COMPARATIVE ANALYSIS OF POLYOMAVIRUS T ANTIGENS IN CELLULAR TRANSFORMATION

by

Nicole Theresa Marie Seneca

B.S. Molecular Biology and Microbiology, University of Central Florida, 2007

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This dissertation was presented

by

Nicole Theresa Marie Seneca

It was defended on

September 18, 2014

and approved by

Neal A. DeLuca, Ph.D., Professor, Microbiology and Molecular Genetics

Paula Grabowski, Ph.D., Professor, Biological Sciences

Paul R. Kinchington, Ph.D., Professor of Ophthalmology, Molecular Genetics and

Biochemistry

Saleem A. Khan, Ph.D., Professor, Microbiology and Molecular Genetics

Dissertation Advisor: James M. Pipas, Ph.D., Herbert W. and Grace Boyer Chair in

Molecular Biology, Professor, Biological Sciences

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Polyomaviruses can induce tumors in animals and transformation of cells in culture by expression of the viral T antigen proteins. The most widely studied polyomavirus, SV40, expresses the large T antigen protein (LT), which interacts with many cellular proteins to transform cells. Several other polyomaviruses also express a LT protein that is required for transformation; however, previous research has hinted that these polyomavirus LTs may not transform cells by the same mechanisms. The aim of this work is to uncover the differences in molecular mechanisms of transformation by LTs of two human polyomaviruses, BKV and JCV, and a primate polyomavirus, LPV, as compared to SV40 LT.

The LT proteins contain discrete structural domains that are also associated with different functions of the protein. These domains can be removed to isolate individual functions without greatly altering those functions. Expression of a short fragment of the LT antigens known to interact with the cell cycle regulating family of pRB proteins showed that this region confers the same transformation capabilities between the four polyomaviruses. The BKV and JCV fragments, however, also conferred a growth advantage in the dense focus assay, suggesting additional activities of this region of BKV and JCV LT other than pRB inhibition. In a separate line of investigation, a region of LT that is only present in SV40, BKV, and JCV LTs known as the variable linker and host range region (VHR) was removed. Very little is known about how this region of LT effects transformation. Transformation assessed by growth in low serum was reduced by VHR truncation of the SV40, but not the JCV, LT. Conversely, anchorage

independent transformation was enhanced only by truncation of the JCV VHR. This is the first report to link the SV40 or JCV VHR region to transformation potential. Expression of the both VHR LT truncations resulted in changes in T antigen expression and cellular p53 post-translational modifications but interaction with the pRB pathway was unaffected. Future studies will focus on identifying the cellular protein interactions with each LT domain that contribute to the differences in transformation identified here.

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PREFACE

Many people have helped get me to this point, whether they know it or not. To my mother, I have always looked to you as a model of resilience and persistence. You have shown me that no matter how bad it gets, you must keep your chin up and focus on the future. To my father, your support has been invaluable.

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1.0   INTRODUCTION

Polyomaviruses (PyV) are part of a group of viruses known as DNA tumor viruses. Tumor viruses can induce cancer in their natural hosts or in experimental animal systems. To date, all of the mammalian PyVs that have been tested, a total of 8, have this property. Some only induce tumors in non-host animal models, while others can induce tumors in the native host *and* animal models. In humans, only one PyV has been shown to be a causative agent of cancer, others can cause diseases in immunocompromised patients that are un-related to cancer, while most of the discovered human PyVs have no known role in disease. The viral proteins that are responsible for tumor formation are also critical for other diseases induced by PyVs, and they are required for normal replication. Thus, the study of the mechanisms of induction of tumors by PyV proteins provides insight into how a normal cell becomes a tumor cell, and these mechanisms may also be important in other PyV diseases and for the basic biology of the virus.

1.1   POLYOMAVIRUSES AND DISEASE

To date, 101 polyomaviruses (PyVs) have been found in an array of mammalian species, as well as birds. Most of these viruses have *not* been associated with disease; however, the remaining few PyVs can produce severe disease in the unfortunate host. Disease is commonly associated with suppression or underdevelopment of the immune system. In addition, with the exception of

one PyV (Merkel cell polyomavirus), tumors do not develop in the natural hosts under natural conditions.

Polyomaviruses have been detected in many mammals; however, only six are the causative agents of disease. Hamster polyomavirus and murine polyomavirus (MuPyV) both induce tumors in younglings of the same species (Habel, 1963; Scherneck et al., 2001); though, this is mainly seen in the laboratory and not in wild populations (Gottlieb and Villarreal, 2001; Rowe, 1961). Two human PyVs, BK polyomavirus (BKV) and JC polyomavirus (JCV), replicate to high levels in immune-suppressed transplant patients and patients with acquired immune deficiency syndrome (AIDS) resulting in pathologies involving severe tissue destruction. Human tricodysplasia spinulosa-associated polyomavirus (TSPyV) causes spike-like growths on the skin of transplant patients. The recently discovered human Merkel cell PyV was found to be a causative agent of a rare skin cancer known as Merkel cell carcinoma. This is the first of the human PyVs to be validated as a cause of cancer in humans. BKV, JCV, TSPyV, and MCPyV are discussed further in section 1.4.

Bird polyomaviruses are distinct from the other PyVs in their relation to disease. Infection with two bird PyVs has been shown to cause acute disease of young birds and is not associated with a compromised immune system (reviewed in Johne and Muller, 2007). Budgerigar fledgling disease virus is the causative agent of the disease of the same name. It is fatal in young Budgerigar parrots, causing hepatitis and accumulation of fluid between organs. Young parrots that survive have chronic disease mainly characterized by feather disorders. Goose hemorrhagic polyomavirus (GHPyV) can cause hemorrhagic nephritis and enteritis of goslings resulting in sudden death. Other bird polyomaviruses have been discovered in tissues of

diseased birds (Bennett and Gillett, 2014; Halami et al., 2010; Johne et al., 2006); however, causal links between these viruses and disease have not been tested.

1.2 POLYