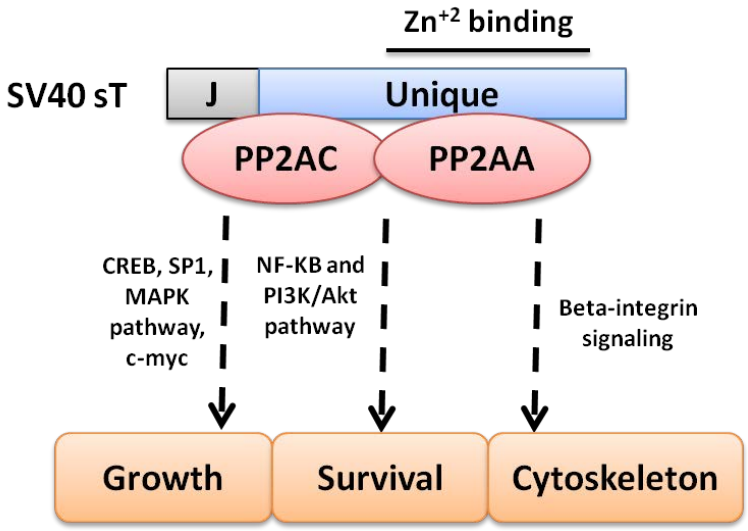


Figure 13: SV40 sT schematic diagram and biological consequences of sT-PP2A interactions.

sT shares N-terminus J-domain with the TAg, while its C-terminus consists of unique sequences. Similar to TAg, it interacts with Hsc70 via its J-domain, however its biological activities arise from its interactions with serine threonine phosphatase PP2A. The sT unique region contains Zn ion binding sites wh ich are critical for its protein stability. sT specifically interacts with A and C subunits of PP2A (PP2AA and PP2AC) and displaces PP2A B subunits, thus preventing holoenzyme formation. sT-PP2A interaction res ults into biological changes which include enhanced cell growth, survival and cytoskeleton changes. The specific pathways affected by sT-PP2A interactions converge to bring these biological changes.

sT interaction with PP2A affects various cellular pathways like mitogen activated protein kinase (MAPK) (Frost, Alberts et al. 1994), NF-KB (Sontag, Sontag et al. 1997), Akt (Zhao, Gjoerup et al. 2003, Skoczylas, Fahrbach et al. 2004), PKC (Skoczylas, Fahrbach et al. 2004), and Rac (Zhao, Gjoerup et al. 2003), and hence regulates cell proliferation, survival and adhesion

(Sablina and Hahn 2008). Interestingly, all the cellular pathways mentioned above are well known downstream effectors of PI-3K (phosphatidyl inositol-3 kinase). In fact, constitutive activation of PI-3K or Akt1 and Rac can substitute SV40 sT in transformation of human primary cells in vitro suggesting inhibition of PI-3K de-phosphorylation as the primary consequence of sT-PP2A interaction (Zhao, Gjoerup et al. 2003). However, these results do not rule out additional pathways which might be altered due to sT-PP2A interaction. For example, sT activates cyclin A and cyclin D expression which allows cell proliferation and foci formation in association with TAg. In addition, sT also induces expression of NF-KB regulated anti-apoptotic genes, and c-Myc target genes (Sontag, Sontag et al. 1997, Skoczylas, Fahrbach et al. 2004).

The above mentioned studies were performed in various cell types which differ in their tissue and species origin. The cellular pathways affected by sT-PP2A interaction are usually involved in cell differentiation and development and affects various other cellular activities depending upon the molecular makeup of the cells. Therefore, depending upon the cellular status, or the presence of other oncogenes, sT-PP2A interaction can result in a different combinatorial affect of these pathways and thus results into a different biological phenotype.

Similar to large T antigen, sT's of MuPyV and SV40 are the most studied and best characterized proteins and only recently MCPyV sT has gained attention due to its unique transformation capabilities. Sequence homology of various polyomavirus sT shows a high degree of similarity in the N-terminal region which considerably decreases in the unique region. This suggests a different role for each sT in viral genome replication or cell transformation. Three of the four PP2A binding sites in SV40 sT are also conserved in MuPy sT, and Co-IP studies have suggested robust binding of PP2A A and C subunits by both polyomavirus sT's. Consistent with this result, comparative analysis of SV40 and MuPy sT's, have shown induction of cellular DNA

synthesis and Cyclin D expression in immortalized NIH3T3 cells (Andrabi, Hwang et al. 2011). However, expression of MuPy sT fails to replace SV40 sTAg in co-operation with SV40 TAg in transforming human fibroblasts. Moreover, MuPy sT but not SV40 sT, prevents differentiation of myoblast and preadipocytes differentiation (Andrabi, Hwang et al. 2011). One explanation for the different biological consequences of SV40 and MuPy sT's in the same cell types could be due to their differential affinity towards specific isoforms of PP2A subunits. Co-IP results from the same study showed that MuPyV sT interacts with both Aα and Aβ isoforms of the A subunit, while SV40 sT preferentially binds only to the Aα subunit (Andrabi, Hwang et al. 2011). Based upon this result, two important hypotheses can be drawn: 1. Due to SV40 interaction with only the Aα isoform, it fails to prevent PP2A holoenzyme formation involving Aβ isoform, and would therefore fail to sequester all the PP2A complexes, and 2. sT needs to compete with the B subunit to interact with the A subunit of PP2A, and their relative affinities would determine which molecule will be found in the PP2A complex. Specific B isoforms may have differential affinities towards different A subunit isoforms, and therefore sT mediated regulation of PP2A may depend upon the expression levels of specific B subunit isoforms.

Lastly, expression of SV40 and MuPy sT's alone in primary cells leads to either senescence or apoptosis and requires a complementary oncoprotein for transformation. In contrast, recently discovered MCPy sT induces cellular transformation on its own. Moreover, while transforming activities of SV40 and MuPy sT's are PP2A dependent, transformation by MCPy sT is PP2A independent. MCPy acts downstream of Akt-mTOR pathway to inhibit a cap dependent translation regulator, 4E-BP1, by inducing its hyper-phosphorylation. In contrast, SV40 sT was shown to decrease phosphorylation of 4E-BP1 (Shuda, Kwun et al. 2011).

In summary, studies performed to characterize sT proteins from polyomaviruses have revealed crucial roles of PP2A in cellular transformation and identification of various down-stream effecter proteins. However, there are several questions regarding sT biology that remain unanswered. For example, 1) What is the mechanism of sT interaction with PP2A, is it active displacement of B subunit from an already formed holoenzyme or does it compete with the B subunit to bind de-novo synthesized A and C subunits. 2). Is J-domain-Hsc70 interaction required for sT-PP2A interaction, 3). How do the different combinations of PP2A regulated pathways govern biology of sT's, 4). are sT mediated alterations of which PP2A downstream pathways is sufficient for its complementary transforming activities with other viral oncoproteins, and 5). Why do the sT's encoded by different polyoma viruses regulate different components of the same molecular pathway?

1.1.12 The Middle Tumor Antigen (mT)

MuPy and Hamster Polyomavirus ER splices into a unique protein called middle T antigen (mT) and of the two, MuPy mT is the most characterized. mT shares the J-domain and additional 112 amino acids with the MuPy sT but has a unique C-terminus sequence comprising of 230 amino acids. Mutations in the ER affecting mT but not the TAg or sT have shown its essential role in cell transformation. Furthermore, mT expression is sufficient to transform established cell types in culture and induce tumors in transgenic animals (Treisman, Novak et al. 1981). However, mT fails to transform primary cells in culture and results into either growth arrest or apoptosis. In such situations, when it is unable to transform cells, co-operation with

viral oncoproteins like MuPyV sT or TAg, Adenovirus E1A or cellular oncoprotein cMyc allows transformation to proceed (Rassoulzadegan, Cowie et al. 1982, Cuzin 1984).

mT is very unique in its localization and mechanism of action. While TAg or sT inhibits cellular tumor suppressor proteins to induce transformation, mT induced transformation results from its induction of various oncogenic growth-signaling pathways. Moreover, while TAg is localized in the nucleus and sT is found both in the nucleus and cytoplasm, mT C-terminus contains a stretch of 22 hydrophobic amino acids which spans the lipid bilayer of plasma membrane and endoplasmic reticulum. The rest of the protein lies in the cytoplasmic region of the cell and mimics cellular receptor tyrosine kinase (RTK acting as a scaffold for signaling molecules and thereby causing activation of cellular oncogenic pathways (Dahl, Thathamangalam et al. 1992, Elliott, Jones et al. 1998).

As mT shares the first 190 amino acids with sT it possesses capability to interact with Hsp70 and Aα and Aβ subunits of PP2A (Pallas, Shahrik et al. 1990). However, unlike sT, mT-PP2A interaction results into recruitment of members of Src family of protein tyrosine kinases (PTKs) (Campbell, Ogris et al. 1994). It interacts with three Src family members, c-Src, c-Yes and c-fyn and specific interactions result in tissue specific activities of mT (Courtneidge and Smith 1983, Kornbluth, Sudol et al. 1987, Cheng, Harvey et al. 1988). mT-Src interaction results into enhanced kinase activity of Src proteins which results into phosphorylation of key serine/tyrosine residues on mT itself: Y-250, S-257, Y-315 and Y-322 (Ichaso and Dilworth 2001). Each tyrosine phosphorylation acts as a scaffold for recruitment and activation of specific adaptor proteins and results into activation of their downstream signaling cascade.

Y-250 phosphorylation is required for interaction with ShcA family of proteins (Campbell, Ogris et al. 1994), and this interaction results into recruitment and activation of adapter protein

complex containing Grb1/Grb2/Sos which in turn activates $p21^{Ras}$ and MAPK pathway (Aronheim, Engelberg et al. 1994). Mutation of Y-250 results into significant reduction in mT transforming activity suggesting a critical role of MAPK pathway in mT mediated transformation. Phosphorylation of mT on Y-257 acts as a docking site for few members of 14-3-3 family of proteins (Pallas, Fu et al. 1994). However, mutations in 14-3-3 binding sites of mT, are not been linked to mT induced tumorigenesis although there is evidence suggesting 14-3-3-mT interactions may be critical for salivary gland tumorigenesis (Cullere, Rose et al. 1998).

The most important transformation function of mT arise from phosphorylation of Y-315 which acts as a docking site for PI-3K (phosphatidyl inositol-3 kinase) (Courtneidge and Heber 1987, Kaplan, Whitman et al. 1987). Mutation of this site significantly reduces tumors of kidney, adrenal gland and mammary gland (Freund, Dawe et al. 1992). However, the same mutation is dispensable for transformation of Rat-1 cells in cell culture (Oostra, Harvey et al. 1983) or induction of tumors in thymus and hair follicle *in vivo*. In other cases the Y-315 mutation enhances mT mediated tumorigenesis, for example in lung and bone marrow. These results emphasize tissue specific roles of oncogenic pathways in tumorigenesis. The mT-PI-3K interaction results into up-regulation of PI-3K phosphorylation products-$PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Gorga, Riney et al. 1990) which further activate other cellular kinases like, PKB, PKC and PDK1 which results into phosphorylation and change in activity of various transcription factors (Marcellus, Whitfield et al. 1991). Interestingly, mutations in ShcA binding site of mT does not affect mT-PI-3K interactions but result in a decrease in $PI(3,4)P_2$ and $PI(3,4,5)P_3$ products and a loss of mT transformation activity. This suggests an essential cross-talk and co-operation between the down-stream components of the two pathways (Ling, Druker et al. 1992).

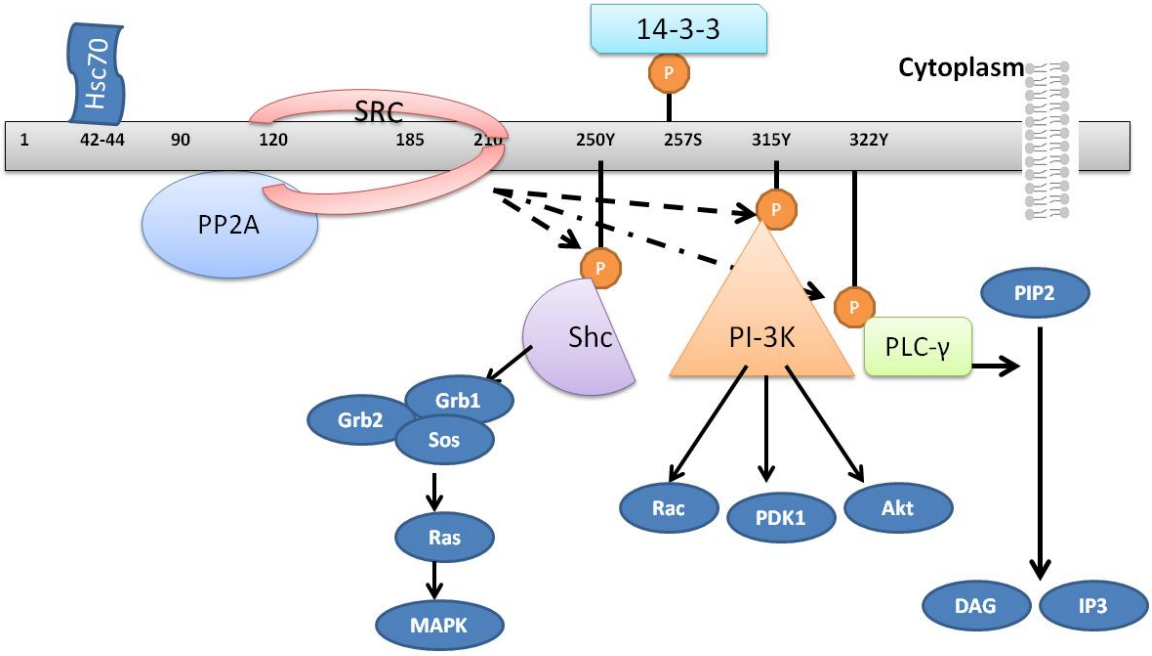

14-3-3
Hsc70
Cytoplasm
SRC
1
42-44
90
120
185
250Y
257S
315Y
322Y
PP2A
P
Shc
PI-3K
PLC-γ
PIP2
Grb1
Grb2
Sos
Ras
MAPK
Rac
PDK1
Akt
DAG
IP3

Figure 14: Schematic of MuPyV mT

Polyomavirus mT localizes in the plasma membrane via its C-terminus hydrophobic residues. Its N-terminus region lies inside the cytoplasm, and is involved in interactions with specific factors belonging to various oncogenic pathways. Similar to TAg or sT, its N-terminus region contains J-domain with which it interacts with cellular Hsc70. Similar to MuPyV, it also binds with A and C subunits of PP2A, and uses it to recruit SRC tyrosine kinase, and thus mimics as a RTK (receptor tyrosine kinase). mT-Src interaction results into phosphorylation of specific tyrosine residues on mT itself, which then acts as docking sites for Shc, PI3K, and PLCγ-1 and activates their downstream signaling cascades.

In addition, mT also contains serine phosphorylation site, which upon phosphorylation acts as docking site for 14-3-3 protein family.

Finally, mT interacts with Phospholipase C-γ1 (PLC-γ1) through its Y-322 phosphorylation and in turn results into phosphorylation of tyrosine residues of PLC-γ1, probably causing activation of its enzymatic activity (Su, Liu et al. 1995). However, phosphorylation of Y-322 of mT has not been linked to tumorigenesis, but again perhaps a tissue specific role of mT-PLCγ1 interaction is yet to be discovered.

In summary, while studies of sT and TAg has helped in discovery and decoding of various tumor suppressor pathways, mT characterization has helped in uncovering various oncogenic signaling pathways. The most important discovery made by studying mT was of PI-3K pathway and its implications in various tumor types. These studies have been helpful in understanding oncogenic roles of various signaling pathwaysand in decoding their tissue specific

roles in cell differentiation and growth. However, we still have several unanswered questions regarding the functioning of mT: 1). How do various signaling pathways interact to result into transforming properties of mT? 2). Does mT-Src interactions serve other roles than just phosphorylation of mT on specific residues? and 3). Does the developmental lineage of a tissue determine the dominance of specific growth signaling pathways in its cancers?

1.1.13 Polyomavirus oncoproteins co-operate with other oncoproteins in cellular transformation and tumorigenesis

Cancer is a multi-step disease involving multiple mutations affecting more than one oncogene, and resulting in oncogenic co-operation. Whereas the study of cell transformation by individual oncoproteins has helped in decoding specific cellular pathways involved in spontaneous tumor formation the study of oncogenic co-operation has helped in decoding complementary and synergistic pathways involved in tumorigenesis. Although the above discussed polyomaviruses oncoproteins are able to induce cellular transformation and tumorigenesis on their own, in certain circumstances they require co-operation of other cellular or viral oncogenes.

TAg is sufficient to transform several mouse, rat and hamster cells in culture and induce tumors in transgenic animals. However, it requires the presence of other oncogenes to induce human fibroblasts transformation. Activated H-Ras (H-Ras^{V12}) and small T antigen of SV40 or MuPyV have been shown to co-operate with TAg to induce transformation of BJ;hTERT and IMR90 cells (Hahn, Dessain et al. 2002). In fact, co-expression of both these proteins have been shown to enhance TAg transformation phenotypes in mouse cells in cell culture and increase

tumor incidence in transgenic model animals. $HRas^{V12}$ over-expression in primary cells expedites their senescence process (Serrano, Lin et al. 1997), while the $HRas^{V12}$ induced senescence is rescued in the presence of TAg. Moreover, $HRas^{V12}$ expression in TAg expressing cells, leads to a significant enhancement of transformation phenotypes as tested by foci formation, anchorage independence, and xenografts in model organisms (Arbiser, Moses et al. 1997). These results suggest involvement of complementary pathway(s) affected by the two onco-proteins.

Expression of two different TAg truncation mutants T^{N121} and $T^{C257\text{-}708}$ are individually capable of rescuing the $HRas^{V12}$ induced cellular senescence, and enhance foci formation phenotype. Further characterization of the two truncation mutants suggested that binding and inhibition of RBs is required in the N-terminal truncation mutant while p53 inhibition in the C-terminal mutant is not (Cavender, Conn et al. 1995). Point mutations in HPD or LXCXE motifs in T^{N121} abrogate its co-operation with $HRas^{V12}$ in foci formation assay, while several point mutants in the region 382-411 in the $T^{C257\text{-}708}$ context fail to co-operate with $HRas^{V12}$ but retain p53 inhibitory activity. Moreover, point mutations in the HPD or LXCXE motifs in the context of full length TAg allow co-operation with $HRas^{V12}$ suggesting modulation of the same pathway by the two truncation mutants (Cavender, Conn et al. 1995).

1.2 INTRODUCTION SUMMARY

The ultimate goal of cancer biologists is to identify individual tumorigenic pathways and then decode their inter-relationships as well as their tissue type specificity. Different research

groups use different strategies to understand these cancer causing molecular and cellular pathways and using naturally evolved viral oncoproteins is one but very effective strategy.

RB-E2F pathway is the central regulator of cell proliferation and is estimated to be mutated in most human cancers. For this reason, several translational studies are focusing on designing cancer therapeutic drugs, targeting different components of this pathway. Previous studies have divided RBs and repressor E2Fs as tumor suppressor proteins, and activator E2Fs as oncogenes. However, the recent contrasting results about the roles of activator E2Fs in normal cell proliferation have clouded this characterization. Moreover, these studies strongly suggest presence of an alternative mechanism to regulate cell cycle genes expression and cell proliferation. Therefore, there is a requirement for a more specific model about RB/E2F function and their role in cell proliferation.

SV40 TAg is a highly characterized oncoprotein, with several truncation and point mutants available to perform desired genetics. Current "textbook" model for TAg induced transformation considers activator E2Fs as essential downstream oncogenes, however there is a dearth of studies directly testing their roles and requirements of activator E2Fs in TAg biology. Therefore, TAg serves as a great molecular tool to dissect the RB-E2F pathway components and understand their relationships with other growth controlling pathways. Therefore, in one section of this study I am focusing on the role of activator E2Fs in SV40 large T antigen induced proliferation and transformation of primary cells.

Several of the cancer hallmarks are also activated during viral infections, and study of viral oncoproteins has shown alterations in the same cellular pathways as observed in spontaneous human tumors. Polyomavirus ER encoded oncoproteins are among the best studied and better characterized viral proteins, which have enabled us to discover and decode several

tumorigenic pathways. However, the use of these viral oncoproteins to study cancer heterogeneity is still limited due to our focus on only few model oncoproteins. Virus-host relationship employs fight for survival with both the host and the viruses dynamically evolving to surpass each other. With the advancement in sequencing technology several new polyomaviruses with varied host range have been discovered. According to the cancer heterogeneity model, the same cellular pathway may have different intracellular connections and roles in different tissues and cell types. Hence, viruses infecting different hosts (cell types and species), may require modifications of the same cellular processes for their replication and persistence and they may employ different strategies to achieve that.

In fact, the study of different polyomavirus oncoproteins has enabled discoveries of different tumorigenic pathways. While study of SV40 encoded oncoproteins has helped in discovering tumor suppressor p53 and decoding RB-E2F and PP2A tumor suppressor pathways, the study of MuPyV mT has helped discover oncogenic Protein Tyrosine Kinase (PTK) and Phosphoinositide 3-kinase (PI3K) pathways. Therefore, the comparative study of distantly related homologous polyomavirus oncoproteins can help understanding the complex relationships between different molecular pathways, identification of molecular hubs and their cell/tissue type specific roles. The other part of this study involves characterization and comparison of viral oncoproteins expressed by two polyomaviruses SV40 and Lymphotropic Papovavirus (LPV).

In summary, this study is targeted to better characterize a well known tumorigenic pathway, discover novel cell transformation pathways, understand innate immune regulation by polyomavirus oncoproteins, and test small molecule drug candidates on inhibiting TAg ATPase activity as a potential therapeutic target.

2.0 MATERIALS AND METHODS

2.1.1 Cell culture, Transfection and Transduction

All the cell types used in this study were grown at 37° C in the presence of 5% CO_2.

Mouse Embryonic Fibroblasts (MEFs) were obtained from mice embryos at E13.5 days using the protocol described in (Markovics, Carroll et al. 2005). MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). DKO MEFs were obtained from Prof. Gustavo Leone's lab, and were generated as described (Wu, Timmers et al. 2001).

Human diploid lung fibroblasts, IMR90 cells were obtained from ATCC (American Type Culture Conditions), fulfilling all the material transfer guidelines. The cells were grown in MEM (Gibco Catalog # 30-2003)supplemented with 10% FBS, and 1% P/S. Human diploid foreskin fibroblasts, BJ;hTERT cells were provided by Dr. William Hahn's lab, and were cultured in DMEM, supplemented with 10% FBS, 1% P/S and 1% L-Glutamine.

c-Myc control cell lines HO15.19 (c-Myc knockout) and Myc-3 (c-Myc over-expressing) were obtained from Dr. John Sedivy's lab at Brown University, and were cultured in the same medium as MEFs. Phoenix cells used for retrovirus production and African green monkey

kidney cells (CV1) used in viral replication assays were cultured in the same conditions as MEFs. 293FT cells used for the production of lentiviruses were cultured in MEF medium supplemented with 1% L-Glutamine, and 1% Non-essential amino acids (NEA).

Transfection: Transfection grade DNA was prepared using the plasmid DNA purification Maxiprep kit from (Qiagen corp.). Concentration and purity of plasmid DNA was determined using Nanodrop spectrophotometer. For transfection of Human and Mouse fibroblasts, cells were grown to a cell confluency of 60-70% in 10cm culture dishes. Transfection reagentFugene (Promega) was incubated with purified plasmid DNA at the ratio of 3:1 according to the recommendations of the manufacturer, and incubated at RT for 30 minutes. The incubation mix was added to the cells growing in cell culture medium deprived of antibiotics. The cells are incubated for 24-36 hrs and transfection media was replaced with normal growth media.

Retrovirus/Lentivirus Production and Transduction: The packaging cell lines Phoenix-eco and 293FT were used to produce retroviruses and lentiviruses respectively. Transfections of packaging cell lines with specific plasmids were performed using lipofectamine (Invitrogen) by following the manufacturer guidelines. Phoenix cells were transfected with retroviral plasmids containing the gene of interest under the control of the moloney murine leukemia virus (MMLV) promoter and a drug resistant gene under the SV40 promoter. 293FT packaging cells were transfected with an expression plasmid containing the gene of interest under the control of the human cytomegalovirus (CMV) promoter and drug resistant gene under the SV40 early promoter. In addition to the expression plasmid, 293FT cells were co-transfected with three other plasmids containing the structural and replication protein coding genes (gag-pol, vsvg and rsv). Both the retro and lenti expression plasmids also contain the 5'LTR and 3'LTR virus packaging signals.

The cells were incubated with the plasmids and transfection reagents for 24-36 hrs and transfection media was replaced with fresh growth media. Viruses were collected after 48 and 72 hrs of transfection, and cell debris or live cells were separated by centrifugation at 3000g for 15 minutes. The supernatant was then used for transduction. Mouse or human fibroblast cells at 60-70% confluency were infected with the virus-containing media supplemented with 8 ug/ml polybrene. After 24 hrs, the cells were infected again with 10ml of virus containing medium. Twenty four hours after infection, virus containing media was replaced with normal media and selection with puromycin (3 ug/ml) or blasticidin (3 ug/ml) was carried out for 5 days.

2.1.2 SDS-PAGE and Western Blot

Adherent cells were trypsinized and collected in a 15ml conical tube using FBS containing growth medium. Cell pellets were prepared by centrifugation at 500g for 5 minutes. Media supernatant was aspirated and the pellet was washed once with ice cold PBS, and cells were again pelleted via centrifugation. PBS was aspirated, and cell pallets were either frozen on dry ice and stored at -80° C, or were directly used to prepare protein extracts.

the protein extract preparation and every step for SDS-PAGE sample preparation was performed on ice. Two different lysis buffers were used, depending upon the experimental requirements. *Brantons lysis buffer:* (50uM HEPES pH7.9, 0.5mM EDTA, 0.1% NP40, 400mM KCl and 5-10% glycerol) and *RIPA lysis buffer:* (10mM TRIS-Hcl, 1mM EDTA, 0.5mM EGTA, 1% Triton-X-100, 0.1% Sodium deoxycholate, 0.1% SDS and 140 mM NaCl). Both the buffers were maintained at pH 8 and were stored at 4° C. Before use, 5% glycerol, protease (Boehringer Manheim) and phosphatase inhibitors were added to the following concentrations: Leupeptin=

5ug/ml, Pepstatin= 0.7 ug/ml,NaF=50mM, and NaOVa=1mM. To lyse the pelleted cells, lysis buffer was added at 3-4X the volume of the cell pellet and a homogenized solution was prepared by pipeting several times. The dissolved pellet was incubated on ice for 40 minutes with 10 sec. vortexing every 10 minutes. Full speed centrifugation was carried out for samples prepared in Brantons buffer for 15 minutes and supernatant was collected in 1.5 ml eppendorff tubes on ice. For extracts prepared using the RIPA buffer, sonication was performed at frequency 4 three times for 10 seconds in a cold room. Later, the extracts were centrifuged at 13,000 rpm for 15 minutes and supernatant was collected similar to the extracts prepared using Brantons buffer. The protein extracts were stored at -80°C .

Protein concentration was determined with the Bradford reagent (BioRad) using the protocol described by the manufacturer. For SDS-PAGE analysis, 30-100 ug of total protein extract was mixed with sample buffer containing SDS and DTT, and were then incubated at 95 degrees Celsius for 10 minutes. Proteins in each sample were separated using 10% SDS-PAGE gels, and were transferred onto PVDF membrane and incubated with appropriate primary antibodies (Table 4) to detect the specific proteins of interest. Appropriate HRP conjugated secondary antibodies were used and proteins were detected by chemiluminescence with Luminata Forte (Millipore).

Table 4: List of antibodies used in this study

Protein	Primary Antibody	Secondary Anibody	Company/ Institute	Conditions
E2F1	Rabbit sc-193	GAR	Santa Cruz	1:100 O/N
E2F2	Rabbit sc-9967	GAR	Santa Cruz	1:500 O/N
E2F3	Mouse: 05-551	GAM	Millipore	1:400 O/N
E2F4	Rabbit sc-1082	GAR	Santa Cruz	1:2000 O/N
p53	HRP-p53 sc-6243	None	Santa Cruz	1:1000 3 hrs
$p21^{Cip}$	Rabbit sc-471	GAR	Santa Cruz	1:1000 O/N
pRb	Mouse 554136	GAM	BD Pharmingen	1:500 O/N
p130	Rabbit- sc-317x	GAR	Santa Cruz	1:1000 O/N
p107	Rabbit sc-318	GAR	Santa Cruz	1:1000 O/N
c-Myc	Rabbit sc-764	GAR	Santa Cruz	1:500 O/N
HRas	MouseSAB14059	GAM	Sigma Aldrich	1:1000 O/N
GAPDH	Mouse	GAM		1:100,000 O/N
SV40 TAg	Mouse PAb416, PAb419 and PAb901	GAM		1:2500 O/N
LPV TAg	Mouse: Xt-7 and Xt-10	GAM	Chris Buck @ NIH	1:100 2 hrs
SV40 sT	Mouse PAb416, PAb419 and PAb901	GAM		1:2500 O/N
LPV sT	Mouse: Xt-7 and Xt-10	GAM	Chris Buck @ NIH	1:100 2 hrs
LPV LT'	Mouse: Xt-7 and Xt-10	GAM	Chris Buck @ NIH	1:100 2 hrs
SV40 N136	Mouse PAb416, PAb419 and PAb901	GAM		1:2500 O/N
LPV N200	Mouse: Xt-7 and Xt-10	GAM	Chris Buck @ NIH	1:100 2 hrs

2.1.3 Polymerase Chain Reaction (PCR)

Reverse Transcription-PCR Cell pellet was collected as described above. Total RNA was isolated from approximately $1x10^7$ cells using the Qiagen RNeasy mini kit under manufacturer's guidance. On column DNase treatment was performed, and RNA concentration was determined using Nanodrop (NANODROP 2000, Thermo Scientific). cDNA was performed using 1ug of total RNA using poly-dT primers and Superscript II (Invitrogen) reverse transcriptase as described (Chong, Wenzel et al. 2009). Samples were incubated with 1.5U RNaseH (Promega) at 37°Cfor 20 minutes.

PCR reactions were carried out using specific primers and conditions as described in the **Table 5 and 6,** using total cDNAs prepared as described above. GoTaq DNA polymerase containing master mix (Promega) was used for PCR reactions.

Table 5: Primers and conditions used for RT-PCR analysis of human and mouse ISGs.

Genes	Primers	Conditions
Rpl5	F TATTTTGGCAGTTCGTGTGC	59º 30''
	R ATTATGCTCGGAAACGCTTG	
Ifi27	F GCCTCTGCTCTCACCTCATC	55º 30''
	R ATCTTGGCTGCTATGGAGGA	
Ifi44	F TTCGATGCGAAGATTCACTG	53º 30''
	R CCCTTGGAAAACAGACCTC	
GTPase	F TTTGGCGAATGCAATGATAA	56º 30''
	R GTGGGCACTGGAGTAAGCTC	
Rsad2	F TCAAAAGCTGAGGAGGTGGT	54º 30''
	R GCGCTCCAAGAATCTTTCAA	
Oasl2	F GGGACAGAGATGGCACTGAT	55º 30''
	R AAATGCTCCTGCCTCAGAAA	
Oas2	F AATGCCAGTCCTGGTGAGTT	55º 30''
	R CAGCGAGGGTAAATCCTTGA	

* Primer sequence and conditions for corresponding mouse genes are as described in Rathi A.V, et al 2010

Table 6: Primers used for RT-PCR analysis in chapter 3 and chapter 4.

Genes		Primers	Conditions	
E2F1	F	TTGCCTGTCTGTTTGCTGAGCC	60º	30''
	R	CGGAGATTTTCACACCTTTCCCTG		
E2F2	F	TTCGCTTTACACGCAGACGG	60º	30''
	R	AATGAACTTCTTGGTCAGGAGCC		
E2F3	F	AGCCTCTACACCACGCCACAAG	60º	30''
	R	ATCCAGGACCCCATCAGGAGAC		
LPV ER/LT/LT'	F	ACCATGGACCAAACGCTGTCTAAG	55º	3'
	R	TTACATTTGTTCTTCAATTACAATTCC		
V5tag	R	ACCGAGGAGAGGGTTAGGGAT		
LPV sT	F	ACCATGGACCAAACGCTGTCTAAG	59º	40''
	R	AAAGCTGGGTCTTAGAATCCCAGTT		
LPV LT	F	CCTTCCCCAGAAGAAGAGGA	55º	1'
	R	GCAGGAAGTCATCCATTTCA		
SV40 ER/LT	F	ACCATGGATAAAGTTTTAAACAGAGA	54º	3'
	R	TTATGTTTCAGGTTCAGGGG		

Semi-quantitative Real time PCR: Total cDNA was diluted 1:5 in reverse transcription buffer. Real-time semi-quantitative PCR was performed using Maxima SYBR Green (Thermo Scientific) and 7300 Real-time PCR system (Applied Biosystems). Each reaction was performed in quadruples using 5 ul of 1:5 diluted cDNA in 20ul of reaction master mix. Relative amounts of cDNA were normalized to Rpl5. The primers used for real time PCR are given in **Table 7**

Table 7: Primers used in real-time PCR

Rpl5	F	CCAAACGATTCCCTGGTTATGAC
	R	GACGATTCCACCTCTTCTTCTTCAC
Pcna	F	AGGCTCTCAAAGACCTCATCAATG
	R	CCTGTTCTGGGATTCCAAGTTG
Dhfr	F	GTAGAGAACTCAAAGAACCACCACG
	R	TTTTCCTCCTGGACCTCAGAGAG
Mcm3	F	CGCAGGAAGAATGAAAAGAGGG
	R	CTGAGGAAGCAGGAAGTGAGAGTC
Cdc6	F	AGTTCTGTGCCCGCAAAGTG
	R	AGCAGCAAAGAGCAAACCAGG
Pol A	F	CTCTCTCAGGACTTCCCAGCG
	R	TCTTCCCCTTCATTTCTCCCG
Cyclin A2	F	CCCCCAGAAGTAGCAGACTTTTGTG
	R	TGTGACTGTGTAGAGAGCCAAGTGG
Cyclin E	F	GAGCAGGAGACAGAATGACCAAAC
	R	ACAGCAACCTACAACACCCGAG
NoxA	F	GACCATAAATACCCATTGGGCAAG
	R	TTGTTTGGAGACAAGGGTCCC
$p21^{Cip}$	F	CTTGTCGCTGTCTTGCACTC
	R	CTCCTGACCCACAGCAGAAG
Abhd4	F	GTTCCAATCCACTGGCTGTT
	R	AGGACTCCATCATGGCTTTG
Phlda3	F	TTCGCCCGCATCAAAGCCGT
	R	AGGGGGCAGCGGAAGTCGAT
GADD45	F	ACGACATCAACATCCTGCGG
	R	CAAAGTCATCTCTGAGCCCTCG
Pidd	F	GCACCGTGTGAATCTCATTGC
	R	CAGGAAGTGAACCCCGATAAAAG
ymidine Kinase	F	GCTTTCGGCAGCATCTTGAAC
	R	CCCTCAGTTGGCAGAGTTGTATTG

* Primers for Bax and Killer genes are described in Ti

PCR Genotyping: Genomic DNA of approximately $1x10^7$ cells was extracted using Tail PCR lysis reagent (VIAGEN) supplemented with 0.3ug/ml proteinase K. Briefly, the cell pellet was suspended in 150ul of tail PCR mix, vortexed and incubated in a 55°C water bath overnight. Next day, proteinase K was inactivated by incubating the samples at 85°C for 1hr. Later, samples were centrifuged at full speed for 15mins, supernatant was collected and purity of DNA was determined using nanodrop. PCR reaction was carried out using 2ul of purified DNA with primers (see below table) and conditions described previously (Wenzel, Chong et al. 2011).

Table 8: Primers for E2F3 genotyping

Genotyping primers for E2F3 gene		
E2F3	F1	GTGGCTGGAAGGGTGCCAAG
	F2	TGAATCATGGACAGAGCCAGG
	R	GATTGATTCTGGGTTGTCAGG

2.1.4 Immunofluorescence

Cells growing on glass cover slips were fixed with 95% ethanol for 5 minutes in -20 degree Celsius and permeabilized using 0.01% Tween-20 in PBS (PBST) for 15 minutes at room temperature. Cells were incubated with blocking buffer (3% BSA in PBST) for 1hr and then with 1:1000 dilution of a polyclonal hamster anti-Tantigen antibody in blocking buffer for 1-2hrs.

After 3x washing with PBST, cells were incubated for 1hr with goat anti-hamster secondary antibody (Alexa Fluor 488, Jackson ImmunoResearch) diluted 1:500 in blocking buffer. The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted for analysis by fluorescence microscopy.

2.1.5 Transformation assays

Soft-agar assay: Exponentially growing cells were trypsinized and $1.6x10^4$ cells were plated in 0.33% noble agar in duplicate. After every 3-4 days, 0.5ml of MEF media was added on top of the agar layer to prevent it from drying. After 3-4 weeks of plating, colonies were counted by the following method: each plate was divided into 4 quadrants, and movies of colonies formed in each quadrant were made using the ZEN software package (ZEISS Corporation). Each movie captures colonies growing in the different layers of the agar, and the size of colonies was calculated by determining the area under the curve using the same software. Top 99% of colonies by size formed by each cell type were used to generate box plots, and significance was calculated using the Wilcox test in R.

Growth in low serum: $5x10^3$ cells were plated in 12 well plates in normal media containing 10% FBS. After 24hrs, the cells were washed 3x with PBS and low serum (1% FBS) media was added. After every 2 days, two wells per cell type were trypsinized and cells were counted using hematocytometer.

Foci Formation: All the focus formation assays were performed in 10cm cell culture dishes. Underconfluent wild-type (wt) MEFs were trypsinized and counted using hematocytometer. For every sample cell type $5*10^5$ wtMEFs were plated and evenly spread by

gentle rocking of the plates to form a monolayer and cells were incubated overnight. Next day, 10^3 or $5*10^3$ experimental cells were plated on top of the wtMEF monolayer and were incubated for 14 days. The media was replaced every 3-4 days. After 14 days, cells were washed twice with cold PBS without EDTA and were fixed by incubating with 5 ml ice cold methanol for 5-10 minutes at room temperature. Later, methanol was aspirated and the cells were washed with ddH_2O three times. To stain the foci formed by transformed cells, 5 ml of 0.5% crystal violet solution in 25% methanol was added to the cells and incubated at room temperature for 15-20 minutes. Staining solution was removed and the cells were washed with ddH_2O over night on a shaker. Plates were dried at room temperature and photographs were taken.

2.1.6 Proliferation assays

Growth curve: Proliferation curves were generated by plating $2x10^4$ cells in 6 well plates in duplicates on day 0. After 6 hrs of plating the cells, two wells per cell type were counted to normalize the initial number of cells and remove pipetting errors. To generate a growth curve, two wells per cell type were counted every 48hrs and the average of total number of cells was plotted for each day as a function of time.

Senescence assay-Beta Galactosidase (β-Gal) staining: DKO MEFs were transduced with lenti viruses containing either control-shRNA or E2F3 specific shRNA (Sigma Aldrich Mission shRNA) and cells were plated in 6 well plates after 5 days of puromycin (3ug/ml) selection. Beta-Galactosidase staining was performed as described previously (Dimri, Lee et al. 1995). Briefly, cells were washed with PBS and fixed using 2% formaldehyde/0.2% glutaraldehyde solution for 5 minutes at room temperature. After fixing, cells were washed with

PBS and fresh beta-galactosidase stain solution (pH6.0): Bluo-Gal (halogenated-indoyl-beta-D-galactosidase, Invitrogen) as added. After incubating overnight at 37 degrees, blue staining for beta-galactosidase was observed using a light microscope, and all the images were taken at the same magnification as control cells.

wtMEFs were transduced with retroviruses containing pBabePuro empty vector or SV40 sT. Cells were selected with puromycin and 10^3 cells were plated in 6 well plates and beta galactosidase staining was performed as described above.

2.1.7 Cloning and Plasmids:

pBABE-puro-E2F3a and pBABE-puro-E2F3b: E2F3a and E2F3b cDNAs were subcloned from baculovirus expression vectors pBS-E2F3a and pBS-E2F3b respectively, into pBABE-puro-SV40LT vector by replacing LT gene. Briefly, E2F3a and E2F3b cDNAs were amplified using the same reverse primers but specific forward primers, shown below. The primers were designed to have EcoRI restriction site at the 5' end and a Kozak sequence. After amplification and quality testing, EcoRI digestion was performed and products were column purified. pBABE-puro-SV40LT plasmid was linearized by BamHI digestion and the overhangs were filled using Quick Blunt Kit (NEB). The linearized plasmid was gel purified and EcoRI digestion was carried out to remove the SV40LT gene. The pBABE-puro vector was again gel purified and used in a T4 ligation reaction with E2F3a or E2F3b PCR amplified and purified products in a 1 to 3 ratio with the vector. The ligation reaction was transformed into TOP10 competent cells and 5-10 isolated bacterial colonies were tested for the correct orientation and sequence of the inserts.

Table 9: E2F3 cloning primers

Cloning primers for E2F3 gene			
cDNA		**Primers**	**Conditions**
E2F3a	F	GCCACCATGAGAAAGGGATCCAG	60º 1.5'
	R	CGCGAATTCTCAACTACACATGAAGTCCT	
E2F3b	F	GCCACCATGCCCTTACAGCAGCAG	60º 1.5'
	R	CGCGAATTCTCAACTACACATGAAGTCCT	

LPV LT and LPV LT': The cDNA of LPV LT and LPV LT' were cloned into pLenti6.3/V5-blasticidin vector (Invitrogen) by TOPO cloning. Briefly, LT and LT' cDNAs were amplified from MEFs and BJ;hTERT cells expressing LPV ER. Total RNA was extracted from these cells and cDNA was prepared as described in previous section. The primers used to amplify the LT or LT' cDNA were designed by Paul Cantalupo to produce blunt end PCR products. After amplification, the PCR products were resolved through a 1% agarose gel to separate the two splice products. LT and LT' were gel purified and sequence verified before TOPO cloning into the vector following manufacturer's instructions. The reaction mixture was transformed into Stbl3 (Invitrogen) E.coli competent cells and 5-10 isolated bacterial colonies were analyzed by PCR and DNA sequencing to confirm the insert.

2.1.8 Site directed mutagenesis:

LPV sT PP2A binding sites were analyzed by comparing amino acid sequences with SV40 sT and MuPy sT. Three putative PP2A binding sites in LPV sT identified were K7, L142 and W156. Mutagenesis primers were designed using Agilent primer design software (www.agilent.com/genomics/qcpd) to create single amino acid substitutions of the above mentioned amino acids to Alanine (A) in LPV sT cDNA insert in pBABE-puro backbone. The primers used are provided below. The mutagenesis reaction was carried out using QuickChange II XL site directed mutagenesis kit (Agilent Technologies) following the manufacturer guidelines. Briefly, the pBABE-puro-LPVsT plasmid DNA was PCR amplified with primers containing substitution mutations for K7A, L142A or W156A. The parental DNA was digested by adding DpnI restriction enzyme and 2-3 ul of the remaining PCR reaction was used for transformation into XL-10 Gold competent cells (Agilent Technologies). Later, 5 isolated bacterial colonies were PCR amplified using forward (5' end of sT) and reverse (V5 tag in the vector) primers described in the previous section. The colonies with clear bands were sent for sequencing to verify the mutation.

Table 10: Mutagenesis primers

Mutagenesis primers for LPV sT		
K7A	F	tttctctcctccgcagacagcgtttggtccatggtgg
	R	ccaccatggaccaaacgctgtctgcggaggagagaaa
L142A	F	gcaggtacaaaaaaaaatttaaaaaagccatgtgcagtctggggagaatgt
	R	aacattctccccagactgcacatggctttttttaaatttttttttgtacctgc
W156A	F	atcctcaggaaagccaaacgctactaaataacatttgtagcaccaaca
	R	tgttggtgctacaaatgttatttagtagcgtttggctttcctgaggat

2.1.9 Extraction of SV40 genomic DNA from CV1 cells and quantification

CV1 cells were grown to 90% confluency in 10 cm culture dishes, and were infected with SV40 virus at an MOI of 6. After 2 h of infection, virus containing media was replaced with fresh media containing appropriate drug concentrations or appropriate volume of DMSO. Five dilutions were used for each Bisphenol and Hexachlorophene and each dilution was used in triplicates. After 24hpi, media was replaced with fresh media containing appropriate drug dilutions. Cells were collected at 48hpi and viral DNA was extracted using the modified Qiagen miniprep protocol (Cantalupo. P et al 2005). Viral DNA was eluted with 50µl of TE buffer. Concentration for purified viral DNA was determined using nanodrop, and 10ul of the purified DNA was incubated with BamHI for 2 hrs at 37 degrees to linearize the SV40 DNA. Later, the total restriction digestion reaction was run on 0.8% agarose gel, with 1kb DNA ladder. Gel

images were taken at same resolution and same exposure time and the bands were quantified using Image J software. Purified viral DNA concentration was determined using nanodrop and 10µl DNA was linearized using BamHI restriction enzyme. Later, the total restriction digest reaction was loaded on 0.8% agarose gel with 1kb DNA ladder. Each replicate was run on a separate gel, and gel images were taken at the same resolution and exposure times. Image J software was used to quantify the bands on the gel.

3.0 ROLE OF ACTIVATOR E2F'S IN SV40 TAG MEDIATED PROLIFERATIOIN AND TRANSFORMATION.

3.1 INTRODUCTION

Studies of human cancers have shown mutations in components of the RB-E2F pathway leading to enhanced activity of activator E2Fs and causing an up-regulation of E2F target genes (Classon and Harlow 2002). Furthermore, various viral oncoproteins like the large Tumor Antigen (TAg) of polyomaviruses (Zalvide and DeCaprio 1995, Stubdal, Zalvide et al. 1997), E1A of adenoviruses (Whyte, Williamson et al. 1989) and E7 of papillomaviruses (Boyer, Wazer et al. 1996)- have independently evolved to target this pathway, indicating its central role in cell cycle regulation.

The current "textbook" model for TAg induced cell proliferation and transformation suggests that SV40 TAg inhibits RBs and disrupts RB/E2F complexes, which allows "free" functional activator E2Fs to induce expression of E2F target genes leading to cell proliferation. It also says that at least one activator E2F is essential for TAg induced proliferation and hyperplasia *in vivo*. However, recent studies have suggested that the role of activator E2Fs in normal cell proliferation may depend upon the cell types studied and contrary to current paradigm, activator E2Fs may be dispensable for survival and proliferation of certain cell types both in vivo and in

cell culture (Chong, Wenzel et al. 2009). Therefore, in this study I am investigating the role and requirement of activator E2Fs in SV40 TAg induced cell proliferation and transformation.

3.2 SV40 TAG DOES NOT REQUIRE ACTIVATOR E2F'S TO INDUCE PROLIFERATION AND TRANSFORMATION IN MEF'S

3.2.1 Development of cell culture model system

In order to study the role and requirements of activator E2Fs in SV40 TAg induced proliferation and transformation I used MEFs prepared from E2F1 and E2F2 double knockout (DKO) mice in which both the copies of E2F3 gene were floxed ($E2F1^{-/-};E2F2^{-/-};E2F3^{f/f}$). I used this system for the following reasons:

1. Previously characterized cell culture system: Previous studies by Gustavo Leone's group at Ohio State University, showed that the deletion of E2F3 from DKO MEFs results in down-regulation of E2F target genes, growth arrest and cell death (Wu, Timmers et al. 2001). Moreover, they showed that cMyc;HRas induced transformation in MEFs requires at least E2F3. Interestingly, deletion of p53 in E2F1-2-3 triple knockout (TKO) MEFs, rescued the proliferation defects, allowed E2F target genes expression and cMyc;HRas induced transformation (Sharma, Timmers et al. 2006, Timmers, Sharma et al. 2007). SV40 TAg, inhibits p53 via its ATPase domain and therefore theoretically should be able to rescue the proliferation defect of TKO MEFs and also induce transformation.

2. Tissue or cell type specific requirements of activator E2Fs: Previous two studies performed in vivo suggested requirement of at least one activator E2F for TAg induced proliferation and tumorigenesis. However, those two studies were performed in terminally differentiated quiescent cells and as the requirement of activator E2Fs in cell proliferation may depend upon the proliferative state of the cells, I decided to use continuously proliferating cells for this study.

MEFs are primary cells which continuously proliferate in cell culture but with a finite life span and undergo natural senescence. Therefore, MEFs provide a cellular system to study the role of E2Fs in TAg mediated proliferation and transformation in a different cellular context.

I first expressed TAg in E2F1-2 double knockout (DKO) and wt MEFs by retrovirus infection. The levels of E2F1-2 and TAg in the resulting cell pools (DKO;TAg) were evaluated and, as expected, we could not detect any signal for either E2F1 or E2F2. Furthermore, expression of TAg in DKO or control (wt) background was comparable **(Figure 15A)**. Previous studies have shown that TAg-induced proliferation *in vivo* depends upon either E2F1 or E2F2. Therefore, I first determined if TAg can induce proliferation in MEFs lacking E2F1 and E2F2. Cell proliferation assays for wt and DKO MEFs with or without TAg expression, showed that both MEFs lacking TAg proliferate at a similar rate, but TAg expression in either background enhanced cell proliferation (**Figure 15B)**. Moreover, TAg expression in primary cells is known to up-regulate E2F target genes, and my qPCR analysis shows that E2F targets are at least 2 fold up-regulated in TAg expressing wt or DKO MEFs **(Figure 15C)**. I next tested the ability of TAg to induce transformation in DKO MEFs by performing soft-agar assay and as shown in **Figure 15D,** TAg expression in wt or DKO MEF backgrounds induced considerable soft agar colony formation. Therefore, in contrast to previous studies in two different cell types, where activator

E2F1 or E2F2 were found essential, these results show that TAg induced proliferation and transformation in MEFs does not require E2F1 or E2F2.

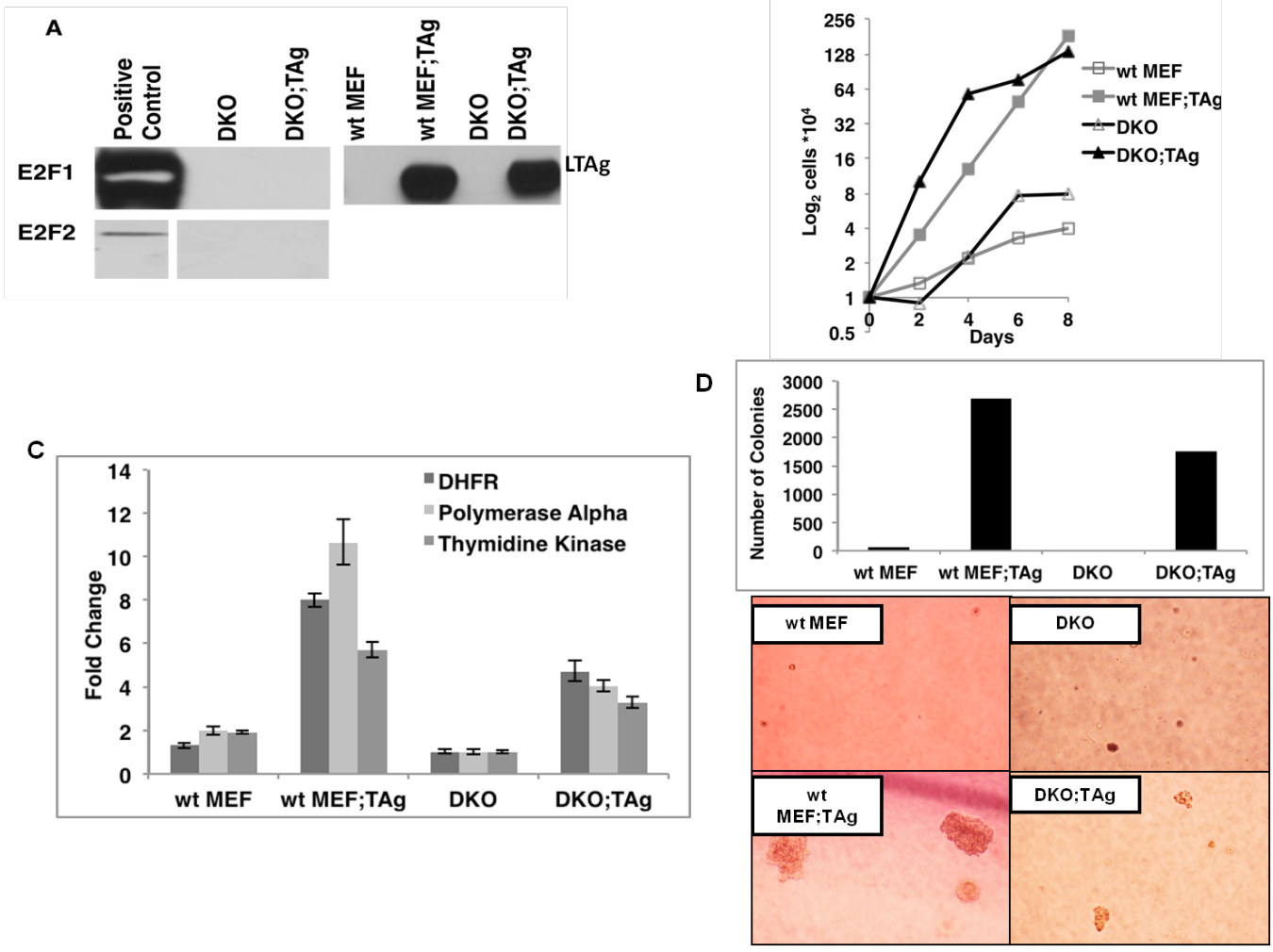


Figure 15: SV40 TAg induces proliferation and transformation in MEFs depleted of activator E2F1-2.

A. Western analysis showing protein expression levels of E2F1, E2F2 and LTAg in different cell types used in this study. **B.** Growth curves comparing proliferation of wt and DKO MEFs in the presence or absence of TAg. Equal number of cells were plated for each cell type and cells were counted periodically in duplicates to obtain the growth curves (N=2). **C.** Real-time PCR analysis of certain canonical E2F target genes in wt or DKO MEFs with or without TAg. The expression levels of E2F targets are given relative to that in DKO MEFs, and cDNAs are normalized against Rpl5 levels. **D.** Soft agar assay testing anchorage independent growth of wt and DKO MEFs with or without TAg expression. Soft agar colonies are formed by TAg expressing wt or DKO MEFs irrespective of the presence of E2F1 and E2F2.

3.2.2 TAg rescues the proliferation defects of TKO MEFs

I next depleted E2F3 from DKO and DKO;TAg MEFs by two different methods: (1) expression of cre-recombinase and, (2) RNA interference using E2F3 specific shRNAs. As mentioned above, DKO cells had E2F3 gene floxed ($E2F3^{f/f}$), and expression of cre-recombinase should knockout the floxed exon of E2F3 gene. Early passage DKO and DKO:TAg MEFs were transduced with retroviruses containing either the cre-recombinase gene or empty vector and cell pools or clones were collected upon appropriate selection **(Materials and Methods)**. I used 4-5 different shRNAs specific for different coding regions of the E2F3 gene and a control shRNA

which would not target any mammalian gene. Upon transduction of DKO or DKO;TAg MEFs with shRNA containing lentiviruses and upon appropriate drug selection, pools of cells were collected and western blot analysis was carried out at early or late passages to determine the extent of E2F3 protein depletion.

In accordance to the previous studies, depletion of E2F3 from DKO MEFs, either by cre-recombinase or by RNAi, resulted in senescence and cell death **(Figure 17).** The cre-treated pools of DKO;TAg MEFs showed no detectable levels of E2F3 protein by western blots in early passage cells however, E2F3 expression re-appeared upon passaging the cells and continued increasing in later passages **(Figure 16A)**. Genotyping-PCR and agarose gel analysis of cre-treated cell pools showed two bands, one corresponding to a $E2F3^{-/-}$ band and the other to a $E2F3^{f/f}$ band. These results suggest either incomplete deletion of E2F3 gene resulting in heterozygous cell population ($E2F3^{f/-}$) or a heterogeneous population of $E2F3^{-/-}$ and $E2F3^{f/f}$ cells or both. Another possibility is the inability of TKO;TAg MEFs to survive and therefore overtime only the cells with at least one copy of the E2F3 gene intact proliferate and take over the population. The efficiency of cre-recombinase mediated homologous recombination is described to be around 10%-15%, which would explain the incomplete deletion of E2F3 gene and therefore we plated cre-treated DKO or DKO;TAg MEFs in high dilution to obtain single cell clonal population. The extent of E2F3 deletion and E2F3 protein and transcript expression in the resulting clones were tested by genotyping-PCR **(Figure 16B)**, western blots **(Figure 16D and E)**, and RT-PCR **(Figure 16C)**. As mentioned above, DKO cells treated with cre-recombinase fail to proliferate and I could not obtain any clones depleted of both E2F3 alleles, however in the presence of TAg TKO clones arose at a frequency of 19.5%. I have analyzed two clones,

TKO;TAg1 and TKO;TAg2 **(Figure 16B lanes 1 and 2)** in this study and further verified the results using pools of shRNA-treated cells.

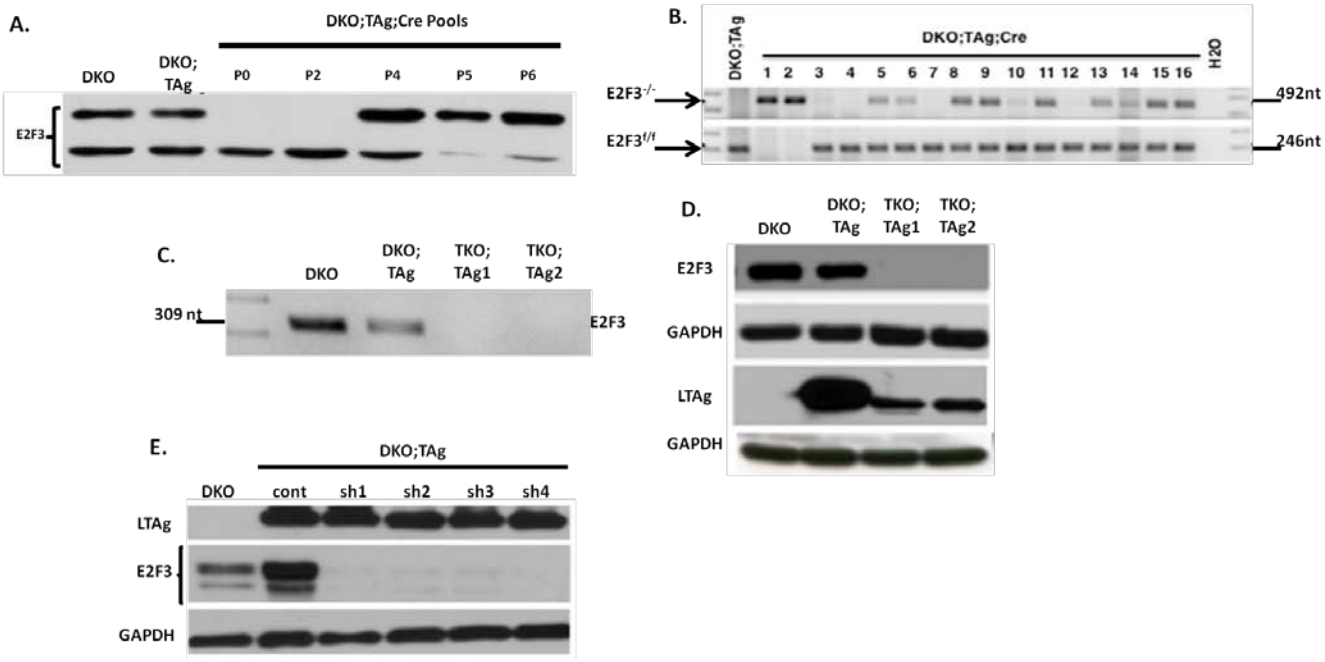
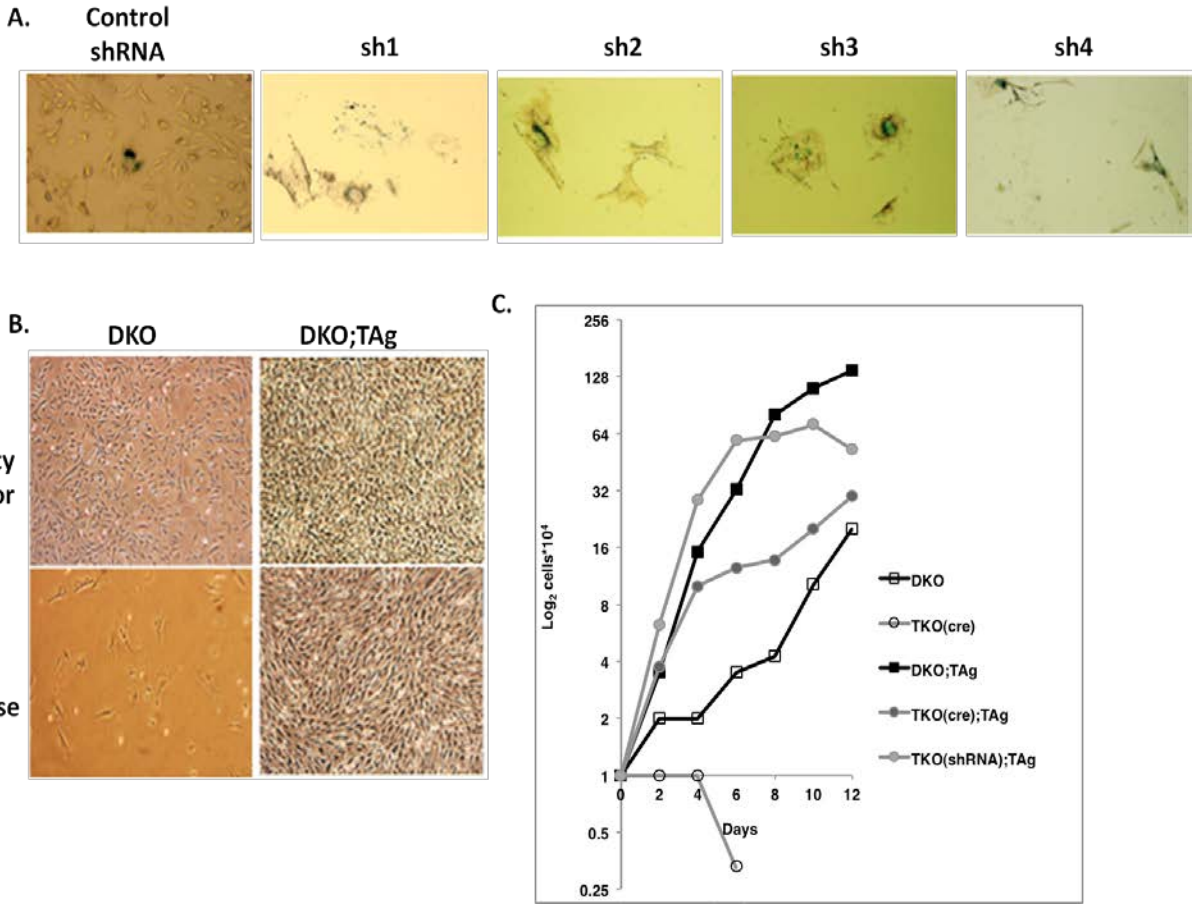


Figure 16: Depletion of E2F3 using cre-recombinase or RNAi.

A. Western blot analysis for E2F3 protein expression in the pools of cre-recombinase treated DKO;TAg MEFs at different passages (P), **B**. Genotyping-PCR to determine the presence or absence of E2F3 in clones of cre-treated DKO;TAg MEFs. Amplification of knockout E2F3 allele ($E2F3^{-/-}$) produce a fragment of 492bp, while the floxed allele ($E2F3^{f/f}$) produce a 246bp fragment. Cells deleted of only one copy of E2F3 gene give both the fragments, **C.** RT-PCR for E2F3 expression in the two TKO;TAg clones obtained by cre-recombinase using primers specific for E2F3a. DKO amd DKO;TAg MEFs shows transcript for E2F3a at the expected size of 309nts, **D**. Western blot analysis of E2F3 and TAg protein levels in the TKO;TAg clones obtained by cre-recombinase. **E.** Western blot analysis of E2F3 and TAg protein levels in pools of DKO;TAg MEFs expressing E2F3 specific shRNAs in comparison to cells expressing control shRNA. Four different E2F3 specific shRNAs (sh1, sh2, sh3 and sh4) were used with a non-targeting control shRNA (cont).

As mentioned above, DKO cells in the absence of LTAg treated with cre-recombinase or E2F3 specific shRNAs fail to proliferate **(Figure 17B and C)** and get into senescence **(Figure 17A)**, in contrast the presence of TAg prevented growth arrest upon removal of E2F3 by either method and the resulting cells proliferated at a similar rate as the DKO;TAg MEFs **(Figure 17C)**. Interestingly TAg protein levels were reduced considerably in the absence of the three activator E2Fs. RT-PCR analysis and MG132 assay suggest that the decrease in TAg protein levels in the TKO background was at both transcriptional and post-translational levels **(Appendix I)**. Next the expression and localization of TAg protein in TKO;TAg MEFs were examined. Immunofluorescence using a specific antibody that recognizes TAg confirmed that the oncogene was uniformly expressed in TKO;TAg MEFs and it was mostly localized in the

nucleus **(Figure 17D),** suggesting the decrease in TAg protein levels was not due to its absence from some cells in the population. These results show that TAg rescues the proliferation defect of TKO MEFs and that TAg does not require activator E2Fs to increase proliferation of MEFs in cell culture.


A.
Control shRNA
sh1
sh2
sh3
sh4
B.
DKO
DKO;TAg
Empty Vector
Cre-recombinase
C.
256
128
64
32
16
8
4
2
1
0.5
0.25
Log2 cells*10⁴
0
2
4
6
8
10
12
Days
DKO
TKO(cre)
DKO;TAg
TKO(cre);TAg
TKO(shRNA);TAg

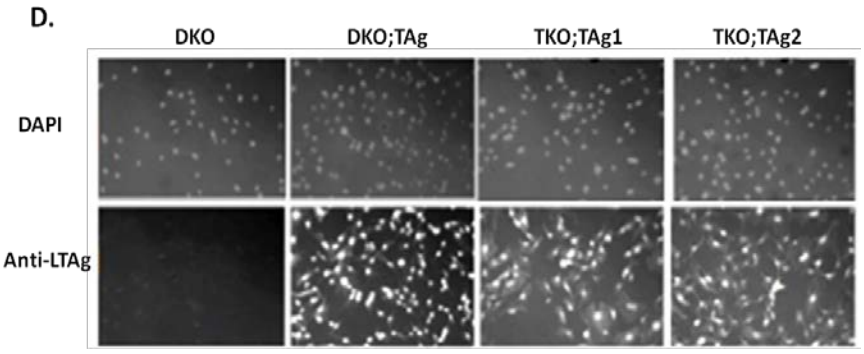

D.
DKO
DKO;TAg
TKO;TAg1
TKO;TAg2
DAPI
Anti-LTAg

Figure 17: SV40 LTAg rescues the proliferation defect of E2F1-2-3 triple knockout (TKO) MEFs.

A. Beta-galactosidase staining to determine senescence in DKO MEFs treated with E2F3 specific or control shRNAs. Blue-violet positive staining and enlarged morphology were observed only in E2F3 shRNA-treated DKO MEFs an d not in control shRNA treated cells. **B.** Comparison of morphology and cell growth after removal of E2F3 by cre-r ecombinase. Images were taken after infecting cells with retro-viruses containing either empty vector (Mock) or cre-r ecombinase at the same time point and same magnification, **C**. Representative examples of cell growth curves upon depletion of E2F3 from DKO or DKO;TAg MEFs, either via cre-recombinase or RNAi. Data shown is an average of two separate experiments. TKO(cre);TAg represents average of the two clones obtained via cre-recombinase and TKO(shRNA);TAg represents average of two pools generated by two different E2F3 specific shRNA s. **D.** Analysis of TAg expression and localization by immunofluorescence using hamster anti-TAg primary antibody and a fluorophore attached anti-hamster secondary antibody. DKO MEFs not expressing TAg are shown as negativ e controls.

3.2.3 LTAg does not require activator E2Fs to induce transformation in MEFs

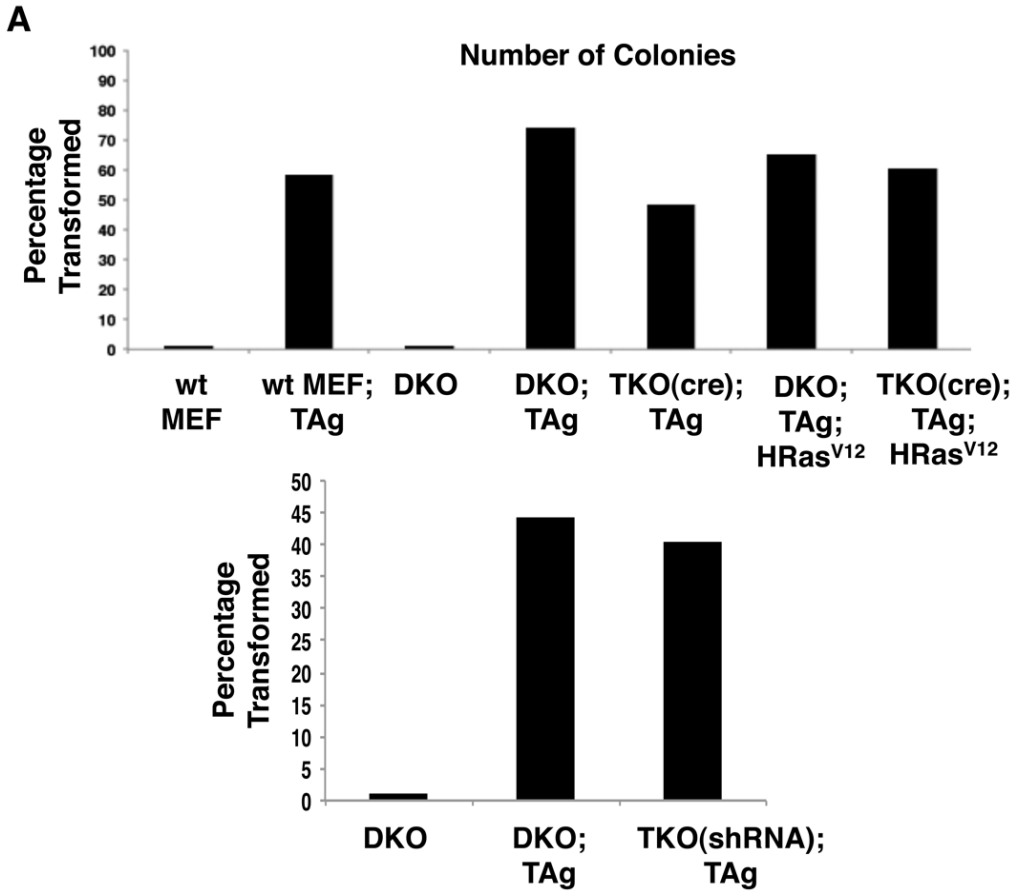
TAg mediated inhibition of RBs is directly linked to the release of activator E2Fs and induction of E2F target gene activation. However, the requirement of activator E2Fs in SV40 TAg mediated transformation of MEFs has never been tested. In order to test the ability of TAg to transform activator E2F depleted MEFs I performed anchorage independence and growth in low serum transformation assays.

Anchorage Independence: Equal number of cells were plated in soft agar and the number and size of colonies formed by TAg-expressing MEFs in the presence or absence of activator E2Fs determined. As expected, both wt and DKO MEFs remained mostly as single cells in soft agar while, TAg expression in wt background induced significant colony formation

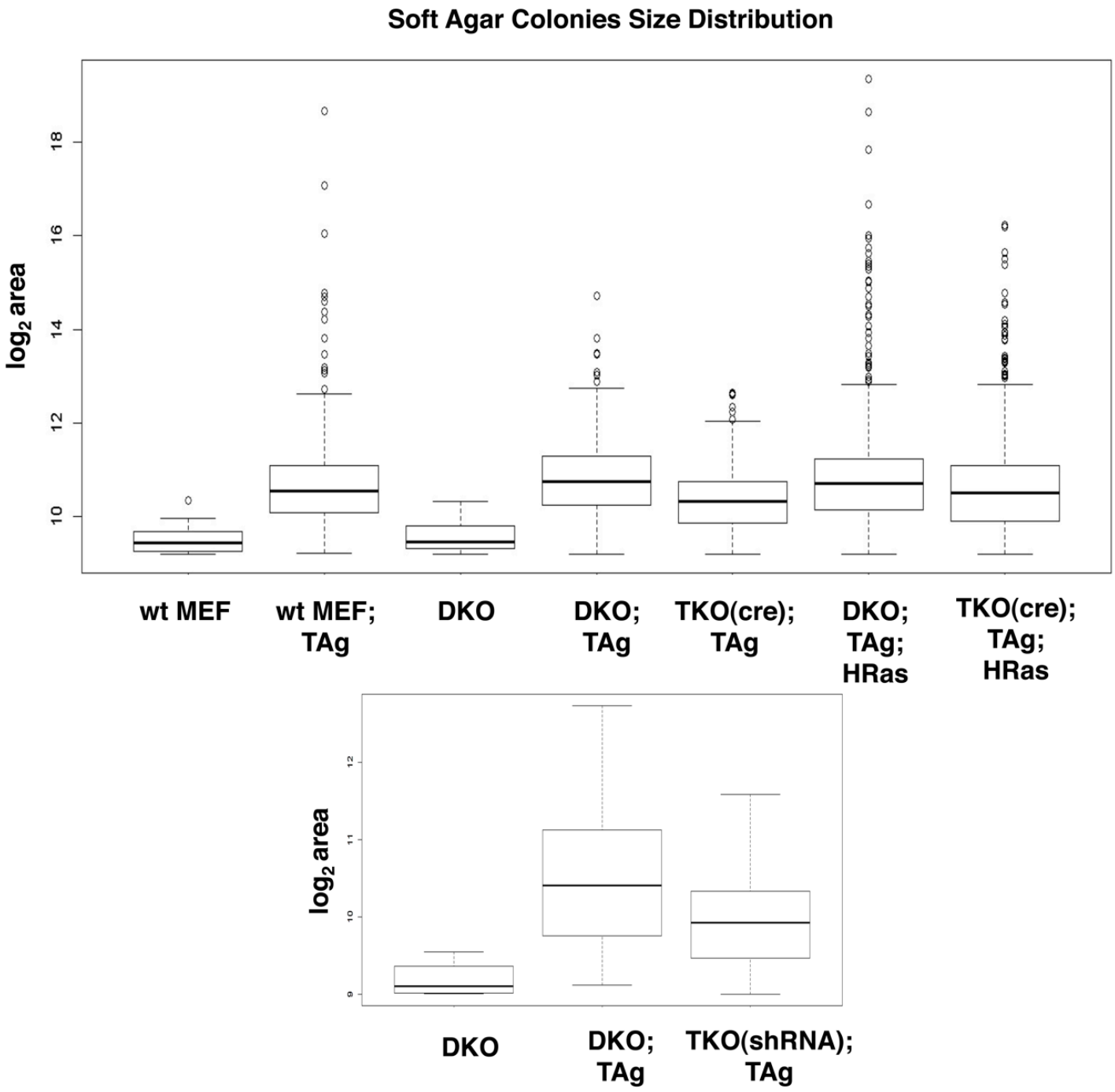
(Figure 18A and B). Moreover, TAg was able to induce growth in soft agar even in the absence of two (DKO;TAg) or all three activator E2Fs **(Figure 18)**. TKO;TAg MEFs generated by two different methods (cre and shRNA) were able to form significant soft agar colonies compared to the control DKO MEFs **(Figure 18)**. While TAg induced soft agar growth in wt and DKO MEFs was similar, there was a significant reduction in the size of soft agar colonies formed in TKO;TAg MEFs as compared to those formed by DKO;TAg MEFs **(Figure 18B)**.

Oncogenic Ras ($HRas^{V12}$) proteins cooperate with TAg to induce transformation (Cavender, Conn et al. 1995, Arbiser, Moses et al. 1997). Addition of $HRas^{V12}$ alone in wt MEFs results in senescence but in the presence of TAg it enhances transformation phenotypes in vitro and results in more aggressive tumors in vivo (Arbiser, Moses et al. 1997). While the phenotype of TAg-HRas cooperation is well documented, very few studies have been done to understand the molecular basis of this cooperation. Therefore, I tested if $HRas^{V12}$ co-operation with TAg is dependent upon the presence of function activator E2Fs. I expressed $HRas^{V12}$ in DKO;TAg and TKO(cre);TAg MEFs by retroviral transduction and determined $HRas^{V12}$ protein expression levels in early (P1) and late passage (P6-7) cells. Comparable levels of $HRas^{V12}$ protein were expressed in both the cells types at early (not shown) and late passages **(Figure 18D)**. I performed soft agar assay to test their oncogenic cooperation and surprisingly, MEFs expressing both TAg and $HRas^{V12}$ not only remained able to grow and form colonies in soft agar **(Figure 18C)** but the size of the colonies were considerably increased in comparison to cells lacking $HRas^{V12}$ expression **(Figure 18C)**. Interestingly, where $HRas^{V12}$ expression increased colony sizes in both DKO;TAg and TKO(cre);TAg MEFs, there was a significant increase of median colony size only in TKO(cre);TAg MEFs **(Figure 18B)**. Overall, these results show that TAg is able to induce significant anchorage independent growth in MEFs even in the absence of

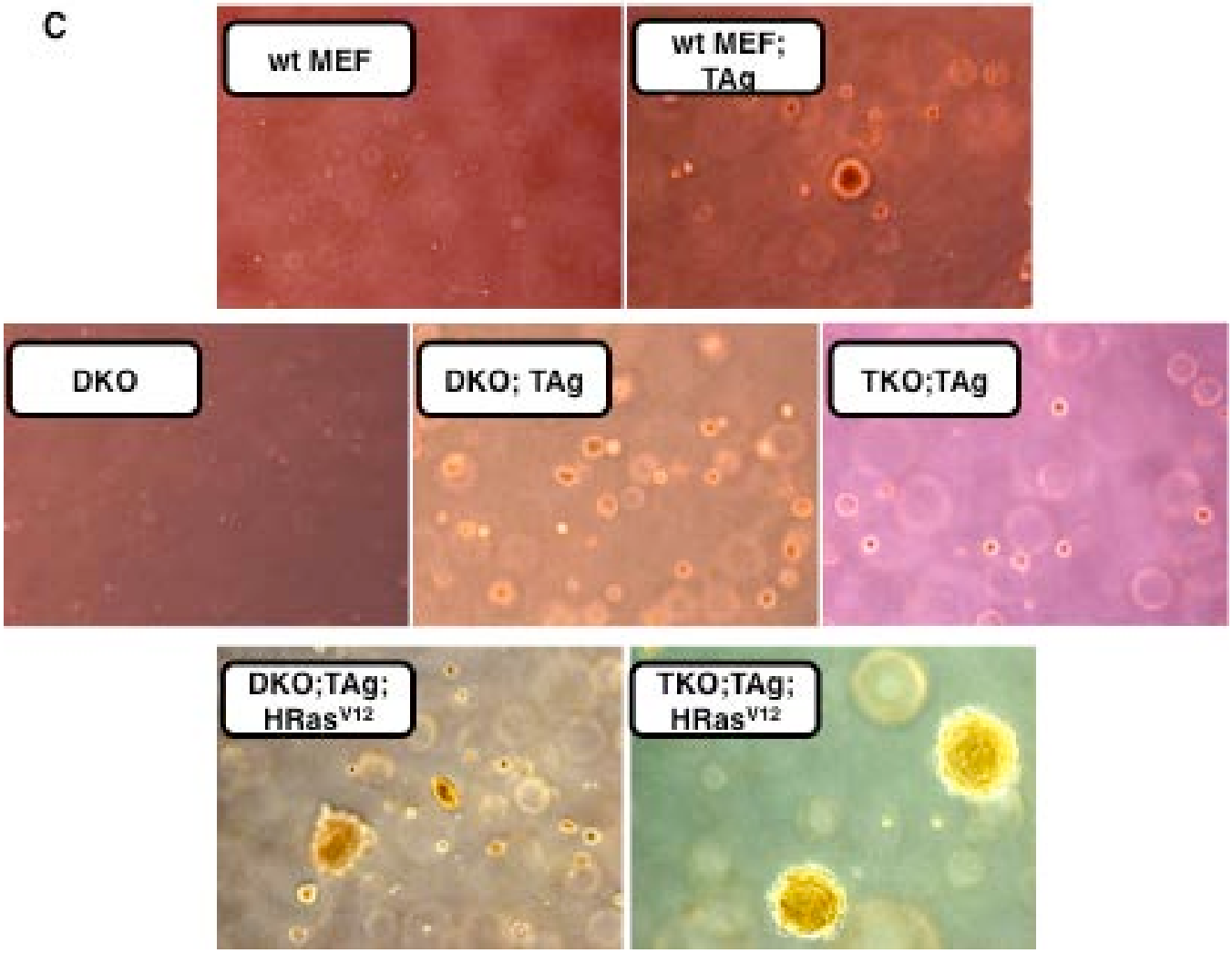
activator E2Fs and that oncogenic cooperation between TAg and $HRas^{V12}$ is also independent of activator E2Fs.



B


Soft Agar Colonies Size Distribution
log2 area
10
12
14
16
18
wt MEF
wt MEF; TAg
DKO
DKO; TAg
TKO(cre); TAg
DKO; TAg; HRas
TKO(cre); TAg; HRas
log2 area
9
10
11
12
DKO
DKO; TAg
TKO(shRNA); TAg

C


wt MEF
wt MEF; TAg
DKO
DKO; TAg
TKO;TAg
DKO;TAg; HRas^{V12}
TKO;TAg; HRas^{V12}

D

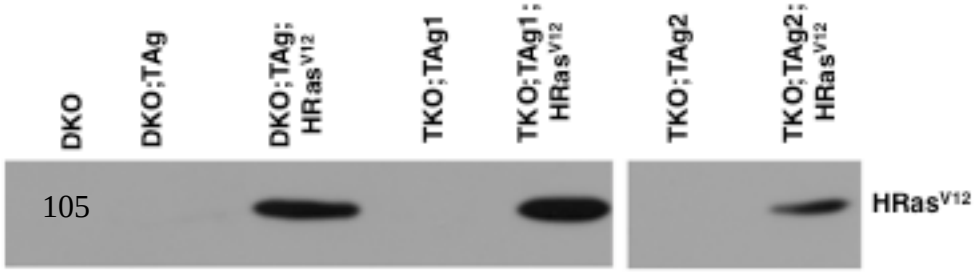
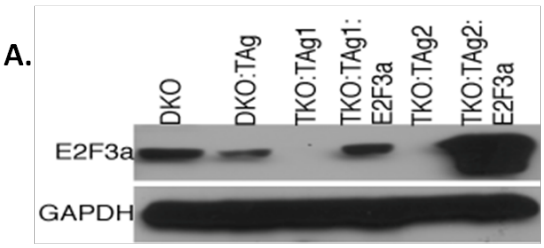

DKO
DKO;TAg
DKO;TAg; HRas^{V12}
TKO;TAg1
TKO;TAg1; HRas^{V12}
TKO;TAg2
TKO;TAg2; HRas^{V12}
HRas^{V12}

Figure 18: SV40 LTAg induced cell transformation is independent of activator E2Fs,

Equal number of cells for all the cell types were plated in soft agar to test anchorage independence and colonies were analyzed after 21 days of plating. Two transforming properties of cells were tested: (1) Percentage of cells within a population able to form colonies; (2) size of the colonies formed. Data represents an average of two independent assays (N=2), performed by using two biological replicates for each cell type. **A.** Bar graph depicting percentage of cells able to form soft agar colonies within different cell types. The criteria used to identify and count colonies is explained in the methods section. Independent experiments were conducted to evaluate colony formation in cells lacking E2F3 through cre-recombinase (upper panel) or RNAi (lower panel). **B.** Box plots showing the size distribution of colonies for each cell type. Each box plot is divided into four quartiles, thick line in the middle of the box represents median for the sample. Significance testing was performed by wilcox test using R programming. Median colonies formed in all the cell types expressing TAg (wtMEF, DKO or TKO) were significantly larger than their control wt or DKO MEFs (p-value<<0.01). Interestingly, $HRas^{V12}$ expression in TKO(cre);TAg cells but not in DKO;TAg caused significant increase in median colony size. p-value for TKO(cre);TAg and TKO(cre);TAg;$HRas^{V12}$ <<0.01, DKO;TAg and DKO;TAg; $HRas^{V12}$ = 0.28. **C.** Representative images comparing the size of soft agar colonies formed by different cell types. **D.** Western analysis showing expression of $HRas^{V12}$ in cells transduced with retroviruses containing $HRas^{V12}$ cDNA.

Lastly, I observed a decrease in TAg protein levels in TKO;TAg(cre) clones **(Figure 16D)**, and previous studies have suggested a direct correlation between TAg protein levels with extent of transformation. Therefore, to differentiate between the effects of lower TAg protein levels versus the effects of E2F3 deletion on the decreased size of soft agar colonies observed in TKO(cre);TAg MEFs, I re-introduced E2F3a cDNA in both the TKO:TAg clones, and tested them for anchorage independent growth. E2F3a expression was verified by western blots and it persisted in these cells even at later passages **(Figure 19A)**. However, restoration of E23

expression failed to restore the size of colonies to the extent observed in DKO;TAg colonies **(Figure 19B)**.



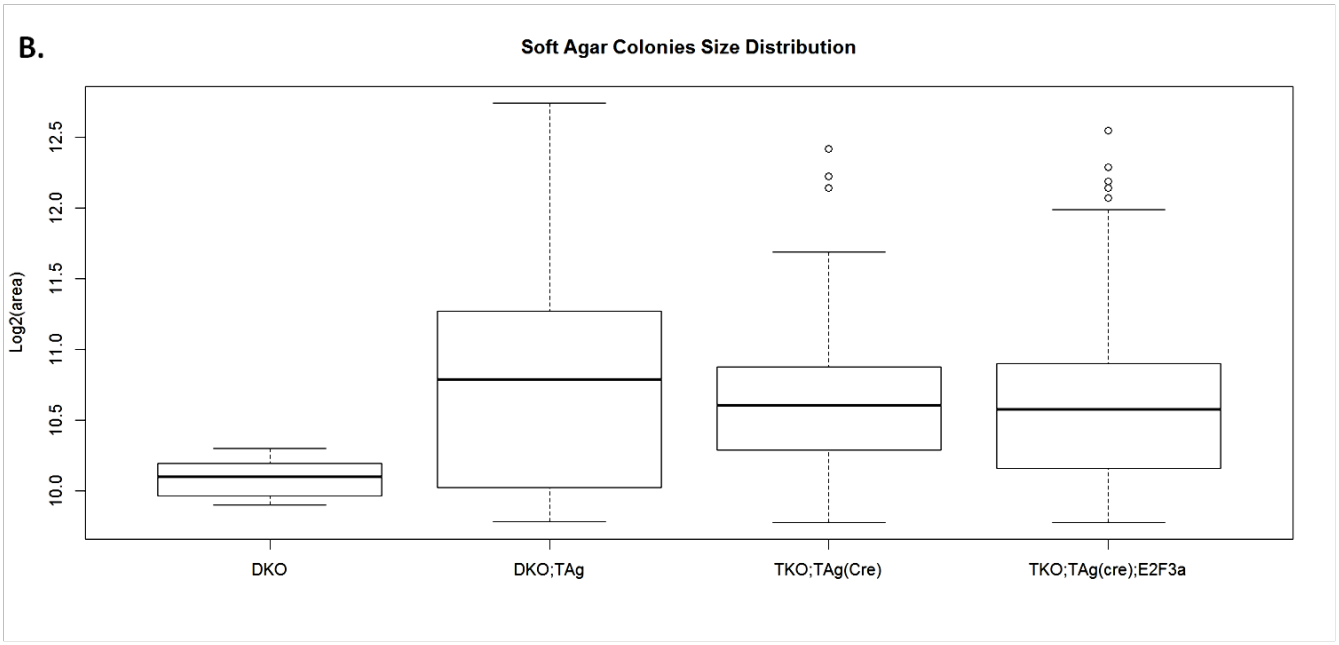


Figure 19: Reintroduction of E2F3a in TKO;TAg MEFs does not enhance the transformation properties of LTAg.

A. Western analysis of E2F3a expression in the two TKO;TAg (passage 6). Reintroduction of the E2F3a gene by retroviral expression results in E2F3a protein levels comparable or higher to those expressed in DKO and DKO;TAg control cells. **B.** Soft agar assay testing anchorage independence upon re-introduction of E2F3a in TKO;TAg clones. Equal number of cells were plated in soft agar and colony formation was determined after 21 days of plating. The criteria we used to identify and count colonies is explained in methods section. Bar graph shows the percentage of cells forming soft agar colonies, while box plots shows size distribution of colonies formed within each cell type. Each box plot is divided into four quartiles and the thick line in the middle of the box represents median for the sample. Significance testing was performed by wilcox analysis using R programming. Median colonies formed in all the cell types expressing TAg (DKO or TKO) were significantly larger than their control DKO MEFs (p-value<<0.05). Re-introduction of E2F3a gene in TKO;TAg(cre) clones failed to increase colony size, p-value for TKO;TAg(cre) and TKO;TAg(cre);E2F3a = 0.94.

Growth in low serum: Primary cells like wt and DKO MEFs do not proliferate in the absence of growth factors while transformed cells do. To test if the depletion of E2F1-2-3 prevents TAg to induce growth in the absence of growth factors equal numbers of all the cell types were plated in normal serum (10%) or low serum (1%)-containing medium and then the number of cells growing in both conditions were counted at regular intervals. In comparison to growth in the presence of normal serum **(Figure 20, left panel)** proliferation in low serum decreased for every cell type **(Figure 20, right panel)**. However, while wt and DKO MEFs ceased to proliferate and started dying in reduced serum conditions, TAg expression in these backgrounds or in TKO MEFs was still able to induce cell proliferation. These results show that MEF cells expressing TAg are able to form colonies in soft agar and grow in reduced serum

conditions even in the absence of two (E2F1-2) of all three (E2F1-2-3) activator E2Fs. In addition, oncogenic co-operation between $HRas^{V12}$ and TAg is independent of the presence of activator E2Fs.

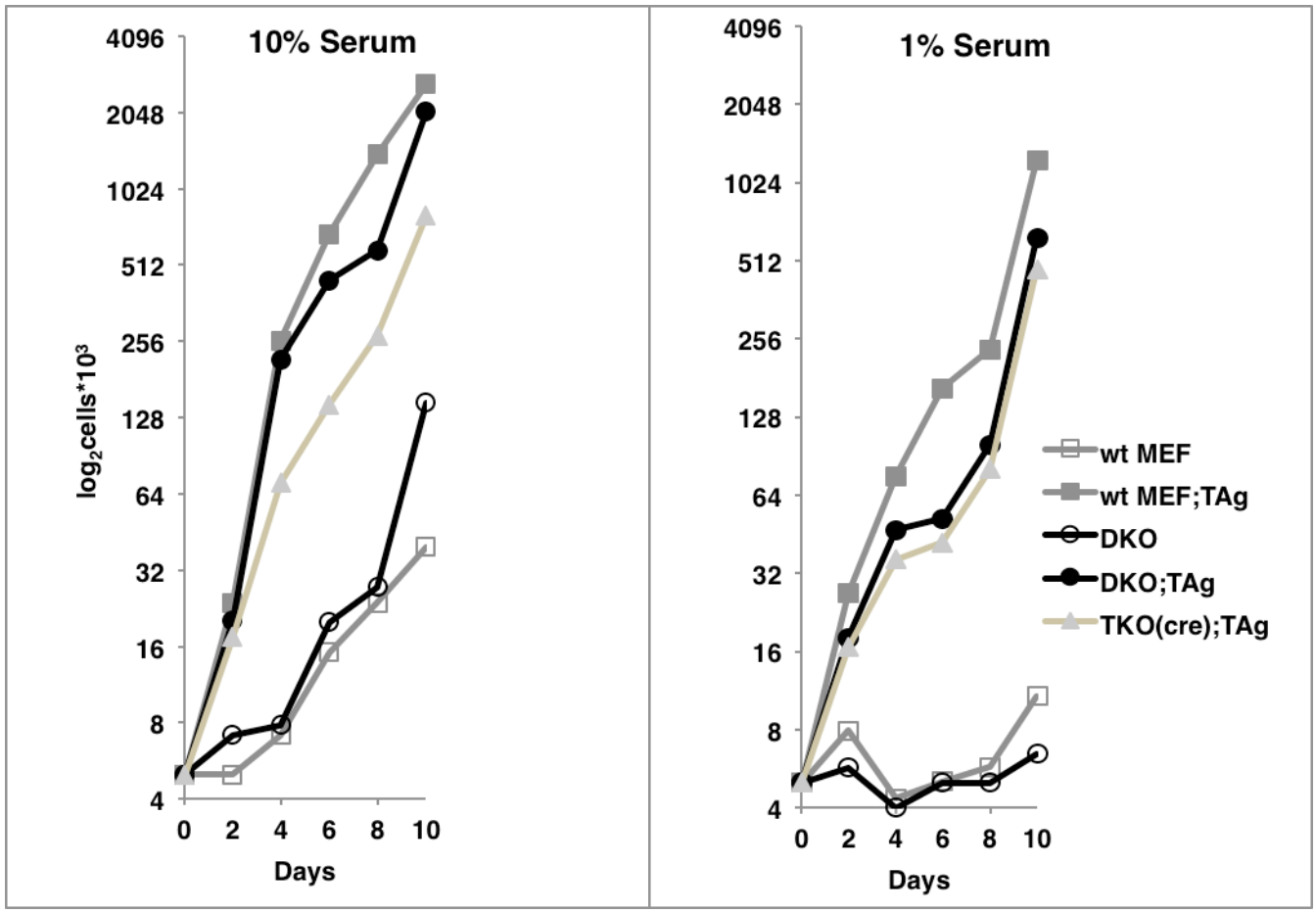


Figure 20: SV40 TAg induced growth in low serum is independent of activator E2Fs,

Equal numbers of cells for each cell type were plated in 6 well plates in media containing either 10% (normal) or 1% (low) serum. Cells were counted periodically in duplicates and growth curves were prepared. Negative (wt MEFs) and positive (wt MEF;TAg) controls were included in each experiment. Each data point is an average of two separate experiments (N=2) and TKO(cre);TAg represents average of the two clones obtained using cre-recombinase.

3.3 SV40 TAG LXCXE MOTIF IS REQUIRED FOR INDUCING PROLIFERATION AND TRANSFORMATION OF TKO MEF'S

In the absence of activator E2Fs p53 is activated and induces expression of its target genes. One of its target gene, $p21^{Cip/Kip}$ is a CDKI and its expression results in the inhibition of CycinD activity and stabilization of p130/E2F4 repressor complexes on the promoters of E2F target genes. This activity was shown to be responsible for G1/S phase growth arrest of TKO MEFs. On the other hand p53 target genes responsible for inducing apoptosis were also expressed resulting in apoptosis. Depletion of $p21^{Cip/Kip}$ from TKO MEFs, allows G1/S cell cycle progression but does not prevent G2/M growth arrest or apoptosis. However, deletion of p53 from TKO MEFs not only rescues cell proliferation but also allows their Myc;Ras oncogenic transformation.

Based upon the previous studies and my results I decided to focus on the activities of TAg responsible for inhibiting the above mentioned cellular pathways. Therefore, I first monitored the two main pathways known to be disrupted by TAg during cellular transformation, namely RB-E2F and p53, in the presence or absence of activator E2Fs.

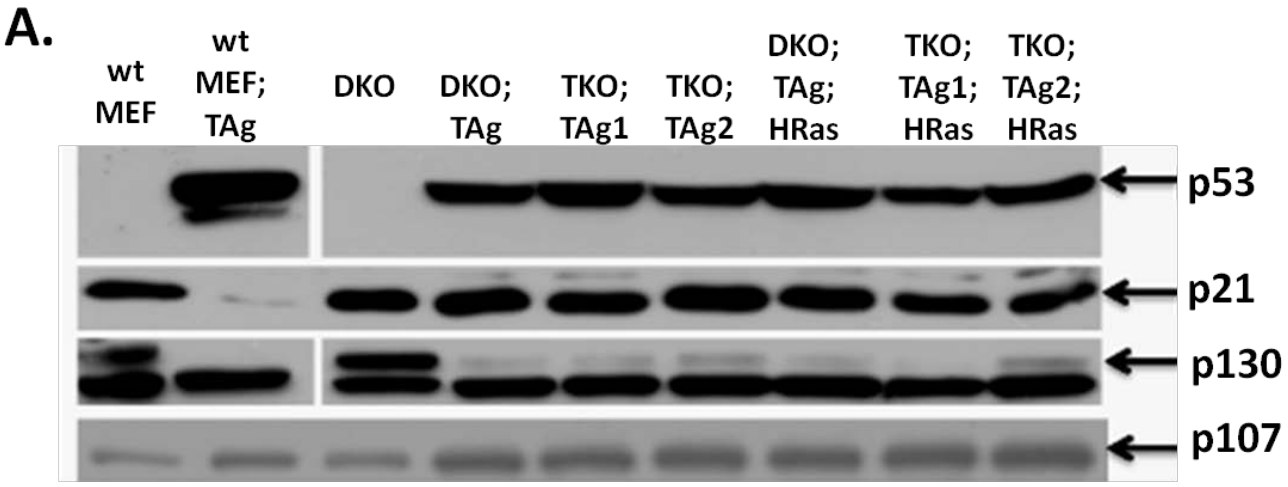
3.3.1 TAg mediated inhibition of RB and p53 pathways is intact in the absence of activator E2Fs

I performed western blots to determine the protein levels of RBs in TAg expressing MEFs in the presence or absence of activator E2Fs. I found that p107 and p130 respond to TAg in a similar way in TKO or DKO background **(Figure 2A)**. TAg mediated inhibition of RBs is expected to increase expression levels of E2F target genes due to the release of activator E2Fs from RB mediated repression. Real time PCR analysis showed an expected increase of canonical E2F target genes in TAg expressing wt, DKO and interestingly, in TKO MEFs. Moreover, while E2F targets were at least two fold increased in DKO;TAg MEFs, they were reduced in comparison to wtMEF;TAg or TKO;TAg MEFs **(Figure 21B).** Possible reasons for this observed phenomenon are discussed later **(Discussion)**. Thus, E2F-target gene activation proceeds in the absence of E2F1-2-3.

TAg binds p53 via its ATPase domain. This process stabilizes and increases the protein levels of p53 while inhibiting its function (An, Saenz Robles et al. 2012). I observed an increase in p53 levels in all cells expressing TAg, in the presence or absence of activator E2Fs (**Figure 21A)**. The levels of p53 were similar in DKO;TAg and TKO;TAg clones regardless different levels of TAg expression.. In all the TAg expressing samples p53 failed to induce expression of selected p53 target genes **(Figure 21C)** (Timmers, Sharma et al. 2007). This suggests that functional inhibition of p53 activity by TAg is retained in the absence of activator E2Fs. The transcript and protein levels of one specific p53-target, $p21^{cip/kip}$ decrease in TAg expressing wt MEFs, and our results confirmed these observations. However, TAg expression in DKO or TKO MEF backgrounds reduced $p21^{cip/kip}$ transcript levels **(Figure 21C)** but the protein levels were

similar to the control DKO MEFs **(Figure 21A)** indicating $p21^{cip/kip}$ protein stabilization upon depletion of activator E2Fs **(Discussion)**. Overall our results suggest that the inhibitory effects of TAg on two central growth regulatory pathways, RB/E2F and p53 are independent of activator E2Fs.

TAg induced transformation of MEFs was enhanced upon expression of $HRas^{V12}$ in the presence or absence of activator E2Fs. I therefore tested if this oncogenic co-operation was due to synergistic effects of TAg and $HRas^{V12}$ activities on the suppression of RB-E2F or p53 pathways. Western blot results show no change in p130, p107, p53 or $p21^{cip/kip}$ protein levels upon expression of $HRas^{V12}$ and no significant change in the expression levels of E2F or p53 target genes. These results suggest TAg and HRas oncogenic cooperation is independent of RB-E2F and p53 pathways.


A.
wt MEF
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DKO
DKO; TAg
TKO; TAg1
TKO; TAg2
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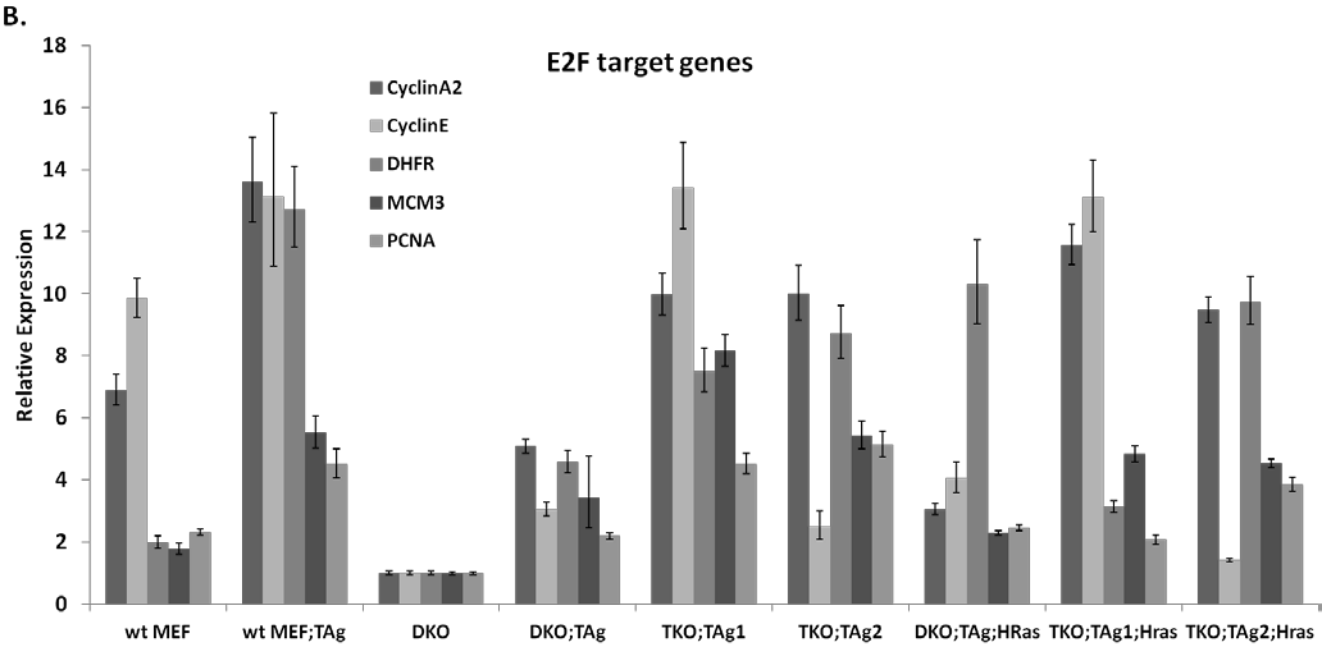
p53
p21
p130
p107


B.
E2F target genes
CyclinA2
CyclinE
DHFR
MCM3
PCNA
Relative Expression
0
2
4
6
8
10
12
14
16
18
wt MEF
wt MEF;TAg
DKO
DKO;TAg
TKO;TAg1
TKO;TAg2
DKO;TAg;HRas
TKO;TAg1;Hras
TKO;TAg2;Hras

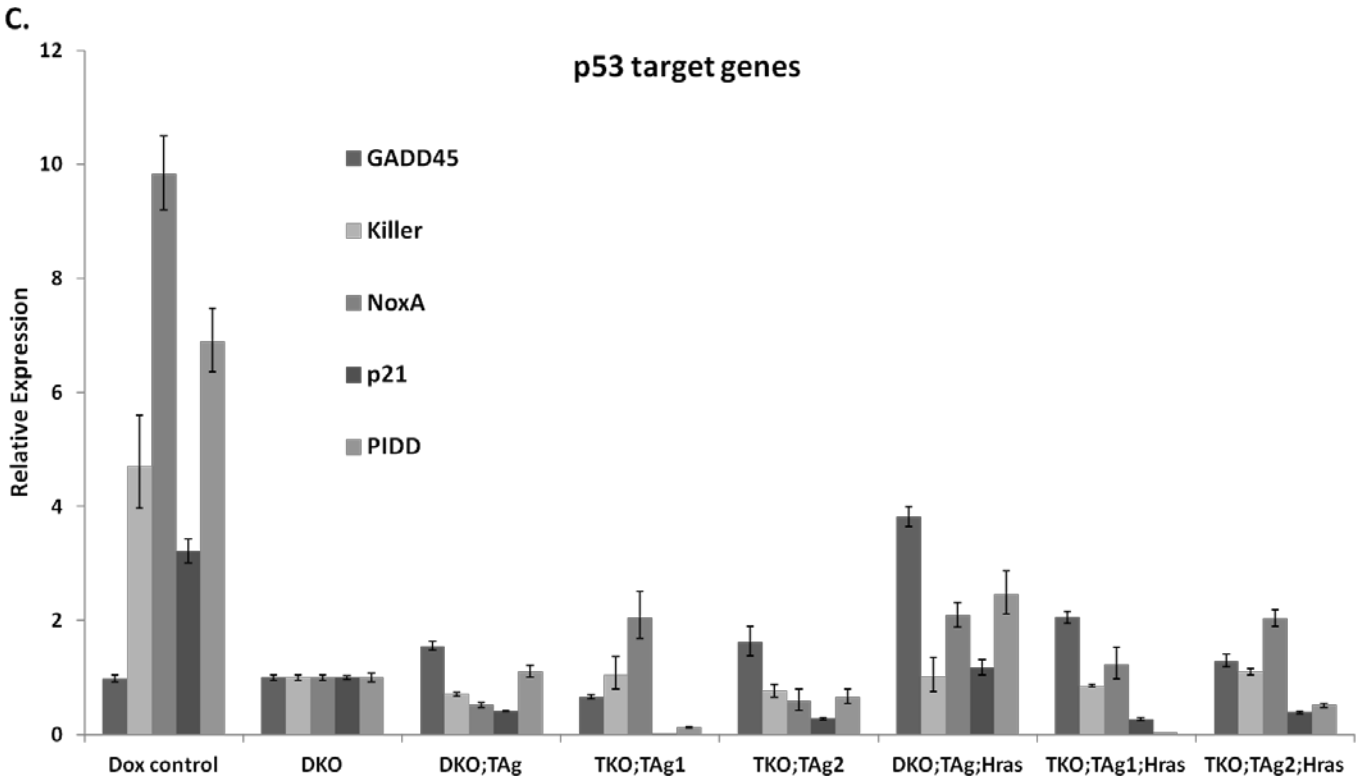
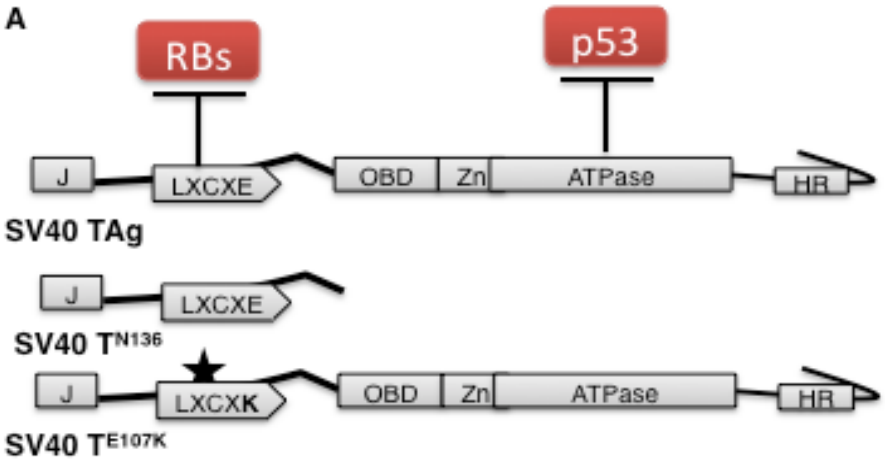


Figure 21: LTAg disrupts the pRB and p53 pathways in the absence of activator E2Fs.

A. Western blot analysis of key cell cycle regulatory proteins in cells expressing TAg, with or without activator E2Fs. wt MEFs and wt MEF;TAg are used as controls. **B.** Real-time analysis of E2F target genes, **C.** Real-time PCR analysis of p53 target genes. Real time PCR was carried out using gene specific primers and Rpl5 as cDNA normalization control. Each gene's expression is compared to the baseline expression in DKO MEFs. Error bars represent standard deviation. TKO;TAg1 and TKO;TAg2 are two clones obtained by cre-recombinase. wt MEF and wt MEF;TAg are used as controls for E2F target genes, while Doxorubicin treated MEFs were used as positive control for p53 target genes expression.

3.3.2 TAg requires LXCXE motif and a C-terminus activity to rescue the proliferation defect in TKO MEF's

To discern between the requirements of TAg mediated inhibition of RBs or p53 in inducing proliferation in TKO MEFs I expressed TAg mutants unable to interact with either RB proteins (T^{E107K}, point mutation in the LXCXE motif) or with p53 (T^{N136}, truncation mutant) in the DKO MEF background **(Figure 22A)**. Protein expression was determined by western blots **(Figure 22B)** and functionality of the mutants was monitored by analysis for p130 levels. As expected, T^{N136} but not T^{E107K} was able to induce p130 degradation **(Figure 22B)**. Next I used cre-recombinase or RNAi to deplete E2F3 in these cells and tested whether TKO MEFs were able to survive and grow in the presence of either mutant.


A
RBs
p53
J
LXCXE
OBD
Zn
ATPase
HR
SV40 TAg
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LXCXE
SV40 T^N136
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ATPase
HR
SV40 T^E107K

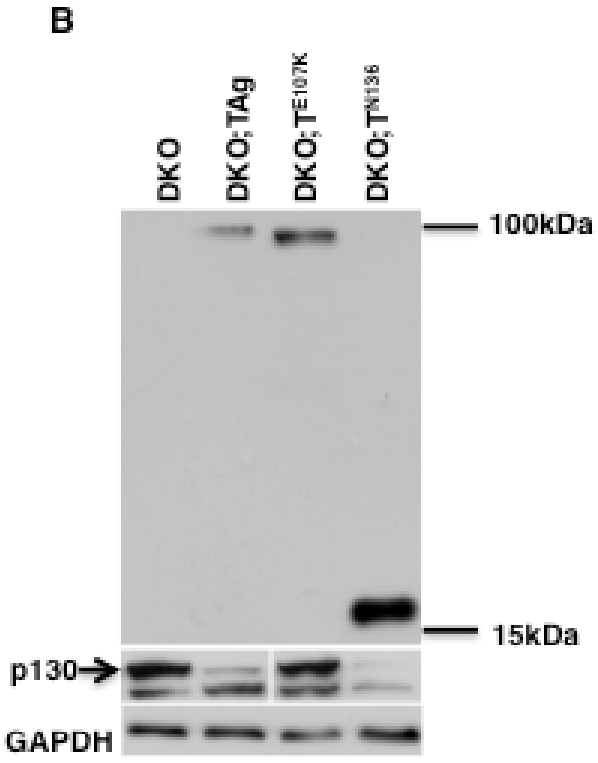

B
DKO
DKO;TAg
DKO;T^E107K
DKO;T^N136
100kDa
15kDa
p130
GAPDH

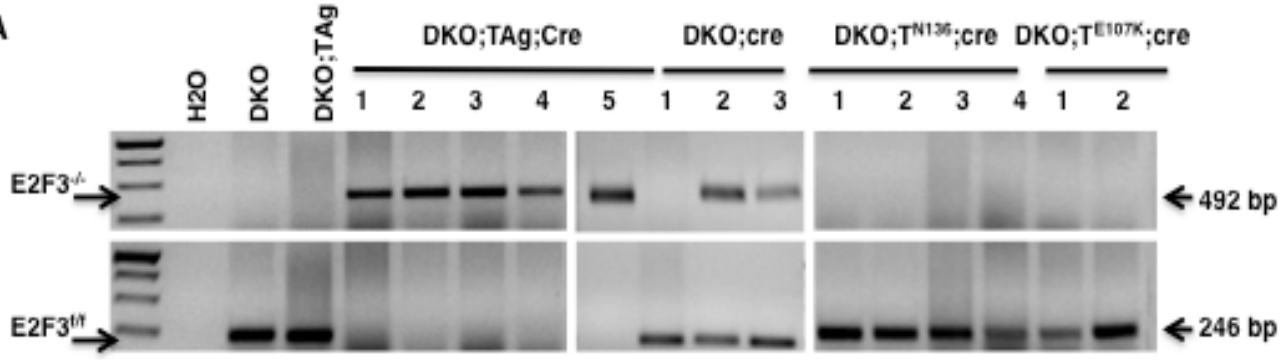
Figure 22: Domain structure and expression of TAg mutants used in this study.

A. Cartoon diagram of the TAg mutants used in this study; wild type TAg, amino-terminus truncation mutant containing RB binding LxCxE motif (T^{N136}) and an RB binding point mutant in full length TAg (T^{E107K}).

B. Western blot results showing expression of wt and mutant forms of TAg in DKO MEFs.

I first treated DKO cells expressing TAg, T^{N136} or T^{E107K} with cre-recombinase and plated them at low density to obtain single cell clones and monitored the depletion of E2F3 under those conditions. While cre-treated DKO;TAg and DKO;T^{N136} gave several colonies upon plating at low density DKO and DKO;T^{E107K} cease to proliferate and very few colonies were obtained **(Figure 23B)**. Similar to the previous results **(Figure 16B)**, I obtained TKO clones of cells expressing TAg but failed to get TKO clones for either T^{E107K} or T^{N136} expressing cells **(Figure 23A and B)**. Failure to obtain E2F3$^{-/-}$ clones of cells expressing TAg mutants suggest that both mutants lacks one or more functions needed to trigger proliferation in the absence of E2F1-2-3. While this result suggests that neither TAg mutant tested is able to rescue the proliferation defect of TKO MEFs, the absence of TKO colonies could also be a result of added stress by growing the cells in "isolation" and thus could result into a false negative. To remove this possibility I used shRNAs to deplete E2F3 from the above mentioned cell types and monitored their proliferation.

A



B

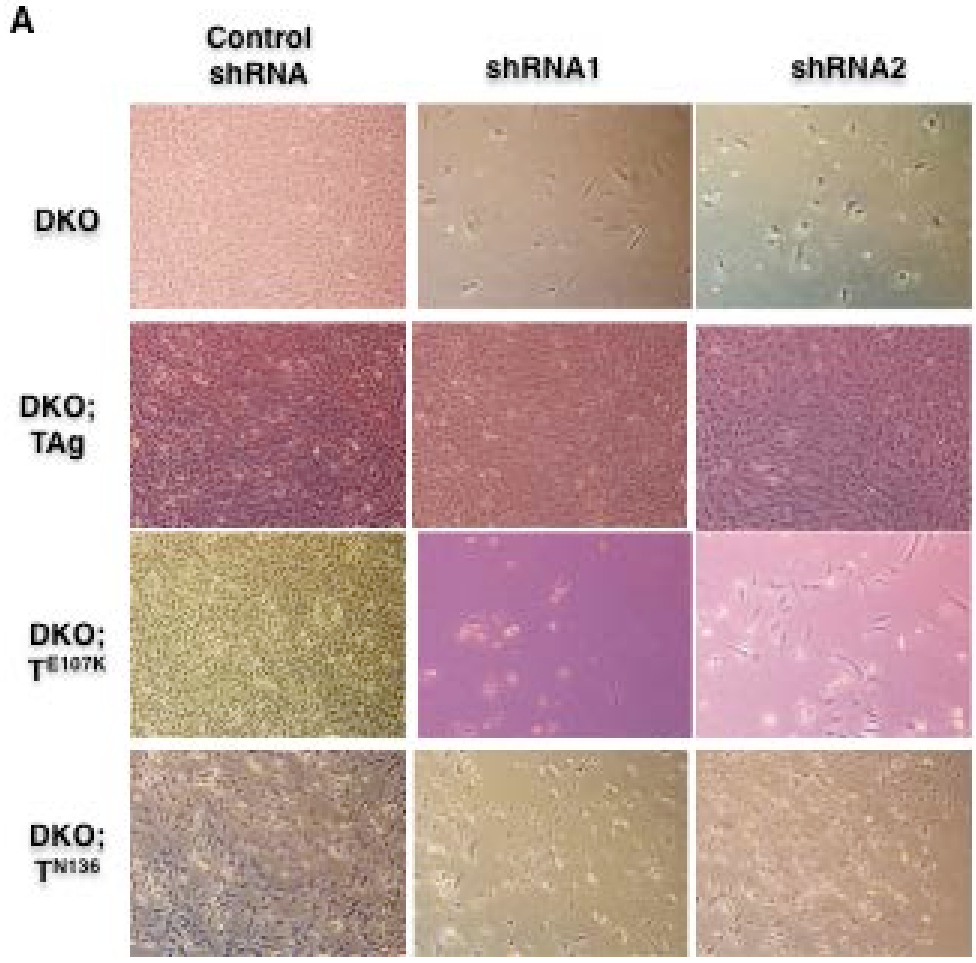
Cell Types	Average number of colonies after low density plating	E2F3$^{-/-}$ Clones
DKO ;cre (n=3)	10	0/25
DKO;TAg;cre (n=3)	>40	5/26
DKO;T^{N136};cre (n=3)	>40	0/30
DKO;T^{E107K};cre (n=2)	8	0/10

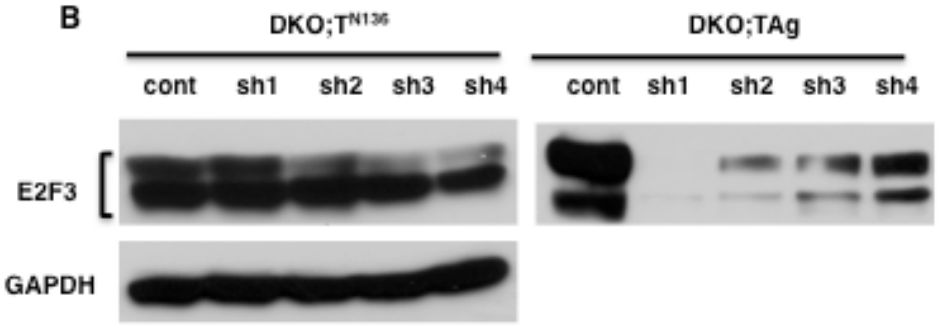
Figure 23: SV40 TAg requires intact LXCXE motif and a C-terminus activity to rescue TKO MEFs proliferation defects.

A. Genotyping-PCR results (as explained in **Figure 16B**) of clones obtained upon low density plating of cre-recombinase treated DKO, DKO;TAg, DKO;T^{N136} or DKO;T^{E107K} MEFs. **B**. Table shows the summary of number of clones obtained after low density plating for each cell type and their genotyping results.

As expected, DKO cells were unable to grow upon removal of E2F3 but DKO cells expressing TAg sustained continuous proliferation. Expression of T^{E107K} was unable to rescue the proliferation of DKO MEFs after E2F3 depletion and these cells morphologically looked similar to shRNA treated DKO MEFs **(Figure 24 A)**. Interestingly shRNA treated DKO;T^{N136} MEFs showed reduced levels of proliferation but with evident cell death **(Figure 24A)**, and the cells which eventually survived had recovered E2F3 expression at levels similar to the control cells **(Figure 24B)**. This outcome suggest two possibilities. Either T^{N136} fails to induce proliferation in $E2F3^{-/-}$ cells and only those cells escaping shRNA-mediated E2F3 depletion survive and grow, or T^{N136} inhibits RNAi activity. To rule out the second possibility I transiently expressed E2F3 specific shRNAs in DKO;T^{N136} MEFs and monitored the protein levels of E2F3 over time by western blots. As shown in **Figure 23C**, after successful initial depletion of E2F3 cells which were able to grow presented increased levels of E2F3 protein. Similar experiment in TAg expressing DKO MEFs showed a consistent depletion of E2F3 levels, even at late passages (data not shown). I therefore conclude that neither T^{E107K} nor T^{N136} mutants are able to rescue the proliferation defect of TKO MEFs.

Moreover, mere inhibition of p53 is not sufficient to rescue the proliferation defect of TKO MEFs and an activity requiring an intact LXCXE motif (most likely RB interaction) is required to rescue the proliferative defect. In addition, some activity in the carboxy terminal region of the TAg, possibly inhibition of p53, is also required, to prevent cell death in the absence of activator E2Fs.


A
Control shRNA
shRNA1
shRNA2
DKO
DKO; TAg
DKO; T^E107K
DKO; T^N136


B
DKO;T^N136
DKO;TAg
cont
sh1
sh2
sh3
sh4
cont
sh1
sh2
sh3
sh4
E2F3
GAPDH

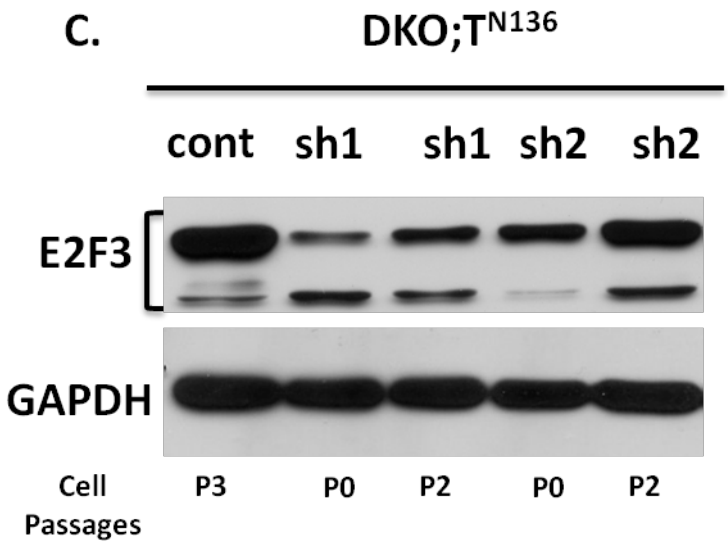


Figure 24: Re-emergence of E2F3 in TKO(shRNA);N136 MEFs allows proliferation.

A. Images comparing cell morphology and density of DKO, DKO;TAg, DKO;T^{N136} and DKO;T^{E107K} treated with either E2F3 specific or control shRNA. All the images were taken at the same time and at the same magnification. **B.** Western blot analysis of E2F3 expression in DKO;T^{N136} and DKO;TAg MEFs shown in Figure 7C. **C.** western blot showing de pletion of E2F3 in DKO;T^{N136} MEFs transiently treated with shRNAs (sh1 (P_0) and sh2 (P_0)), and upon passageing the same cells (sh1 (P_2) and sh2 (P_2)).

4.0 CHARACTERIZATION OF LYMPHOTROPIC PAPOVAVIRUS (LPV) EARLY REGION (ER).

4.1 INTRODUCTION

Polyomaviruses induced cell proliferation and transformation is attributed to the oncoproteins encoded in their early region. The phylogenetic analysis of the large T antigen amino acid sequences from the known human polyomaviruses (blue) and selective primate (green) and rodent (red) polyomaviruses is shown in **Figure 25**. In one cluster primate polyomaviruses SV40 and SA12 are clustered with human polyomaviruses BKV and JCV while the other cluster contains two rodent polyomaviruses MuPyV and HaPyV. While the ER splicing pattern, interactions with cellular factors, effects on growth regulatory pathways and modulation of innate immune system are highly similar in closely related SV40, BKV and JCV polyomaviruses, distantly related polyomaviruses like MuPyV and MCPyV show differences in some or all of the above features. Interestingly, the mechanism of transformation by these viruses also correlate with their phylogenetic grouping. TAg is the essential transforming protein in SV40, BKV and JCV and its expression is required and in most cases sufficient to transform various cell types. On the other hand, mT but not the TAg of MuPyV or HaPyV is the essential transforming protein. In addition, where SV40, BKV and JCV TAg induced transformation requires inhibition of RB/E2F and p53/MDM2 pathways, mT of MuPyV mimics receptor

tyrosine kinase (RTK) and activates various oncogenic pathways to induce cellular transformation.

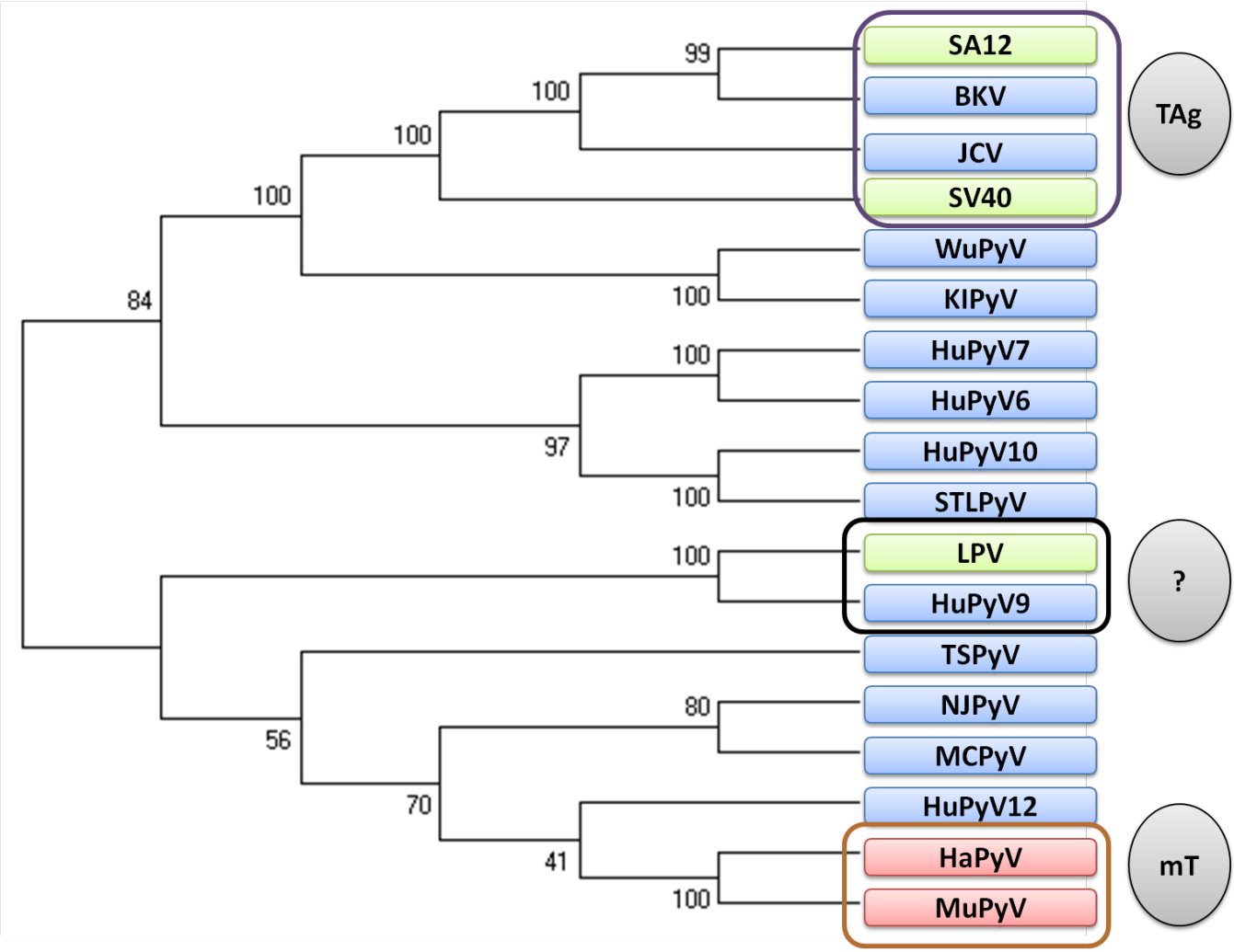


Figure 25: Phylogenetic tree relating human polyomaviruses with specific primate and rodent polyomaviruses.

Viruses isolated from human isolates are shown in blue, while certain well characterized primate and rodent polyomaviruses are shown in green and red respectively. The simplified tree was prepared using amino acid sequences from the large tumor antigen of these polyomaviruses aligned using ClustalW and maximum likelihood. The phylogeny was tested using 100 bootstrap replications and the numerical values shown next to each branch is the bootstrap value suggesting number of replications the two polyomaviruses were grouped together. Cluster I shown in purple box groups four well characterized human and primate polyomaviruses. Cluster II consists of a newly discovered human polyomavirus NJPyV and a primate polyomavirus, LPV. Cluster III, consists of two well characterized rodent polyomaviruses. The essential transforming proteins for cluster I and cluster III are TAg and mT respectively, however the role of oncoproteins encoded by less characterized NJPyV or LPV is unknown. HuPyV (Human polyomavirus), SA12 (Simian Agent 12), SV40 (Simian Virus 40), BKV (BK polyomavirus) and JCV (JC polyomavirus), WuPyV (Washington University polyomavirus), KIPyV (Karolinska Institute polyomavirus), STLPyV (St Louis polyomavirus), TSPyV (Trichodysplasia spinulosa-associated polyomavirus), NJPyV (New jersey polyomavirus), LPV (Lymphotropic Papovavirus), MCPyV (Merkel Cell polyomavirus), HaPyV (Hamster polyomavirus), and MuPyV (Murine polyomavirus).

Lymphotropic Papovavirus (LPV) is a primate polyomavirus that was isolated from B-lymphoblasts of African green monkey (zur Hausen and Gissmann 1979). It is very similar to other known polyoma viruses in terms of genome size, coding potential and genome organization (Pawlita, Clad et al. 1985, von Hoyningen-Huene, Kurth et al. 1992). Fluorescent Antibody (FA) tests performed using human and non-human primate serum showed prevalence of Ab against LPV or a similar virus in ~30% human population and in almost all the non-human primates (Takemoto and Segawa 1983).

Amino acids sequence comparison of LPV TAg with SV40 TAg suggests an overall 41% similarity and conservation of many domains and motifs required for interaction with cellular proteins. However, LPV TAg is structurally closer to MuPyV than SV40 as it contains a longer N-terminal linker region and lacks the C-terminus host range region. In fact, based upon TAg sequence homology LPV is distantly grouped from both SV40 or MuPyV clusters **(Figure 25)** and is grouped with a recently discovered human polyomavirus 9 (HuPy9) (Scuda, Hofmann et al. 2011). This may also explain its cross-reactivity with the human sera.

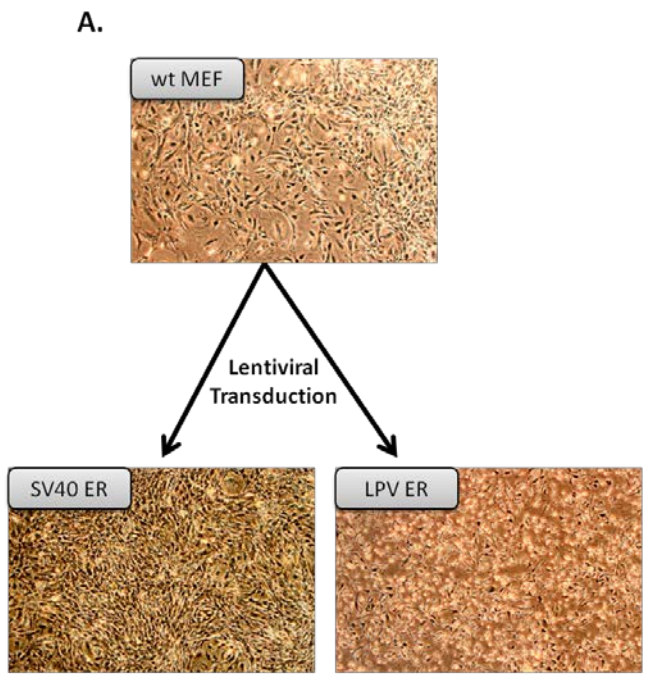
Several attempts to grow this virus in different cell types from different animal models have failed and its host range is highly limited to B-lymphoblast cells of human and primate origin. Similar to other polyomaviruses, its early region encodes a large T antigen (LPV TAg) and a small T antigen (LPV sT) and is known to transform hamster embryonic cells in culture (Takemoto and Kanda 1984, Kang and Folk 1992) and induce tumors of choroid plexus in transgenic mice (Chen, Neilson et al. 1989). Compared to other model polyomaviruses relatively little is known about transforming mechanism of LPV ER or its ER encoded products. Therefore, the study of LPV transforming properties and characterization of its ER might enable discoveries of new oncogenic pathways. Moreover, in the absence of a full genomic clone of HuPy9 the study of LPV ER serves as a surrogate to determine transforming capabilities of HuPy9. I hypothesized that LPV ER encoded oncoproteins would transform primary rodent and human fibroblasts using a different mechanism than SV40 encoded oncoproteins. In this study I characterize and compare the transforming capabilities of LPV ER to SV40 ER in mouse and human fibroblast cells.

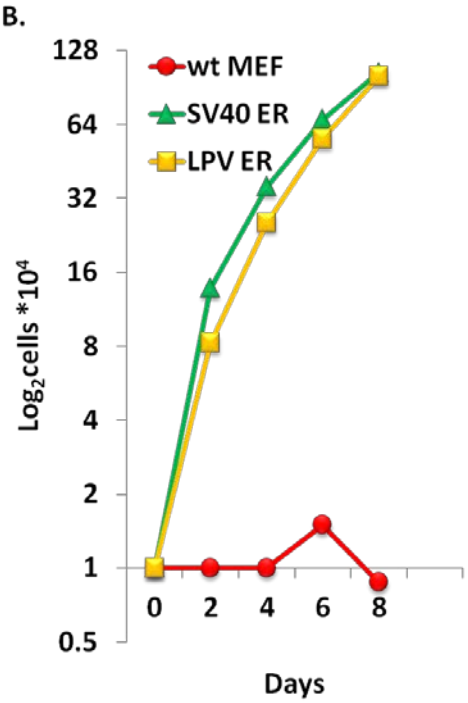
4.2 LPV ER TRANSFORMS PRIMARY MEFS IN CELL CULTURE.

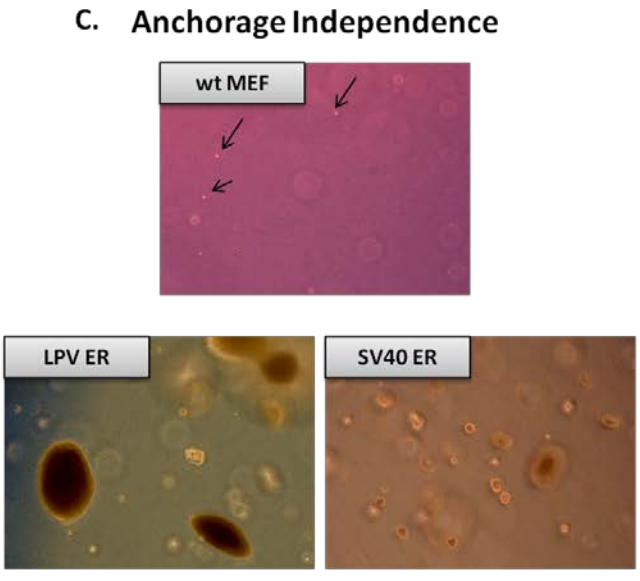
Several different studies have shown that LPV ER is highly potent in inducing cellular transformation in cell culture and induces tumorigenesis in transgenic model animals. Transgenic mice expressing LPV ER or SV40 ER under the same promoter show robust neoplastic growth in choroid plexus, thymus and spleen, and lympho proliferative disorders (lymphoma and leukemia). Interestingly, transgenic mice expressing ERs of either SV40 or LPV form tumors of choroid plexus, however the LPV ER transgenic mice show a higher kinetics of neoplasia formation (Chen, Neilson et al. 1989, Chen and Van Dyke 1991). Similarly, infection of hamster embryonic (HE) cells in cell culture with LPV induce anchorage independent and low serum growth and upon inoculation in newborn hamsters formed tumors (Kang and Folk 1992). Although these studies show robust transformation capabilities of LPV ER both *in vivo* and in cell culture the mechanism of transformation by LPV ER is still not understood.

I expressed LPV ER or SV40 ER in primary MEFs under the CMV promoter and as shown in **Figure 26A**, expression of both the ERs results in the morphological transformation of MEFs. While the SV40 ER expressing cells became smaller and densely packed, LPV ER expressing cells became smaller but highly refractile. I performed proliferation analysis of MEFs expressing LPV ER and SV40 ER as compared to wt MEFs. As shown in **Figure 26B**, expression of either LPV or SV40 ER enhanced the proliferation rate of MEFs to similar levels. Next I performed transformation assays to compare the ability of LPV ER to SV40 ER to induce low serum and anchorage independent growth in MEFs. As expected, wt MEFs failed to proliferate in soft agar conditions and remained as single cells while early region of LPV or SV40 induced considerable colony formation **(Figure 26C)**. Similarly, where wt MEFs failed to

proliferate in reduced serum conditions, expression of ERs from SV40 or LPV induced similar level of proliferation **(Figure 26D)**. Interestingly, while both the ERs induced similar growth in low serum conditions, soft agar colonies formed by LPV ER were considerably larger than those formed by SV40 ER.


A.
wt MEF
Lentiviral
Transduction
SV40 ER
LPV ER


B.
128
64
32
16
8
4
2
1
0.5
wt MEF
SV40 ER
LPV ER
Log2cells *10^4
0
2
4
6
8
Days


C. Anchorage Independence
wt MEF
LPV ER
SV40 ER

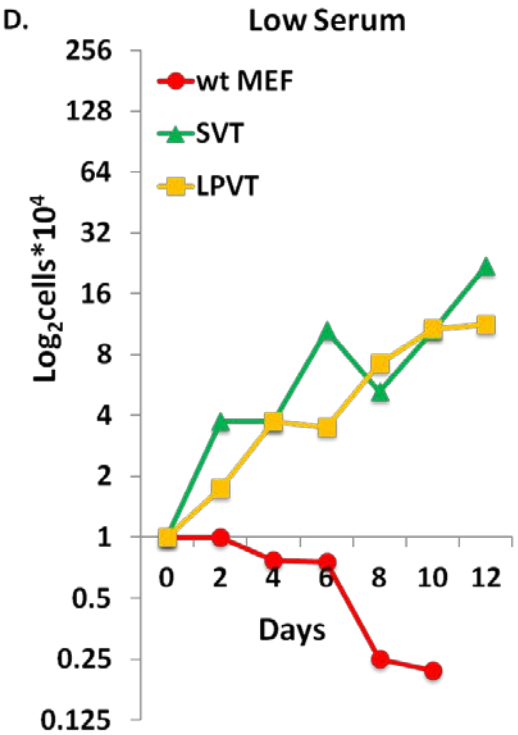

D.
Low Serum
256
128
64
32
16
8
4
2
1
0.5
0.25
0.125
wt MEF
SVT
LPVT
Log2cells*10^4
0
2
4
6
8
10
12
Days

Figure 26: LPV ER transforms MEFs to a similar level as SV40 ER.

Early regions of SV40 and LPV were cloned in pLenti-blasticidin vectors and MEFs were transduced with lentiviruses containing either SV40 ER or LPV ER. A). Expression of SV40 ER or LPV ER in MEFs induced morphological transformation. B). Growth curve analysis of MEFs expressing SV40 or LPV ER compared to wt MEFs. Equal numbers of cells from each cell type were plated in duplicates in 12 well plates and counted at specific time points. C). Equal number of cells from each cell type were plated in soft agar in duplicates and colony formation was determined after 21 days. D). Equal number of cells from each cell type were plated in normal serum at day 0, and the medium containing 10% serum was replaced with low serum media on day 1. Cells were counted periodically and growth curve was prepared.

4.2.1 LPV ER inhibits RB/E2F and p53/MDM2 tumor suppressive pathways

The transformation abilities of SV40 is attributed to TAg mediated inhibition of the two major growth suppressive pathways- RB/E2F and p53/MDM2. Therefore, I assessed the growth inhibitory activities of RB and p53 in SV40 and LPV ER expressing MEFs. Western blot analysis of SV40 ER expressing MEFs showed depletion of p130 protein levels and an increase of p107 protein levels. Similar results were obtained from LPV ER expressing MEFs as well **(Figure 27A)** suggesting similar inhibitory activity of the two ERs on RB proteins. Inhibition of RBs by the TAg is expected to induce E2F target genes expression. Western blots and, real time PCR analysis of MEFs expressing SV40 or LPV ER showed at least 2-fold up-regulation of E2F target genes **(Figure 27B)**. The p53/MDM2 tumor suppressive pathway was also inhibited by both the ERs. Western blot results indicate an up-regulation of p53 protein levels in both LPV or SV40 ER expressing MEFs **(Figure 27A)**. This is a characteristic result of TAg interaction with

p53 protein. Real-time PCR analysis showed prevention of p53 target genes expression in LPV or SV40 ER expressing MEFs, even when p53 protein levels in these cells were considerably higher than the wt MEFs **(Figure 27C)**.

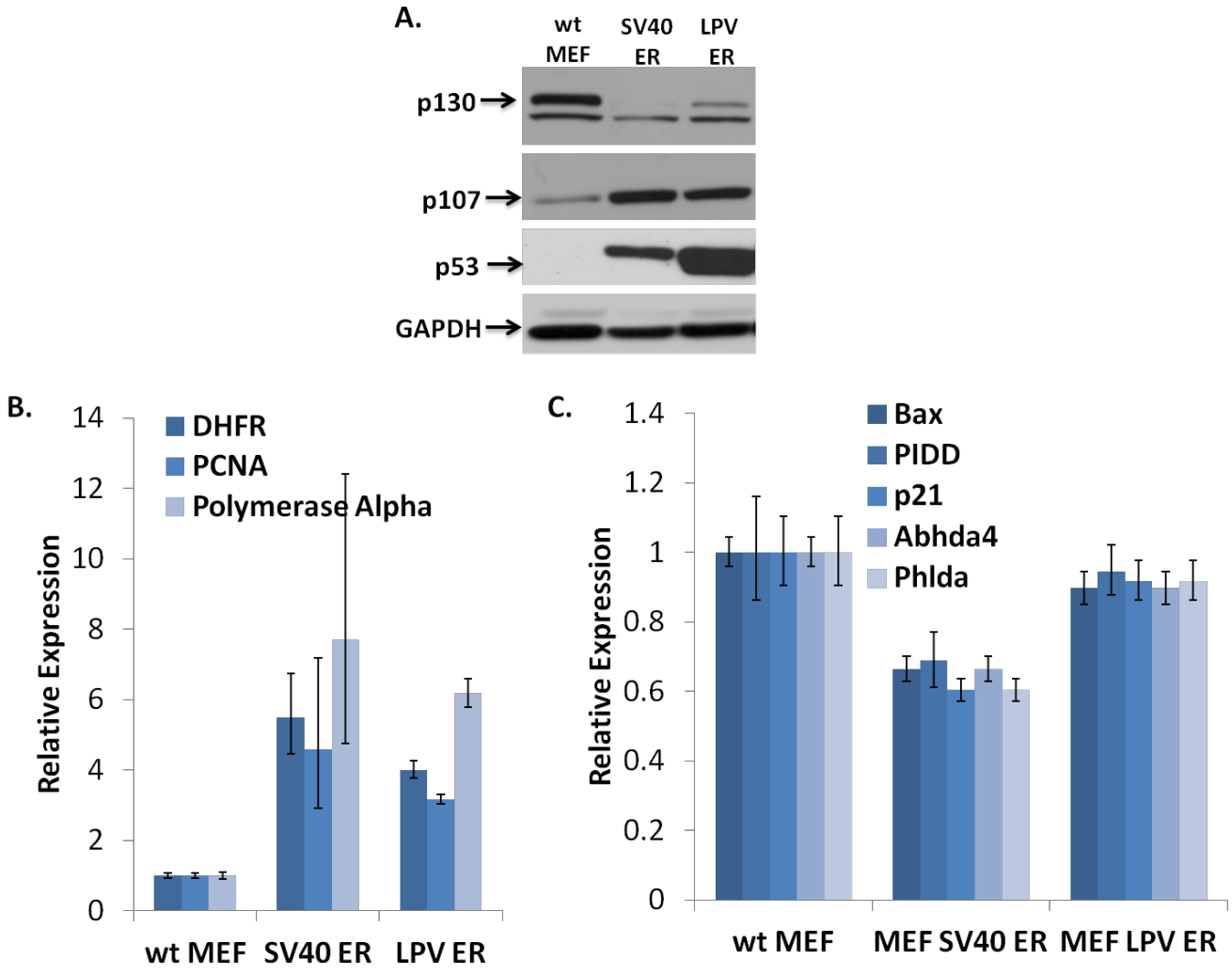


Figure 27: LPV ER inhibits RB/E2F and p53/MDM2 pathways.

A). Western blot analysis of p130, p107 and p53 protein expression in MEFs expressing ERs from SV40 or LPV. Expression of ERs from either polyomaviruses induced degradation of p130 while increase protein levels of p107 and p53. B). Real-time PCR analysis for canonical E2F target genes using gene specific primers. C). Real-time PCR analysis of canonical p53 target genes using gene specific primers.

The gene expression levels are represented relative to the expression level in wt MEFs and Rpl5 expression levels were used for cDNA normalization. The error bars represent standard deviation and each gene for every sample was analyzed in quadruple.

4.2.2 LPV ER encodes four differentially spliced products

Previous studies comparing the transformation capabilities of SV40 and LPV showed that while SV40 ER transformed cell pools selected for high TAg expressing cells, LPV ER transformed cells expressing high amount of TAg became genetically unstable, and there was selection against higher TAg expressing cells (von Hoyningen-Huene, Kurth et al. 1992). These results suggest that the LPV ER transforms cells similar to SV40 ER, but unlike SV40 TAg, LPV TAg may not be the essential oncoprotein. Moreover phylogenetics **(Figure 25)** also suggests that the two homologous proteins are distantly related and may exhibit different activities *in vivo*.

The early regions of all the polyomaviruses studied so far are known to encode 3-5 differentially spliced products. In order to determine the products of LPV ER total RNA was extracted from LPV ER expressing MEFs or human fibroblasts and reverse transcription PCR was performed. In order to amplify the whole ER, I performed RT-PCR experiments using primers specific for the 5' and 3' ends of the ER **(Set A primers shown in Figure 28A)**. I observed a PCR fragment for LPV TAg at the expected size (2094 nts), and this was

subsequently confirmed by sequencing of the gel purified band. I also observed a band of higher intensity around ~750nts **(Figure 28B)** and sequencing of the gel purified band showed that its mRNA was formed upon removal of two introns from the ER. The first splice used same acceptor and donor sites as the TAg mRNA and thus included complete exon1 but only 372bps of exon2. The second splice results in exclusion of most of the remaining TAg exon2 sequence and includes 134 bps from the 3' end of TAg exon2. This second splice results in a change in reading frame, thus introducing a stop codon 17 bps earlier than the TAg stop codon **(Figure 28D)**. Based upon the sequence alignment the protein product encoded by this mRNA shares the amino-terminal 203aa with TAg, while the reading frame shift would result in a 38a.a. unique carboxy-terminal region **(Figure 29B)**. The resulting 241a.a. product has similar splicing pattern as the T primes (T') of JCV, and hence I am calling it LPV LT'.

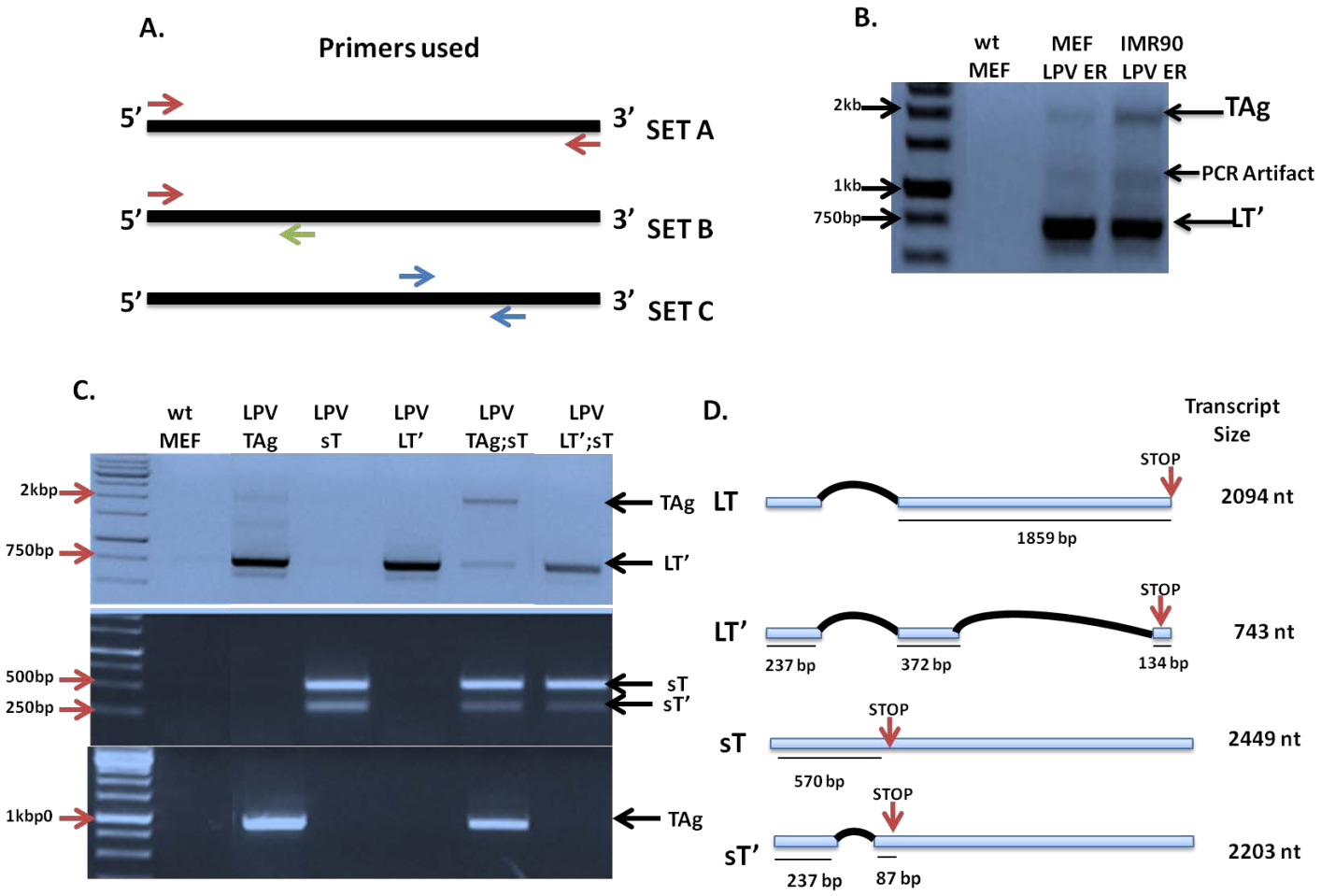

A.
Primers used
5'
3'
SET A
SET B
SET C
B.
wt MEF
MEF LPV ER
IMR90 LPV ER
2kb
1kb
750bp
TAg
PCR Artifact
LT'
C.
wt MEF
LPV TAg
LPV sT
LPV LT'
LPV TAg;sT
LPV LT';sT
2kbp
750bp
500bp
250bp
1kbp0
TAg
LT'
sT
sT'
TAg
D.
Transcript Size
LT
STOP
2094 nt
1859 bp
LT'
STOP
743 nt
237 bp
372 bp
134 bp
sT
STOP
2449 nt
570 bp
sT'
STOP
2203 nt
237 bp
87 bp

Figure 28: LPV ER encodes four differentially spliced products.

A). Schematics of LPV ER shown as the black bar, and three different set of primers used for PCR amplification are shown as arrows roughly placed at their complimentary region along the length of ER. Set A primers are specific for the 5' and 3' ends of LPV ER. SetB forward primer shwn in red is the forward primer from setA, while the reverse primer of set B is specific for the 3' end of LPV sT. Set C primers are specific for the TAg region which is spliced out from LT' and is absent in sT cDNA. B). RT-PCR analysis of human and mouse fibroblasts expressing LPV ER using set A primers. Three bands were observed corresponding to ~2.1kb, ~1kb and ~750bs. The ~1kb band was confirmed as a PCR artifact by sequencing and using purified plasmids as controls. C). RT-PCR analysis of MEFs expressing LPV ER products alone or in combinations. In the top gel image, set A primers were used to amplify TAg or LT' transcripts. In the middle gel image, sT specific, setB primers were used and two bands were observed at ~550b and ~300b corresponding to sT and its spliced product sT' respectively. In the bottom gel image, primers from set C were used, which should specifically amplify TAg transcript. As evident from the figure, only the cells transduced with TAg cDNA show amplification of TAg, while no band is visible in either sT or LT' expressing MEFs. D). Schematic showing splicing pattern of LPV TAg, LT', sT and sT', with the expected transcript size in LPV ER expressing MEFs is shown on the right. Below each bar is shown the length of the segment in nts and the red vertical arrow shows the stop codon for each transcript.

I also observed band(s) around 1kb, however repeated gel purification and sequencing efforts failed to provide a "meaningful" sequence. I later confirmed these bands as PCR artifacts by using purified plasmid containing LPV ER as control. PCR amplification of the ER from purified plasmid gave two bands, one at ~2.5kb (LPV ER and the other at 1kb. I did not observe a band for TAg (2.1kb) or LT' (743bp) with the purified plasmid confirming that they are authentic splice products of the ER. I also used a retroviral vector containing LPV sT cDNA under MMLV promoter (prepared by Paul Cantalupo, Pipas lab) and obtained MEF cell pools expressing LPV sT. LPV sT expression was confirmed by RT-PCR using Set B primers specific

to 5' and 3' ends of LPV sT **(Figure 28A)**. I observed a band of approximately 570bps **(Figure 28C)** specific for sT. This was confirmed by sequencing. I also observed another band of approximately 324bps which upon sequencing appeared to be a spliced variant of sT transcript **(Figure 28C)**. This splice product shares the first 273nts with the sT transcript and splices out 246nts of the sT unique region and adds 87nts at the 3' terminus. I call this sT splice form sT' (small t prime). Based upon sequence alignment, sT' retains the J-domain coding region but lacks the putative PP2A interacting motifs and the Zn^{+2} binding clusters of sT protein. Purified plasmid containing sT cDNA was used as a control for determining the authenticity of sT' and as expected only one band at ~570 bp (sT) marker size was observed suggesting that sT' is a splice product formed *in vivo*.

Next, I cloned cDNAs of TAg and LT' under CMV promoter in lentivirus vectors, expressed them individually in mouse fibroblasts (MEFs) and obtained cell pools.. Next I performed transcript and protein analysis of the MEF pools by RT-PCR and western blot analyses respectively. As shown in **figure 28C** RT-PCR analysis using set A primers show the presence of TAg and LT' transcripts in MEFs transduced with TAg cDNA. This was expected as the second splice site for LT' is still present in the TAg cDNA. As observed in MEFs expressing LPV ER the band intensity of TAg was consistently lower than the LT' band intensity and similar results were also obtained with MEFs transduced with TAg cDNA **(Figure 28C)**. MEFs expressing LT' cDNA show a single band of the expected size **(Figure 28C)**.

I also expressed the various LPV ER splice forms in different combinations to obtain pools of cells expressing sT;TAg, and sT;LT'. Since the TAg cDNA retains the capacity to express LT' I did not co-express these constructs. The combinatorial expression of the early region products was confirmed by RT-PCR **(Figure 28C)**. Finally, in order to verify expression

of the TAg transcript alone I used another set of primers **(Set C, Figure 28A)** which were specific for TAg sequences. PCR amplification using set C primers shows the presence of TAg transcript only in cells expected to express TAg while no signal was obtained in either the LT' or sT cDNA expressing MEFs **(Figure 28E)**.

Next I determined the protein expression encoded by the spliced products in MEFs by western analysis using monoclonal antibody Xt-7 developed and provided by Dr. Christopher Buck (NIH). This antibody is specific for epitopes present in the J-domain and thus is expected to recognize the proteins encoded by all four spliced products formed from the LPV ER. LPV ER expressing cells show strong expression of TAg, LT' and sT, but not of sT' **(Figure 29A)**. No sT' protein signal was observed in MEFs transduced with sT cDNA (LPV sT or LPV TAg;sT) while a strong signal for sT protein was observed in both the cell types. Consistent with the RT-PCR results, MEFs expressing TAg cDNA alone or in combination with sT show strong bands for both LT and LT' while cells expressing LT' cDNA alone show only one band corresponding to the predicted size of LT'. I also observed two additional bands corresponding to sizes ~50kDa and ~25kDa. These two bands could be degradation products of the TAg or unidentified products originating from the TAg transcript due to differential splicing or alternate start codons. Since the antibodies used in the western analysis recognize epitopes in the J-domain both unidentified proteins must contain J-domain. In conclusion, my results show that the LPV ER encodes at least four differentially spliced products: LT, sT, LT' and sT'. However, in the two cellular systems used in this study the sT' transcript was observed but no protein product corresponding to its predicted size was detected by western analysis.

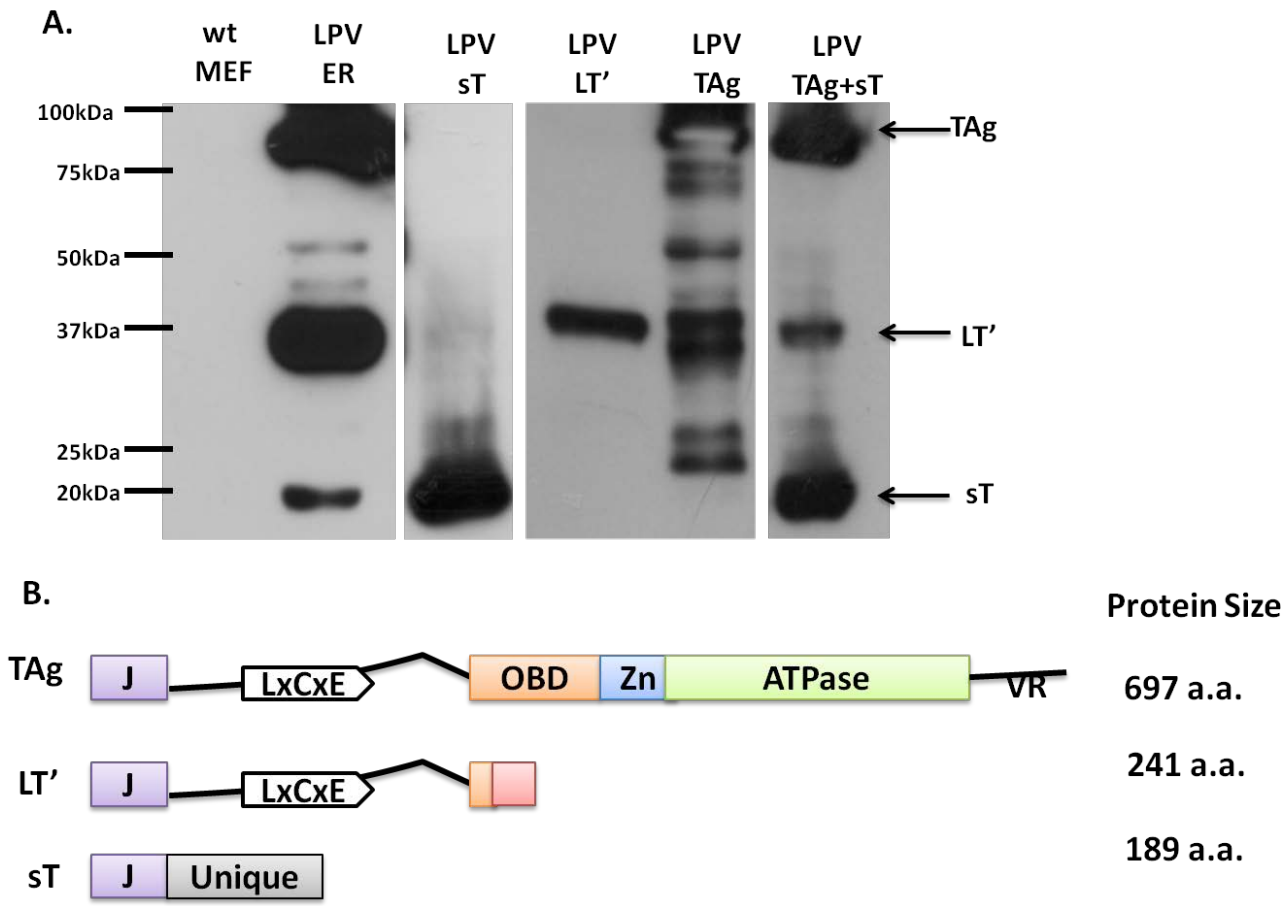

A.
wt MEF
LPV ER
LPV sT
LPV LT'
LPV TAg
LPV TAg+sT
100kDa
75kDa
50kDa
37kDa
25kDa
20kDa
TAg
LT'
sT
B.
Protein Size
TAg
J
LxCxE
OBD
Zn
ATPase
VR
697 a.a.
LT'
J
LxCxE
241 a.a.
sT
J
Unique
189 a.a.

Figure 29: Protein analysis of MEFs expressing either LPV ER or different splice products.

A). Western blot analysis of MEFs transduced with LPV ER, LPV sT cDNA, LPV LT' cDNA, LPV TAg cDNA or combinatorial expression of LPV TAg and sT cDNAs. Robust expression of TAg, LT' and sT protein was observed in LPV ER expressing MEFs but no signal for sT' protein was detected in either LPV ER or cells transduced with sT cDNA. Protein bands for TAg and LT' was observed in MEFs transduced with TAg cDNA, while only LT' protein signal was detected in LPV LT' MEFs. Similarly, sT protein expression was present in LPV sT and LPV TAg;sT MEFs. There are other bands observed in cells expressing LPV ER (~50kDa) or LPV TAg cDNA (~50kDa and ~25kDa). Interestingly, these extra bands disappeared from LPV TAg cells when LPV sT was transduced in these cells (LPV TAg;sT). B). Schemetic representation of LPV TAg, LT' and sT protein domains. based upon sequence homology with SV40 TAg. LPV TAg contains similar domains and unstructured regions as SV40 TAg, but lacks the C-terminal HR region and possess a longer linker region. LPV LT' contains a unique C-terminus region formed due to the change in reading frame but it shares the J-domain, linker region and 4 amino acids of the OBD domain with the TAg, LPV sT shares the J-domain with LT' and TAg, but contains a unique C-terminus region which contains putative PP2A interaction sites and Zn ion binding sites. The protein length of each LPV oncoprotein is shown in the right hand side in term of the number of amino acids.

4.3 DIFFERENTIAL EFFECTS OF LPV ER SPLICE PRODUCTS ON CELL PROLIFERATION AND TRANSFORMATION

Results described in the previous section show transcript expression of at least four different ER spliced products and the corresponding proteins of three of these were detected. In order to

determine the contributions of each splice product in LPV ER transformation of MEFs I analyzed the growth promoting activities of each product individually or in combinations.

4.3.1 LPV LT' induces growth arrest in MEFs.

I first examined the ability of LT' to induce proliferation in MEFs. LPV LT' expression alone resulted in severe growth defects in MEFs. These cells displayed an enlarged senescent like morphology and failed to reach confluency **(Figure 30A)**. The LT' protein has a unique C-terminus region that is abundant in basic amino acids (Lysine) followed by a hydrophobic amino acids tail. This is similar to the sequence of mT encoded by MuPyV. In the case of mT this sequence is required for membrane localization and and for its RTK mimicking activity. In order to determine if the growth arrest phenotype of LPV LT' expressing MEFs is due to the activity of the unique region I used a LT' truncation mutant (N200, prepared by Paul Cantalupo, Pipas lab) which shares the first 200 amino acids with the LT' but lacks the unique C-terminus region. N200 was cloned in pLenti6.3-blasticidin vector and was expressed in MEFs via lentiviral transduction. Whereas LT' induces growth arrest in MEFs, deletion of its C-terminus unique region rescues the growth defect and results in the immortalization of primary MEFs **(Figure 30A)**. **Figure 30B** shows that LT' and N200 are expressed at similar levels. Additional experiments are required to determine the localization of LT' and N200 and the effects of their expression on different growth regulatory pathways.

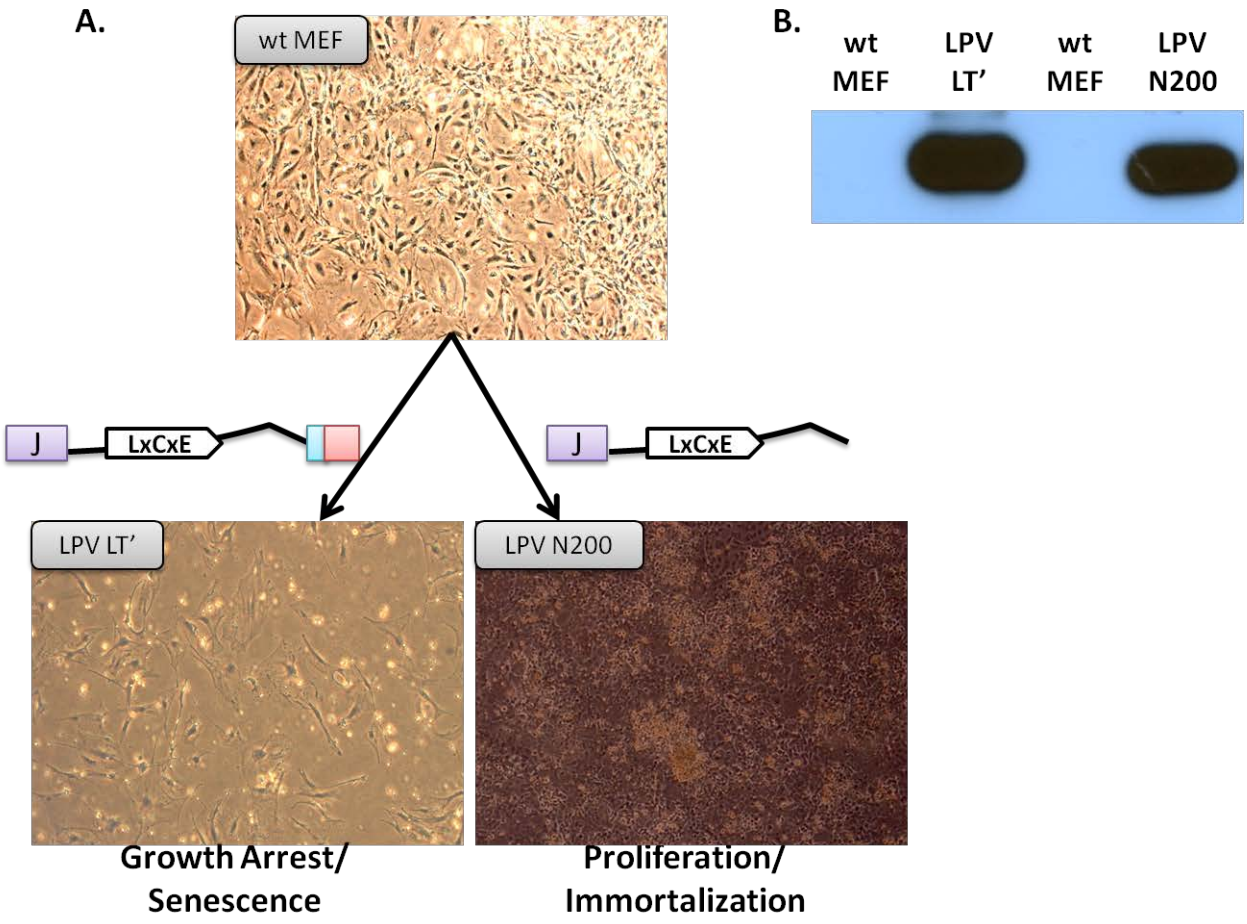


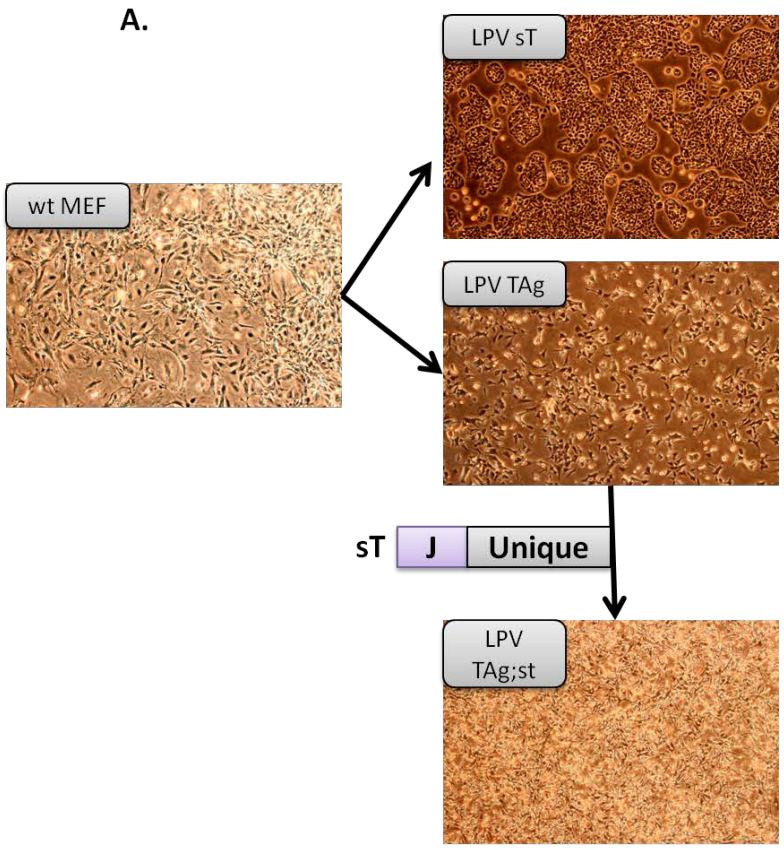
Figure 30: LPV LT' induces growth arrest in MEFs.

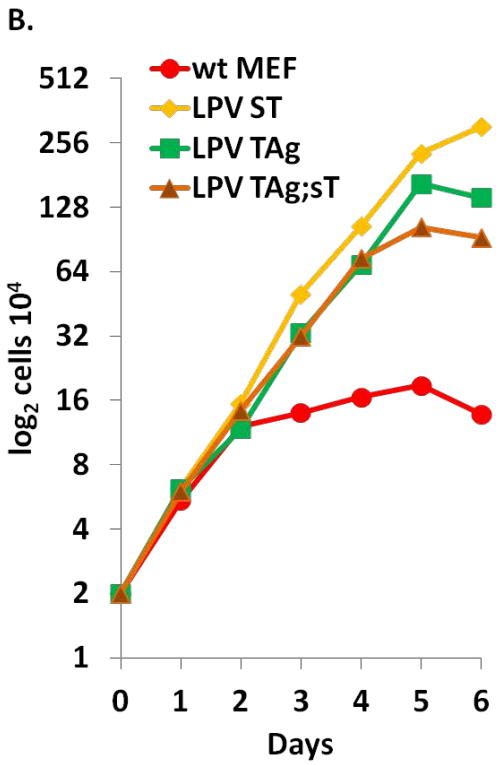
MEFs were transduced with lentiviruses containing LT' or N200 cDNAs. A). Expression of LT/ alone induced growth arrest in MEFs, the cells showed enlarged senescent like morphology. Expression of N200, on the other hand morphologically transformed MEFs and the cells became reflective and smaller in size. In contrast to LT', N200 expressing MEFs proliferate and are immortalized. B). Western blot analysis showing similar levels of LT' and N200 protein expression.

4.3.2 LPV TAg or sT expression is sufficient to immortalize MEFs

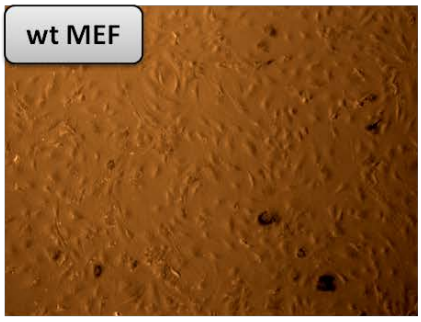
In order to test the abilities of LPV TAg and sT to immortalize MEFs I performed growth curves with cells expressing TAg and sT alone or in combination. As compared to wt MEFs, TAg expressing MEFs display a smaller size morphology with few cells showing a refractile morphology. In contrast, sT expressing cells display dramatic morphological transformation and grow as a "clonal group" of cells. In addition, expression of sT in TAg expressing MEFs resulted in an increase in the number of cells exhibiting a refractile morphology that resembles the morphology of LPV ER expressing cells **(Figure 31A)**. Expression of TAg and sT alone allowed several continuous (>20) passaging of MEFs suggesting that they immortalize MEFs. Growth curves of MEFs expressing sT and TAg alone show a similar enhancement of proliferation rate. Interestingly cells expressing both the oncoproteins (LPV TAg;sT) also display a similar enhancement of proliferation rate **(Figure 31B)**.

These results show that both TAg and sT are able to induce morphological transformation in MEFs, however each of these proteins induce a very distinct morphological change. Moreover expression of TAg and sT in trans results in a recapitulation of morphology exhibited by LPV ER expressing MEFs. Immortalization of primary MEFs by SV40 TAg is well documented (Ahuja, Saenz-Robles et al. 2005) and I obtained similar result with LPV Tag. However while SV40 sT expression alone induces senescence in primary MEFs **(Figure 31C)**, LPV sT alone induced proliferation.

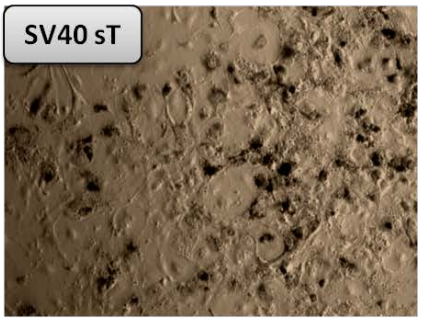




C.



5X magnification



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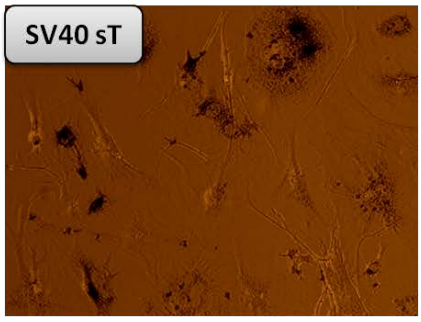


10X magnification

Figure 31: Both LPV TAg or LPV sT induces proliferation in MEFs.

A). Transduction of MEFs with retroviruses containing LPV sT cDNA or with lentiviruses containing LPV TAg resulted in morphological transformation and cell proliferation. Expression of LPV sT in LPV TAg MEFs resulted in further morphological transformation and the resulting cells became more reflective and appeared similar to LPV ER expressing MEFs. B). Equal number of cells from two biological replicates of each cell type were plated in duplicated in 12 well plates and growth curves were prepared by counting cells at regular intervals. MEFs expressing LPV sT or TAg alone or in combination showed considerable increase in cell proliferation as compared to control wt MEFs. C). Beta-galactosidase assay testing senescence in MEFs transduced with retroviruses containing SV40 sT. Control MEFs showed very few cells exhibiting beta-galactosidase activity, which increased considerably in MEFs transduced with SV40 sT containing cDNA. The image taken at 10X magnification shows enlarged morphology of SV40 sT transduced MEFs, another characteristic of senescent cells.

4.3.3 LPV sT is essential and sufficient for growth in low serum

LPV ER expressing MEFs retain the ability to grow in the absence of growth factors and form colonies in soft agar. Expression of LPV sT or TAg alone or in combination are able to induce proliferation and immortalization of MEFs to a similar extent. Therefore, I next assessed their roles in LPV ER induced cell transformation. I performed low serum and soft agar transformation assays with MEFs expressing sT or TAg using wt MEFs as controls.

Figure 32A shows that wt MEFs cease to proliferate in low serum conditions while sT expressing MEFs induce continuous cell proliferation. While TAg alone was able to induce proliferation in MEFs similar to sT expressing MEFs in normal serum conditions, in low serum conditions LPV TAg MEFs failed to proliferate. While expression of sT in TAg expressing

MEFs (LPV TAg;sT), rescued the proliferation defect of LPV TAg MEFs in low serum conditions their proliferation was lower than MEFs expressing sT alone. These results show that LPV sT is necessary and sufficient for transformation of primary MEFs for growth in low serum conditions.

Next I performed soft agar assays to determine the ability of sT and TAg to transform MEFs in anchorage independent conditions. **Figure 32B** shows that while wt MEFs fail to proliferate in soft agar and remain as single cells, expression of either sT or TAg allow MEFs to form soft agar colonies. The colonies formed by either of the two oncoproteins are of similar size but were greatly reduced in comparison to LPV ER expressing MEFs. These results suggest that both LPV sT and TAg possess that ability to transform MEFs in soft agar conditions and that the activities of both are required to fully transform MEFs as determined by the soft agar transformation phenotype.

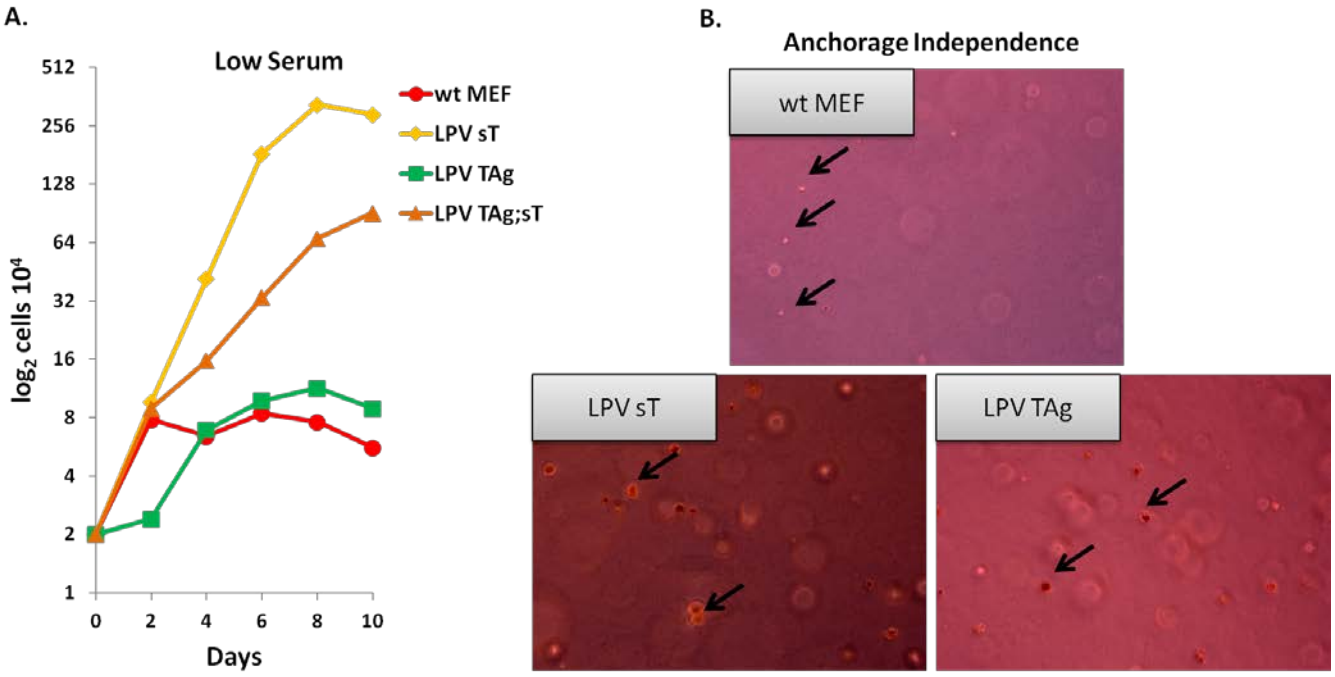


Figure 32: LPV sT is essential and sufficient for growth in low serum.

A). Two biological replicates of MEFs expressing LPV sT or TAg alone or in combination were plated in duplicates in 12 well plates in normal serum containing media on day0. On day 1, 10% serum media was replaced with 1% serum containing media and growth curves were prepared by counting cells at regular time intervals. MEFs expressing LPV sT alone or in combination with TAg continuously proliferate, while wt MEFs or LPV TAg MEFs ceased to proliferate. B). Similar number of MEFs expressing LPV sT or TAg cDNA were plated in soft agar and colony formation was determined after 21 days. Both sT or TAg formed soft agar colonies of similar size, while wt MEFs remained as single cells.

5.0 CONCLUSIONS AND DISCUSSION

5.1 CONCLUSIONS AND DISCUSSION I

The RB/E2F pathway is one of the central regulators of cell proliferation and almost 30% of human cancers bear mutations or deletions of the *RB1* gene while other cancers are expected to bear mutations in other components of the pathway ultimately leading to RB inhibition **(Figure 6)** (Berman, Yuan et al. 2008, Chen, Tsai et al. 2009). While the role of RB proteins as tumor suppressors is well established (Weinberg 1991, Weinberg 1992), we are just beginning to understand the cellular and physiological roles of E2Fs (Chen, Tsai et al. 2009). The current "textbook" model for normal or oncogenic cell proliferation suggests an essential role of activator E2Fs in activating cell cycle genes and promoting cellular replication (Egan, Bayley et al. 1989, Nevins 1992, Sharma, Timmers et al. 2006, Timmers, Sharma et al. 2007, Chen, Tsai et al. 2009, Rathi, Saenz Robles et al. 2009).

SV40 TAg induced proliferation and transformation requires its interaction with RBs and this interaction was shown to inhibit RBs growth inhibitory activity. TAg mediated RB inhibition is suggested to be required for the release of activator E2Fs which in turn, would activate cell cycle genes and drive cells into proliferation. Consistent with this model, gene expression analysis both *in vivo* and in cell culture have shown up-regulation of E2F target genes in the cells expressing TAg which is significantly reduced in TAg mutants for RB binding or Hsc70 binding

(Rathi, Saenz Robles et al. 2009). Other studies directly testing the role of activator E2Fs in TAg induced proliferation or tumorigenesis also favor the essential and oncogenic role of activator E2Fs (Pan, Yin et al. 1998, Saenz-Robles, Markovics et al. 2007) in TAg induced tumorigenesis.

The study of E2F proteins is challenging due to their high functional redundancy and antagonism, and several studies have provided contrasting results regarding their cellular functions (Chen, Tsai et al. 2009). In fact, the recent studies have shown that certain cell types like progenitor cells of retina and lens as well as mouse embryonic stem cells can proliferate in the absence of activator E2Fs (Chong, Wenzel et al. 2009, Wenzel, Chong et al. 2011). Moreover, studies of various human tumors have also suggest that activator E2Fs can act as both oncogenes or tumor suppressors depending upon the cancer types studied (Chen, Tsai et al. 2009) indicating that the role of activator E2Fs in normal and oncogenic cell proliferation is cell context dependent. Consistent with the recent advances in our knowledge regarding cell context role of activator E2Fs in normal cell proliferation, my results in this study show that SV40 TAg induced cell proliferation and transformation in MEFs is independent of activator E2Fs. My results also show that cooperation between two strong oncogenes, TAg and $HRas^{V12}$, is also independent of activator E2Fs and TAg is able to induce expression of E2F target genes even in their absence.

5.1.1 How are TKO;TAg MEFs proliferating in the absence of activator E2Fs?

The proliferation defects observed in TKO MEFs were attributed to the activation of p53. Activated p53 induces transcription of its target genes which either causes cell cycle growth arrest or apoptosis or both. p53 on one hand activates its target gene the CDKI $p21^{Cip/Kip}$, which

leads to the stabilization of p130/E2F4 repressor complexes on the promoters of E2F target genes (Sharma, Timmers et al. 2006, Timmers, Sharma et al. 2007). This increase is linked to G1/S phase growth arrest and the inhibition of p53 but not $p21^{Cip/Kip}$ is sufficient to rescue the proliferation defect of TKO MEFs (Sharma, Timmers et al. 2006, Timmers, Sharma et al. 2007). On the other hand, other p53 target genes like NoxA, GADD45 or Pidd are also up-regulated and result in apoptosis.

Based upon my results, I propose a model in which TAg disrupts p130/E2F4 complexes and inhibits p53, thereby allowing cell proliferation in the absence of activator E2Fs **(Figure 33).** My results show that p53 activity is inhibited by TAg in all the MEF backgrounds tested and subsequently $p21^{Cip/Kip}$ transcript levels are considerably reduced upon TAg expression **(Figure 21A and C)**. However, $p21^{Cip/Kip}$ protein levels are reduced by TAg expression only in the wt background but not in DKO or TKO MEFs, suggesting a dual regulation of $p21^{Cip/Kip}$, transcriptionally by p53 and translationally by activator E2F1-2. A similar observation was reported for $p19^{ARF}$ in TKO MEFs where depletion of activator E2Fs did not affect its transcript levels but resulted into a considerable increase in protein levels (Timmers, Sharma et al. 2007). The stabilization of $p21^{Cip/Kip}$ protein in TAg expressing MEFs should not have a negative impact on cell cycle progression as TAg acts down-stream of $p21^{Cip/Kip}$ by directly inhibiting RBs. Similar results are expected with the TAg mutant T^{N136} which retains a functional LXCXE motif and disrupts p130/E2F4-5 repressor complexes. However T^{N136} lacks the carboxy terminal region required for inhibiting p53. Therefore, p53 mediated apoptosis would still take place in TKO;T^{N136} MEFs. The inability to produce TKO clones with T^{N136} supports the role of p53-$p21^{Cip/Kip}$ axis in TKO MEFs growth inhibition which is consistent with previous studies (Sharma, Timmers et al. 2006). However, involvement of other cellular proteins which interact

with the missing C-terminus region of the TAg cannot be ruled out. This issue requires a more exhaustive analysis of different TAg truncation mutants.

Moreover, consistent with the previous reports, my results also suggest that p53 inhibition is essential to rescue TKO proliferation defect. However, my results which show that the TAg mutant retaining p53 inhibition activity but lacking a functional LXCXE motif (T^{E107K}) is unable to rescue the proliferative defects observed in TKO MEFs, suggest that p53 inhibition is essential but not sufficient to rescue the proliferation defect of TKO MEFs. Although, $p21^{Cip/Kip}$ cell cycle inhibitory activity in TAg or T^{N136} expressing MEFs should not affect cell cycle progression due to the presence of LXCXE motif, it might have inhibitory effects in TKO;T^{E107K} cells and this was verified in F**igure 22B**, where T^{E107K} was unable to decrease p130 protein levels.

Overall, my results suggest that inhibition of both the RB/E2F and p53 pathways is required for TAg induced proliferation and transformation of MEFs. This is consistent with previous reports indicating that induction of cell proliferation and transformation by TAg can be achieved through at least two different functions, one within the amino and one within the carboxy terminal region of the oncogene (Cavender, Conn et al. 1995, Saenz Robles, Shivalila et al. 2013).

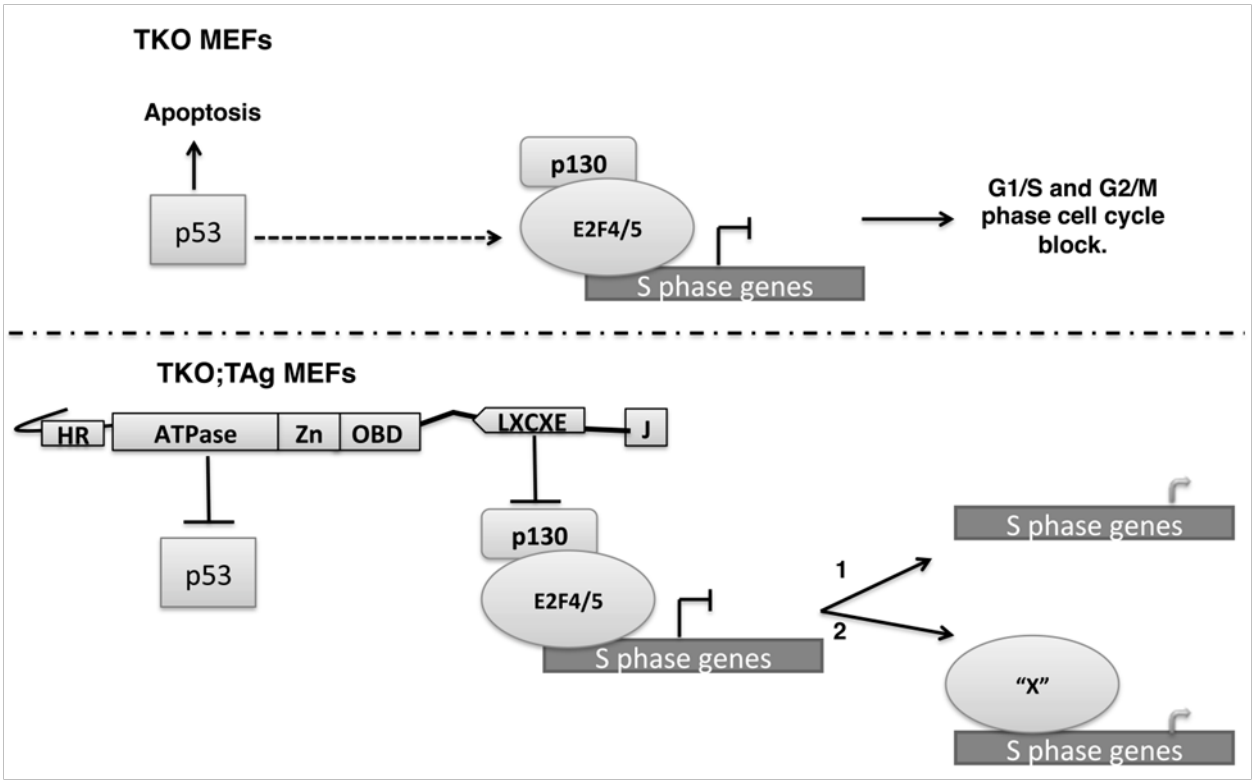


Figure 33: Proposed model for SV40 TAg mediated cell proliferation in the absence of activator E2Fs.

Depletion of all three activator E2Fs leads to p53 mediated cell cycle arrest or apoptosis. TAg inhibits p53 via its ATPase domain, and prevents p53 target genes expression. TAg also inhibits RB/E2F repressor complexes via its LXCXE motif, removing them from the promoters of cell cycle genes, and allowing their expression. Expression of cell cycle genes in TAg expressing TKO MEFs can be a result of (1) de-repression of promoters or (2) the activity of some other transcription factor(s).

5.1.2 How are the E2F target genes up-regulated in TKO;TAg MEFs in the absence of E2F activators?

Under non-proliferative conditions, E2F promoters are occupied by p130-E2F4/5 as part of the DREAM repressor complexes (Sadasivam and DeCaprio 2013). TAg expression triggers p130 degradation and disruption of the DREAM complexes, thus clearing up the promoters of E2F target genes. TAg mediated disruption of RB/E2F complexes is well documented and my results also verify that TAg's characteristic inhibitory activity on RB is present even in the absence of activator E2Fs. Therefore, expression of E2F target genes in the absence of E2F1-3 could result from mere de-repression of E2F targets.

There is sufficient evidence towards the existence of such a mechanism. As mentioned above, depletion of dE2F1 caused cessation of DNA synthesis and larval growth defects in Drosophila, but a simultaneous deletion of dE2F2 (repressor E2F), rescued both these defects (Asano, Nevins et al. 1996, Du, Xie et al. 1996). While this result suggests antagonistic role of activator and repressor E2Fs, it also suggests that depletion of the repressor E2F, dE2F2 is sufficient for the expression of E2F target genes even when the activator is absent. In another study, expression of a dominant negative E2F-DP protein, which lacks E2F transactivation domain, but retains the ability to compete and occupy E2F DNA binding sites, allowed cell proliferation and E2F target gene expression (Bargou, Wagener et al. 1996). Therefore, in these studies removal of repression or de-repression of the cell cycle genes promoter is sufficient to induce their expression and activator E2Fs are not directly involved in cell cycle gene transactivation, but in fact act as "repressors" of repressor E2Fs. This ability of activator E2Fs

would depend upon their competition with repressor E2Fs in binding to the promoters of E2F target genes, and thus preventing p130/p107-E2F4/5 repressor complexes to be formed.

However, one would expect that "de-repression" of E2F target gene promoters by TAg would allow basal level of gene expression in the absence of activator E2Fs. Instead I observed an even higher level of gene expression in TKO;TAg MEFs compared to DKO;TAg MEFs. This observation suggests the presence of some other factor(s) on the TAg mediated "cleared" promoters. Therefore, another possibility is that, once the cell cycle promoters are de-repressed, some other transcription factor now occupies the promoters and induces their expression. This is an exciting possibility as the discovery of such a protein(s) and its regulatory pathway(s) can lead to a significant contribution towards understanding of cell cycle regulation. There are at least two possible candidates other than activator E2Fs which have been shown to induce cell cycle genes. First, other members of E2F family can functionally replace activator E2Fs, as they all are known to bind the same consensus promoter sequence. E2F4 is constitutively expressed, and can interact with all three RB family members and DP proteins. However, unlike activator E2Fs, it lacks a NLS, and instead possess a bipartite NES, which is responsible for its exit from the nucleus during cell cycle progression. Its nuclear entry is dependent upon its interactions with either the RB family members or DP. In fact, over-expression studies and studies of certain mice tumors have shown that E2F4, can act as an activator in special circumstances (Souza, Yin et al. 1997, Puri, Cimino et al. 1998, Wang, Russell et al. 2000, Lee, Bhinge et al. 2011).

Second, other unrelated transcription factor(s) might functionally replace the activator E2Fs. In fact, one strong candidate is the c-Myc transcription factor. Several of the E2F target gene promoters also contain c-Myc binding sites, and over-expression of c-Myc has been shown to induce expression of E2F target genes (Dang 1999). A recent unpublished study by the Pipas

lab in collaboration with Dr. Gustavo Leone's lab at Ohio State has indicated c-Myc to be a down-stream effecter of pRb. In the absence of pRb, c-Myc has been observed to regulate G1-S phase cell cycle progression in mice intestinal cells. The same study has also suggested that both E2F activators and c-Myc might control parallel pathways of cell proliferation and act synergistically in activating cell cycle genes (Liu et al, submitted). Perhaps disruption of the RB pathway by TAg leads to c-Myc redeployment on the promoters of E2F target genes and thus allows their expression. More studies are needed to elucidate the molecular mechanisms controlling E2F target genes expression in the absence of E2F1-2-3.

The ability of TAg to up-regulate E2F target genes and to induce proliferation and transformation in E2F1-2-3 depleted MEFs contradicts the current model of TAg dependency on activator E2Fs. These results question the central role of E2F activators as essential drivers of tumorigenesis and suggest that alternative pathways and/or mechanisms are at play. Viral onco-proteins can open the door to understand the induction of transformation, cell cycle regulation and cell survival in the absence of activator E2Fs.

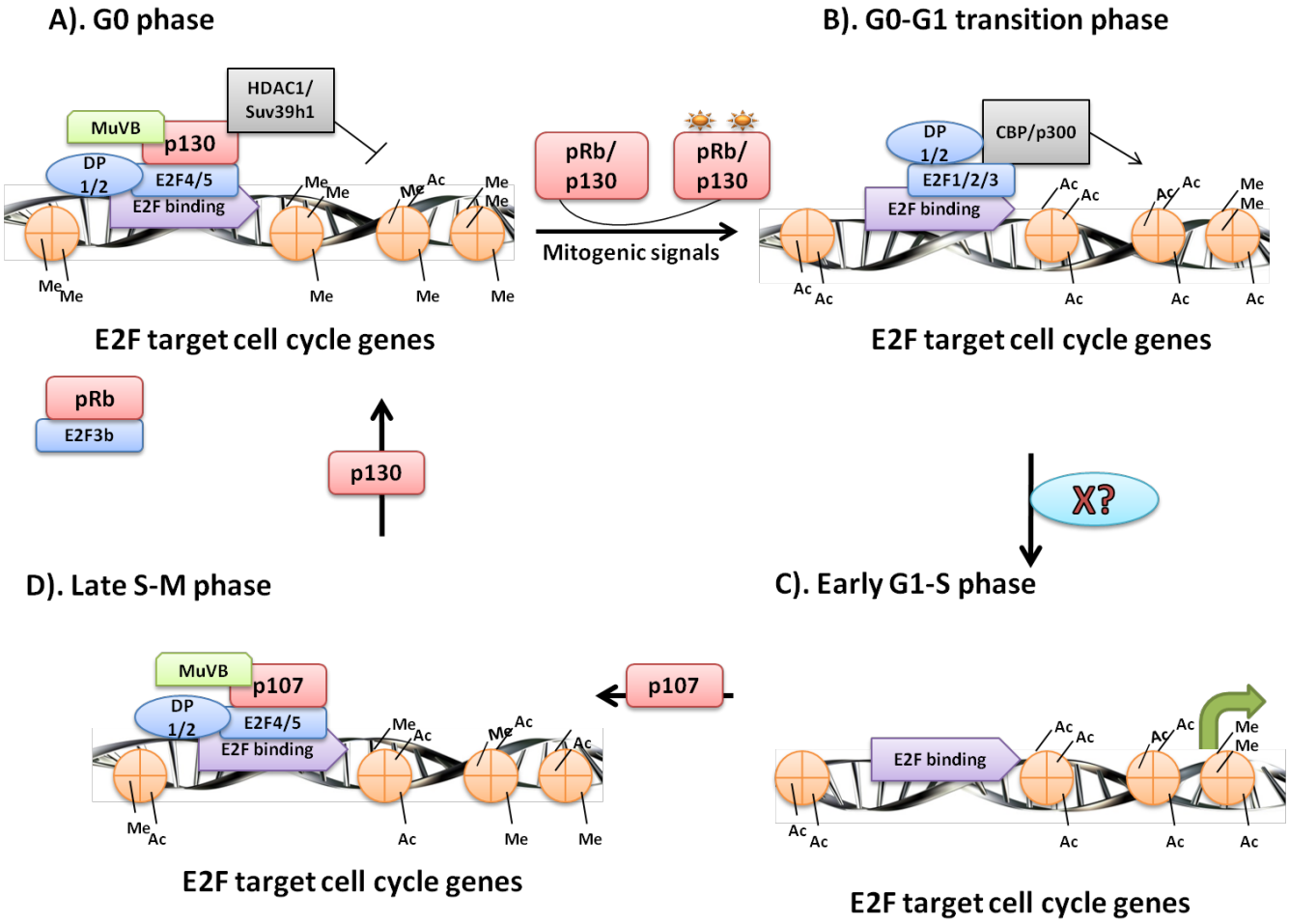

A). G0 phase
B). G0-G1 transition phase
HDAC1/
Suv39h1
MuVB
p130
DP
1/2
E2F4/5
E2F binding
pRb/
p130
pRb/
p130
Mitogenic signals
DP
1/2
CBP/p300
E2F1/2/3
E2F binding
E2F target cell cycle genes
E2F target cell cycle genes
pRb
E2F3b
p130
X?
D). Late S-M phase
C). Early G1-S phase
MuVB
p107
DP
1/2
E2F4/5
E2F binding
p107
E2F binding
E2F target cell cycle genes
E2F target cell cycle genes

Figure 34: Activator E2Fs as "repressors" of repressor E2Fs

A). In G0 phase of cell cycle, E2F target genes are repressed due to the occupation of promoters by repressor DREAM complexes and RB mediated chromatin modifications. Mitogenic signals induce RB phosphorylation and results into disruption of DREAM complexes and also inhibits pRb to release activator E2Fs. B). In this model, activator E2Fs would compete with repressor E2Fs to occupy the E2F target gene promoters in early G1 phase of the cell cycle, and will also attract chromatin modifiers to activate the promoters. C). Activator E2Fs become dispensable for cell cycle gene expression once the histone modifications have changed. The cell cycle genes are expressed either due to de-repression of the promoters or by some yet unidentified transcription factor shown as "X". D). Finally, p107 levels increase during late S-M phase, and bind to repressor E2Fs to form repressor complexes and occupy the promoters and prevent gene expression.

5.1.3 Why there are discrepancies in the results obtained in previous studies as compared to this study?

To my knowledge there are only two studies to date, where the role of activator E2Fs in TAg induced proliferation and transformation was directly tested. Both these studies were performed *in vivo* using mice depleted of specific activator E2Fs and expressing either wt TAg (in intestinal villi cells) or its N-terminus truncation mutant N121 (in choroid plexus and lymphoid tissues), which can bind and inhibit RBs. Both these studies showed that expression of TAg in control animals resulted into hyperplasia, which was significantly reduced in the absence

of specific activator E2Fs. In the study where TAg was expressed in intestinal villi cells, E2F2 depletion resulted into significant reduction of proliferative cells, suggesting an essential role of activator E2F2 in mediating TAg induced proliferation. The other study, where N121 was expressed in mouse choroid plexus, deletion of E2F1 significantly reduced proliferation of choroid plexus epithelium cells and resulted into lower tumor formation kinetics. One difference between this study and the previous two studies is the use of different systems to study the dependence of TAg on activator E2Fs. In contrast to this study, the previous two studies were performed *in vivo* using transgenic mice, and thus the dispensable nature of activator E2Fs observed in this study could be a cell culture artifact. The complex organ architecture present *in vivo* could result into a more complex role for activator E2Fs which may be dependent upon the signals obtained from the tissue environment. Primary MEFs when initially extracted from the mouse embryos are composed of heterogeneous cell population, but upon growth in cell culture they select for cells which adapt to grow in artificial conditions.

Apart from the cell culture and *in vivo* differences, this study also differs from the previous two studies in terms of the types of cells studied. While activator E2Fs are considered essential for cell proliferation, their requirement has also been shown to be dispensable for certain cell types. Previous studies have shown that continuously proliferating progenitor cells of retina, lens and intestinal crypts *in vivo*, and mouse embryonic stem cells in cell culture do not require activator E2Fs for cell cycle genes activation or proliferation. In growth arrested cells (terminally differentiated cells *in vivo* or serum deprived cells in culture), the cell cycle genes promoters are suppressed due to the occupation of promoters by p130/E2F4-5 containing DREAM complexes, and "repressive" histone modifications (Gonzalo, Garcia-Cao et al. 2005). For cell cycle re-entry, these two types of repressions need to be removed. Therefore, activator

E2Fs will be required for exiting the growth arrested state of the cells. On the other hand, continuously proliferating cells skip the G0 phase of cell cycle, and enter the cell cycle directly from the M phase. These cells also have low repressive histone modifications, and instead contain higher histone acetylation marks. Therefore, I propose that the requirement of activator E2Fs for cell proliferation may depend upon the initial proliferative state of the cells (differentiated/quiescent vs. proliferating/immortalized) and they are required for cell cycle genes expression in the growth arrested cells, but become dispensable in continuously proliferating cells which skip the G0 phase **(Figure 35)**. This model would explain why they are dispensable for the proliferation of continuously proliferating stem or progenitor cells, but are required for the proliferation of serum deprived MEFs in culture.

Therefore in addition to the *in vivo* and cell culture difference between this and previous studies another and possibly the more relevant difference is of the cellular systems used. The previous studies which showed the essential nature of activator E2Fs in TAg induced proliferation were performed in terminally differentiated quiescent cells, while this study made use of continuously proliferating MEFs. Hence, the requirement of activator E2Fs in TAg induced proliferation may also depend upon the initial proliferative status of the cells.

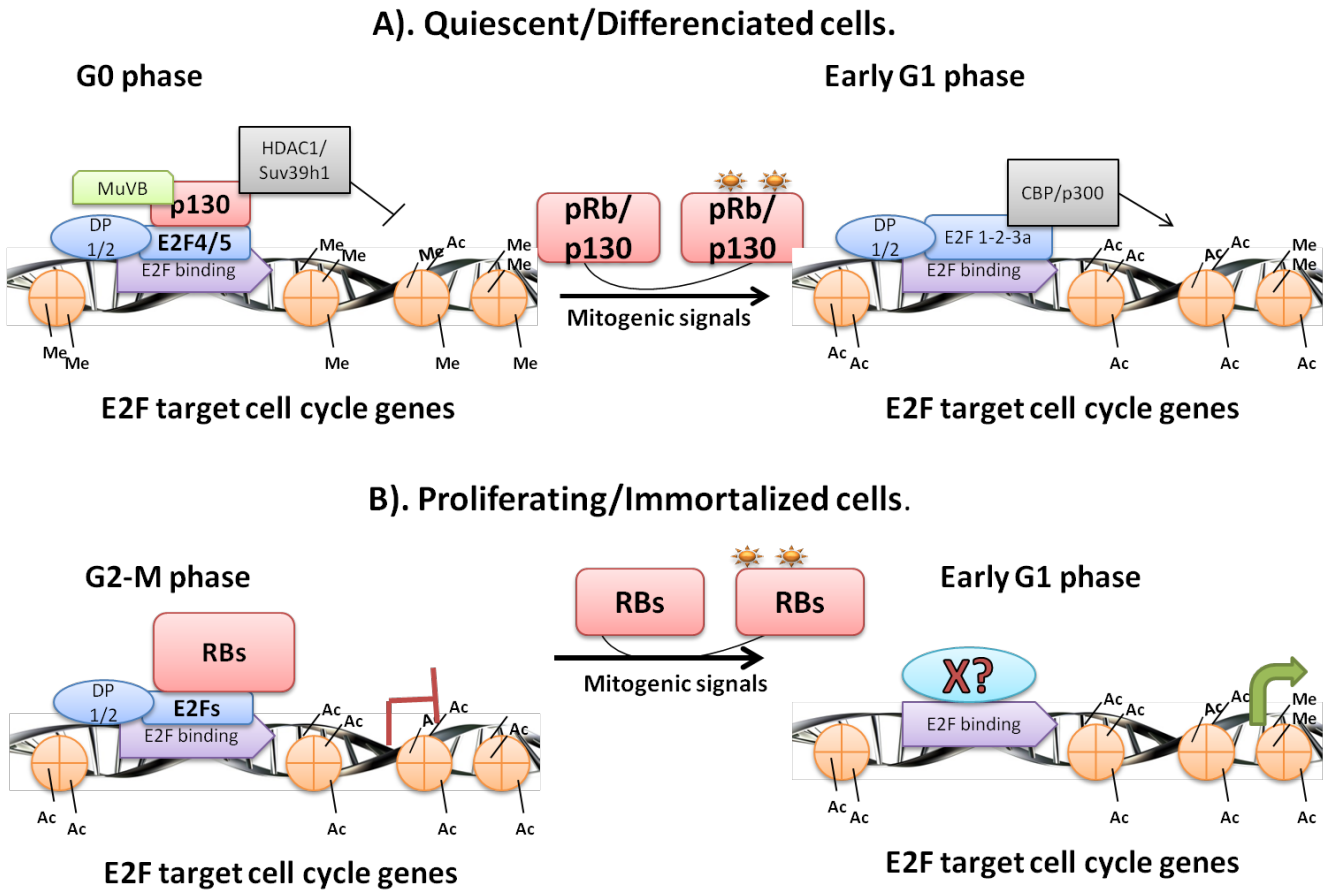
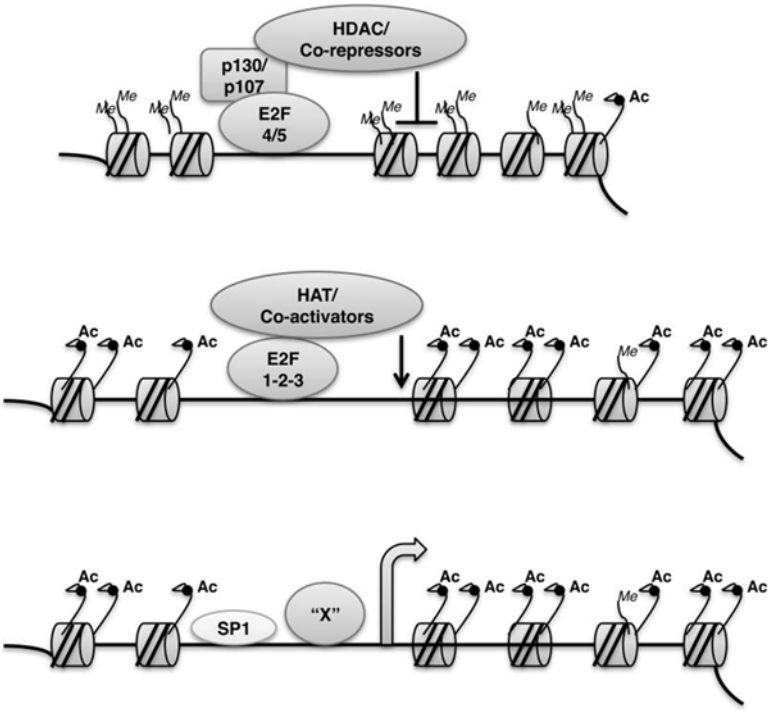

A). Quiescent/Differenciated cells.
G0 phase
Early G1 phase
HDAC1/
Suv39h1
MuVB
p130
DP
1/2
E2F4/5
E2F binding
pRb/
p130
pRb/
p130
Mitogenic signals
CBP/p300
DP
1/2
E2F 1-2-3a
E2F binding
E2F target cell cycle genes
E2F target cell cycle genes
B). Proliferating/Immortalized cells.
G2-M phase
Early G1 phase
RBs
RBs
RBs
DP
1/2
E2Fs
E2F binding
Mitogenic signals
X?
E2F binding
E2F target cell cycle genes
E2F target cell cycle genes

Figure 35: Cells proliferation state specific requirement of activator E2Fs.

A). In growth arrested cells (terminally differentiated cells *in vivo* or serum deprived cells in culture), the cell cycle genes are suppressed due to the occupation of promoters by p130/E2F4-5 containing DREAM complexes, and "repressive" histone modifications (Gonzalo, Garcia-Cao et al. 2005). For cell cycle re-entry, these two types of repressions need to be removed. Therefore, in these cell types, activator E2Fs are required to transactivate the E2F target genes, possibly via their interactions with histone modifiers. However, it is possible that they become dispensable for gene expression, once the chromatin modification is complete. B). On the other hand, continuously proliferating cells like stem cells or progenitor cells skip the G0 phase and enter the cell cycle directly from the M phase. These cells have low to undetectable levels of DREAM complexes present on the promoters of E2F target genes (Sadasivam and DeCaprio 2013) and consistent with this result the E2F target promoters contain less "repressive" histone modifications, and instead contain higher histone acetylation marks (Takahashi, Rayman et al. 2000). The E2F target gene repression in G2/M phase occurs mainly due to the promoter occupation by RB/E2F repressor complexes. Therefore, in such cell types activator E2Fs become dispensable and during the next cycle the gene expression can occur as a result of mere "de-repression" of the promoters due to RB inhibition, or it could be mediated by the presence of another transcription factor “X”.

A. **Quiescence/ Differentiation**



Cell Cycle Genes

Repression: Promoter occupation, Histone Methylation and Chromatin compaction.

De-repression (TAg requires activator E2F): Promoter clearance from repressor complexes, Histone acetylation and chromatin relaxation.

Gene Activation: Does not require activator E2Fs

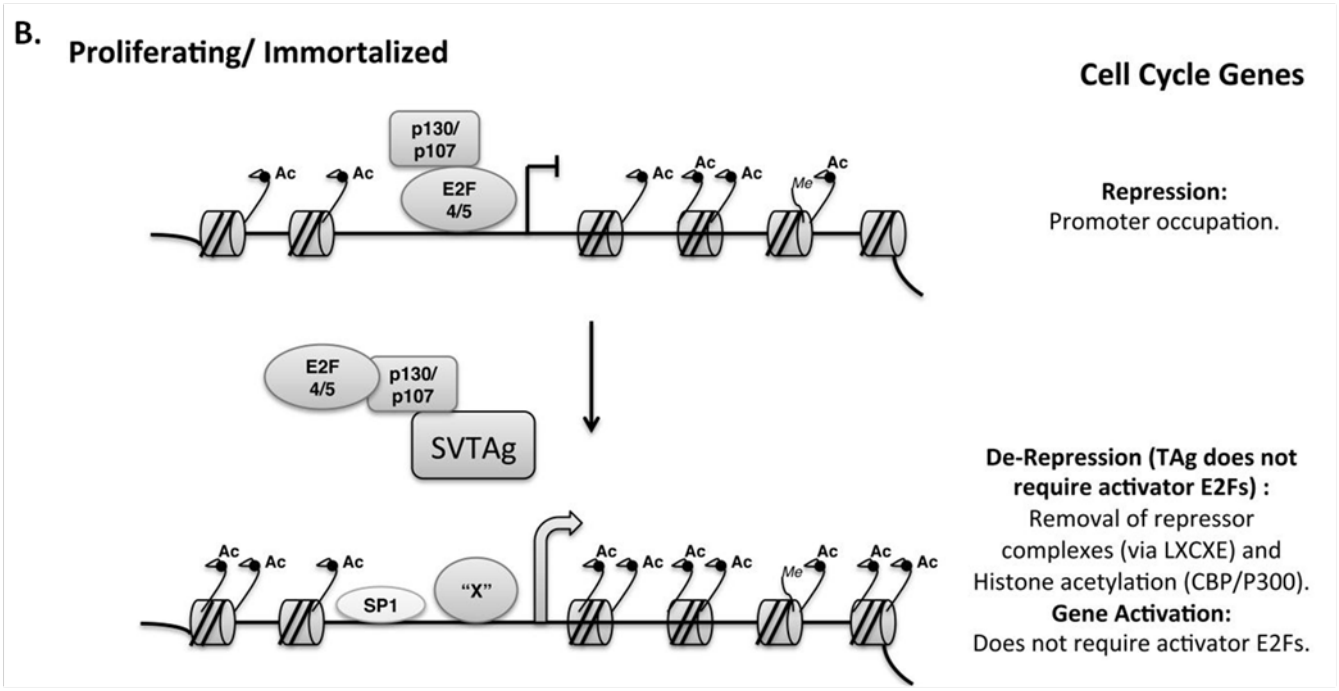

B.
Proliferating/ Immortalized
Cell Cycle Genes
p130/
p107
E2F
4/5
Ac
Me
Repression:
Promoter occupation.
E2F
4/5
p130/
p107
SVTAg
SP1
"X"
De-Repression (TAg does not require activator E2Fs) :
Removal of repressor complexes (via LXCXE) and Histone acetylation (CBP/P300).
Gene Activation:
Does not require activator E2Fs.

Figure 36: Requirement of activator E2Fs by TAg in activating cell cycle genes depend upon the initial proliferation status of the cells.

(A) Previous studies determining the role of activator E2Fs in TAg induced proliferation were performed in terminally differentiated or queiscent cells. In these cells, the E2F target genes are suppressed due to the repressive action of chromatin modifiers and promoter occupation by p130/E2F4 containing DREAM repressor complexes. Activation of E2F target genes in these cells require removal of the RB/E2F repressor complexes from promoters of these genes and transactivation of these genes by co-activators bounded to activator E2Fs. These cells therefore, require activator E2Fs to transactivate their target gene promoters by the action of activating chromatin modifiers like CBP/P300. This action of activator E2Fs is perhaps required to drive the cells out of quiescent stage of the cell cycle, and after that activator E2Fs may become dispensable.

(B) Continuously proliferating cells like stem cells or primary MEFs used in this study, do not require activator E2Fs as they skip G0 phase of the cell cycle. The E2F target genes are shut down only due to the promoter occupation by p130-p107/E2F4-5 repressor complexes, and mere removal of these repressor complexes by TAg LXCXE motif is sufficient for gene activation.

5.2 CONCLUSIONS AND DISCUSSION II

Previous studies have shown transformation of hamster cell lines expressing LPV ER and robust tumorigenesis in transgenic mice. However, no study has ever determined the different oncoproteins encoded by the LPV ER or the role and contribution of the ER products in cell transformation. In this study I have extended current knowledge of the splice products of the LPV ER and their individual contributions in cell transformation.

Previous studies detected expression of large T antigen in LPV ER expressing cells. My RT-PCR results show that the LPV ER encodes at least four different splice products: large T antigen (TAg), small T antigen (sT), a large T antigen splice form (LT', large T prime) and a small T antigen splice form (sT', small T prime). However, I detected protein expression of TAg, sT and LT' but not of sT'. This suggests either sT' protein is highly unstable or is present in amounts to low to be detected by western blot. It will be interesting to determine if these products are expressed in B-lymphoblast cells during LPV infection and their individual role in viral life cycle.

The LPV ER induces transformation in MEFs to similar levels as SV40 ER and shows similar inhibitory effects on RB/E2F and p53/MDM2 tumor suppressor pathways. While these results suggest a similar mechanism of action for SV40 and LPV ER encoded oncoproteins in cell transformation a deeper analysis of individual LPV oncoproteins showed major differences. While the TAg of SV40 is the essential oncoprotein and is sufficient for inducing the low serum growth of MEFs in cell culture, my results show that the LPV TAg requires the cooperation of LPV sT. The transforming functions of SV40 TAg are attributed to its ability to bind and inhibit RB and p53 tumor suppressor proteins and I did observe inhibition of RB/E2F and p53/MDM2

tumor suppressor pathways in LPV ER expressing MEFs. However, the contribution of LPV TAg in inhibiting these pathways is still undetermined. LPV TAg was previously shown to bind RB proteins (Dyson, Bernards et al. 1990) but its interaction with p53 protein is still unclear as different groups have provided contrasting evidences (Symonds, Chen et al. 1991, Kang and Folk 1992). The previous studies showing contrasting results regarding LPV TAg-p53 interactions could be due to the use of different cellular systems or species specificity. While one study was performed *in vivo* using transgenic mice the other study was performed in cell culture using hamster embryonic cells. More molecular and genetic analysis is required to determine the mechanism of p53 pathway inhibition in LPV ER expressing MEFs.

Another possibility for the restricted transforming ability of LPV TAg could be attributed to the expression of LT' in TAg cDNA expressing MEFs. My results show that LT' cDNA expression induces growth arrest in MEFs and initial results suggest that its growth inhibitory activity resides in its unique C-terminus region. Western blot analysis shows that LT' protein expression is similar in LPV LT' MEFs and LPV TAg MEFs, but in contrast to LT' MEFs, TAg MEFs are immortalized. On one hand this result suggests prevention of LT' mediated growth arrest by TAg. On the other hand the growth inhibitory effects of LT' may also suppress TAg transforming abilities. Further studies using TAg mutants defective in expressing LT' are required to determine the proper growth regulatory activities of LPV TAg.

The role of LPV or SV40 sT in cell immortalization and transformation is also remarkably different. While SV40 sT cooperates with TAg in transforming human fibroblasts and enhances TAg transformation phnotype in cell culture, its expression alone in primary or immortalized cells fail to induce proliferation and results into growth arrest (Andrabi, Hwang et al. 2011). Consistent with these results, I found that expression of SV40 sT in primary MEFs

results in senescence while the expression of LPV sT is sufficient to immortalize and transform MEFs. The expression of LPV sT enhances the proliferation of MEFs in normal or low serum conditions and rescues the proliferation defects of LPV TAg MEFs in low serum conditions. Both LPV sT and TAg induce similar colony sizes in soft agar. However, the size of the colonies formed are considerably reduced as compared to LPV ER expressing MEFs. These results suggest that LPV sT alone is sufficient to induce certain transformation phenotypes in MEFs. However, sT requires TAg cooperation for recapitulation of soft agar colony size observed in LPV ER expressing cells.

Initial studies suggest that sT of a recently discovered human polyomavirus MCPyV exhibits transforming functions (DeCaprio and Garcea 2013) and it has been shown to be essential for MCPyV induced transformation (Shuda, Kwun et al. 2011). However, these studies were performed in immortalized cell types. The ability of LPV sT expressing MEFs to proliferate in normal and transformation assays is particularly an important result as till date no polyomavirus sT has been shown to immortalize or transform primary cells in culture. Some of the sT biological activities are attributed to its interactions with tumor suppressor PP2A protein complex and structural and amino acid sequence comparison between homologous sT oncoproteins from different polyomaviruses shows high conservation among various motifs and domains suggesting a similar mechanism of action. However, major differences have been observed. For instance, sT's of SV40, MuPyV and MCPyV all bind to PP2A but the biological effects of their interactions are significantly different. While SV40 sT oncogenic activity depends upon its inhibition of PP2A mediated dephosphorylation of Akt, MCPyV sT mediated transformation is independent of PP2A interaction (Shuda, Kwun et al. 2011). Instead it bypasses the Akt-mTOR pathway and acts downstream to increase phosphorylation of 4E-BP1, a cap-

dependent translation regulator (Shuda, Kwun et al. 2011). In contrast, SV40 sT induces dephosphorylation of 4E-BP1 via Akt-mTOR signaling. These results show that the sT's from distantly related polyomaviruses, while interacting with the same cellular protein, can affect downstream pathways at different steps and in contrasting fashions. The phylogenetic analysis shown in **Figure 25**, places LPV closer to MCPyV than SV40 or MuPyV. Similarly, TAg of MuPy and MCPyV are also shown to lack transforming capabilities when expressed alone, and I obtained similar results with LPV TAg. However, more studies are required to completely understand the molecular pathways affected by LPV TAg and its role in LPV ER mediated soft agar growth.

5.3 IMPLICATIONS

5.3.1 What activity of TAg is sufficient to rescue the proliferation defects of TKO MEFs?

TAg interacts with the RB/E2F repressor complexes via its LXCXE motif and requires ATPase activity of J-domain bound Hsc70 to disrupt these complexes (Zalvide and DeCaprio 1995) and release E2Fs. My model suggests that in order to de-repress promoters of cell cycle genes in TKO MEFs, TAg requires an intact LXCXE motif to bind and inhibit p130/E2F4 repressor complexes. Similarly, based upon the previous studies, p53 inhibition is essential for proliferation of TKO MEFs (Sharma, Timmers et al. 2006) and T^{N136} mutant analysis in my study also supports this hypothesis. In accordance to the proposed model in this study, TKO MEFs expressing TAg mutant incapable of binding p53 (T^{D402N}) (Ahuja, Rathi et al. 2009) should fail to rescue the TKO MEFs proliferation defects, and p53 knockdown should be sufficient to induce proliferation in TKO MEFs expressing T^{N136} or T^{D402N} mutants. Similarly the TAg mutant (T^{D44N}) incapable of binding Hsc70 should also fail to rescue the TKO MEFs growth arrest and, p130 knockdown in either T^{E107K} or T^{D44N} mutants expressing TKO MEFs should be sufficient to induce proliferation.

In order to understand the role of TAg-Hsc70 and TAg-p53 interactions in the proliferation of TKO;TAg MEFs, I tried to obtain DKO MEF pools expressing T^{D44N} or $T^{C257\text{-}708}$ (TAg truncation mutant sufficient to bind p53) mutants. In both the cases DKO MEFs were

transduced with lenti viruses containing either of the TAg mutants, and drug resistant cell pools were obtained. Interestingly, even after various attempts I failed to obtain DKO MEFs expressing either of the two mutants as no protein signal could be detected upon western analysis of the cell pools.

5.3.2 Does oncoproteins from other polyomaviruses require activator E2Fs for cell transformation?

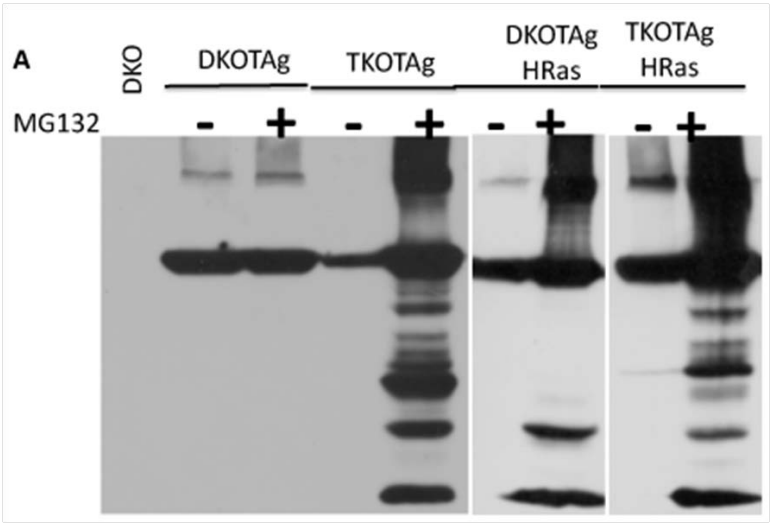
Distantly related polyomavirus have been shown to use diverse mechanisms to induce proliferation and transformation in primary cells. **Figure 25** shows phylogenetic analysis based upon sequence homology of TAg from selective polyomaviruses. In this analysis, SV40, BKV and JCV are grouped together, while MuPyV and HaPyV are clustered together, but distantly from the SV40 group. Interestingly, the essential or dominating transforming protein and the mechanism of transformation for the polyomaviruses belonging to the same group are similar, and differ from the polyomaviruses belonging to the distant group. While the TAg of SV40, BKV or JCV is the essential oncoprotein, mT is required for MuPyV or HaPyV induced cell transformation. Transformation by TAg of SV40, BKV and JCV requires interaction with both the RB and p53 proteins, while the MuPyV mT induced transformation requires activation of growth signaling pathways. While the TAg of all these viruses are shown to bind and inhibit RBs (Dyson, Bernards et al. 1990), but TAg of MuPyV or MCPyV lack the ability to bind p53. Similarly, there are contrasting evidences for LPV TAg to bind p53.

My results in this study show that SV40 TAg requires at least two activities to bring proliferation in MEFs depleted of activator E2Fs. Based upon my model shown in **Figure 33**,

inhibition of RB/E2F repressor complexes and p53 inhibition are essential for proliferation of TKO MEFs. Therefore, based upon the phylogenetic analysis and my model, I would expect TAg from BKV or JCV to induce proliferation and transformation in TKO MEFs, but the TAg from MuPyV or MCPyV would not. Similarly, it is critical to determine if polyomaviral oncoproteins other than the TAg have devised mechanisms to circumvent the requirement of activator E2Fs in cell proliferation. In addition, there is sufficient evidence to believe that cell cycle genes are regulated by alternate pathways. TAg induced activation of E2Fs by directly inhibiting RBs is one mechanism of inducing cell cycle genes, however, in the absence of activator E2Fs other pathways can get activated and thus their mode of action and regulation are essential for understanding cell cycle regulation. mT of MuPyV (Campbell, Ogris et al. 1994) and sT protein of MCPyV are shown to play essential role in cellular transformation (Shuda, Kwun et al. 2011) and my results show that LPV sT is sufficient to immortalize DKO MEFs and transform wtMEFs in cell culture. These oncoproteins do not directly interact and inhibit RB proteins, and therefore it will be of interest to determine the requirement of activator E2Fs in cell cycle gene induction and cell proliferation in cells expressing LPV sT or MuPyV mT.

APPENDIX A

DEPLETION OF ACTIVATOR E2F'S IN MEF'S AFFECTS TAG TRANSCRIPTIONAL AND POST-TRANSLATIONAL LEVELS



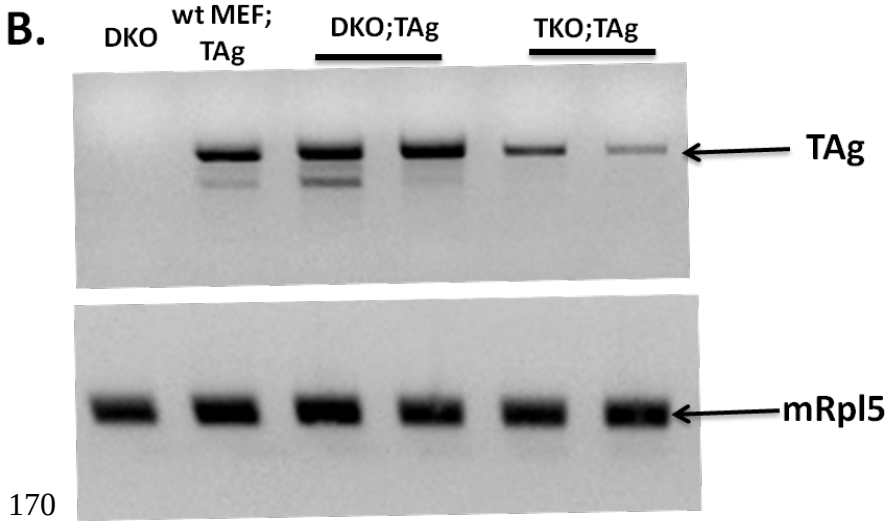
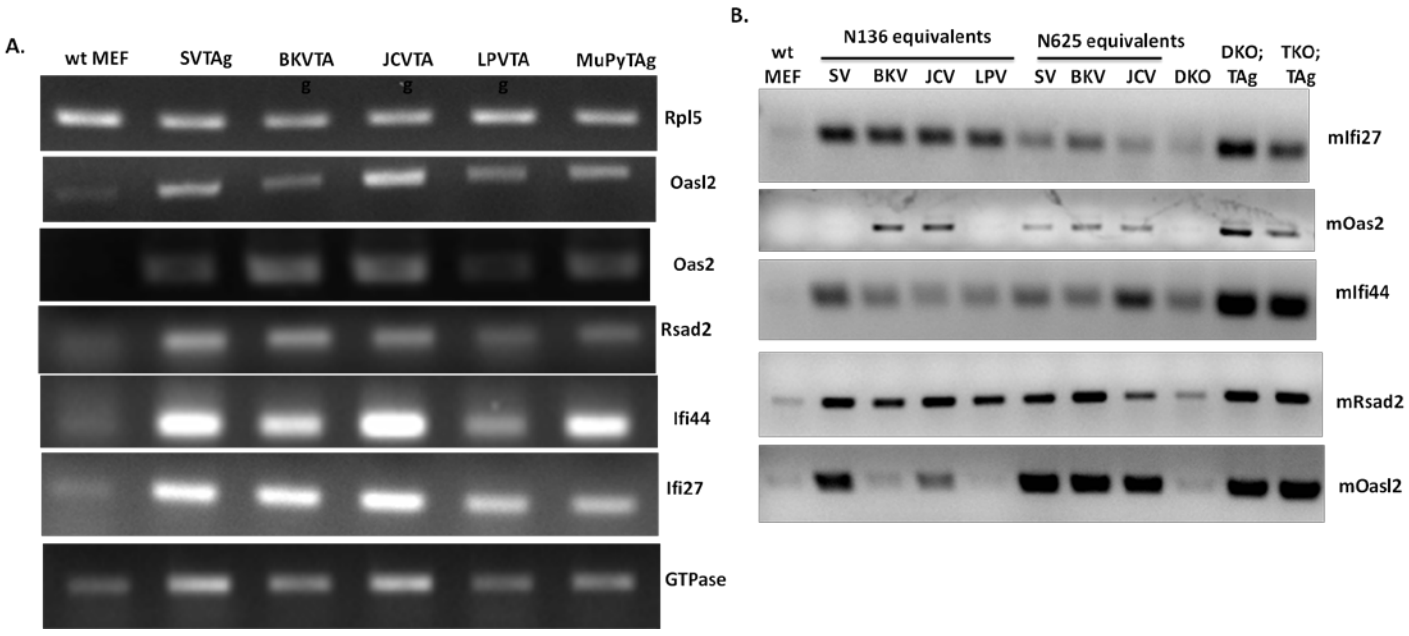


Figure 37: TAg protein and transcript expression levels are reduced in the absence of E2F1-2-3.

A). Western blot results for SV40 TAg expression in DKO;TAg, DKO;TAg;HRas, TKO;TAg and TKO;TAg;HRas MEFs treated with either MG132 or DMSO. B). Transcript level analysis of TAg expression in two pools of DKO;TAg MEFs and two clones of TKO;TAg MEFs. The DKO MEFs and wt MEFs expressing TAg (wtMEF;TAg) were used as negative and positive controls respectively. Primers used were specific for 5' and 3' ends of TAg transcript, and mouse Rpl5 was used as loading control.

APPENDIX B

EARLY REGION AND N-TERMINUS TRUNCATION MUTANT OF VARIOUS POLYOMAVIRUSES INDUCES EXPRESSION OF INTERFERON STIMUTALED GENES (ISG)



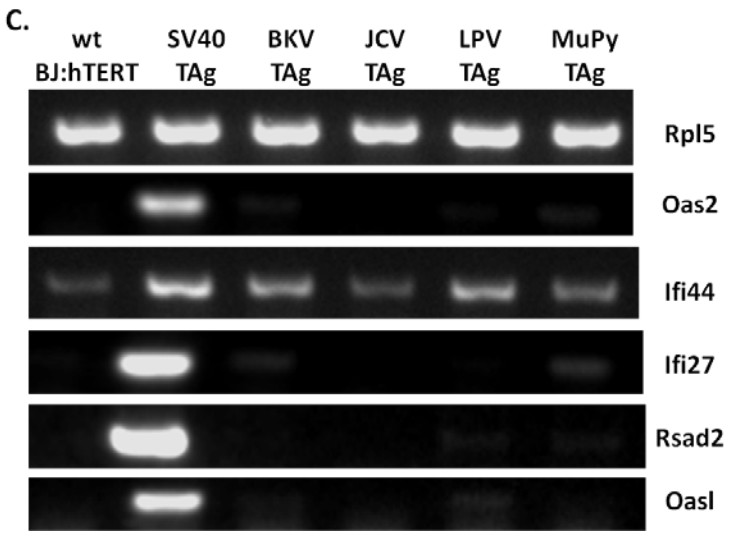


Figure 38: Polyomavirus TAg induces interferon stimulated genes

Transcript levels of different ISGs were analyzed in MEFs (A and B) and BJ;hTERT (C) cells, expressing different polyoma TAgs or their mutants (B). Two biological replicates and two technical replicates were used and the figure above shows a representation of the experimental results. Rpl5 was used as a loading control. RNA was extracted using Qiagen RNeasy kit, while cDNA were prepared using Superscript II reverse transcriptase. Equal amount of cDNA was used to perform PCR using primers specific for the different ISGs.

APPENDIX C

SCREENING OF LARGE T ANTIGEN ATPASE INHIBITOR SMALL MOLECULES AS ANTI-POLYOMAVIRAL DRUGS

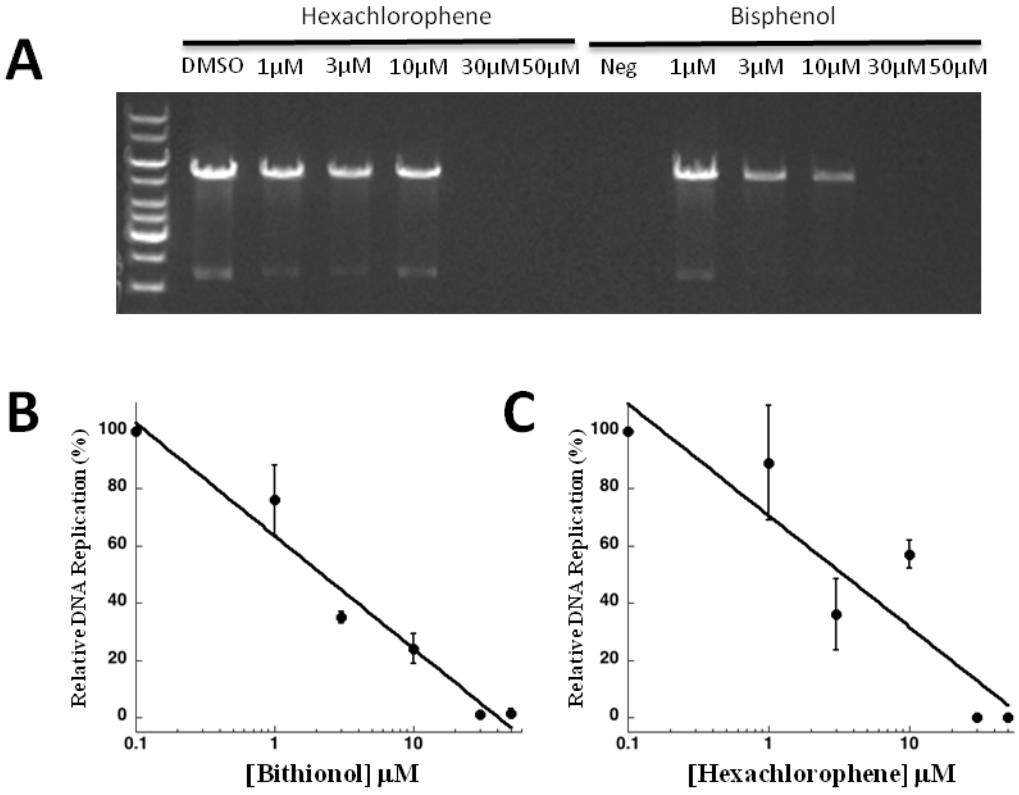


Figure 39: Small molecule inhibition of SV40 DNA replication.

(A) SV40 infected cells were treated with either DMSO or the indicated concentrations of compound, and after 48 h DNA was harvested and processed as described in the Materials and methods. An ethidium bromide-stained gel is shown. "Neg" refers to cells that were mock-infected. This analysis was performed in triplicate, and the averaged results for treatment with bithionol and hexachlorophene are shown in (B) and (C), respectively, ±SD. (Data corresponding to 0.1 mM are equivalent to the DMSO control.) Fitted data for each individual experiment were also averaged and used to obtain an EC-50, which for biothionol and hexachlorophene were 2.2±0.51 mM and 3.2±0.55 mM, respectively

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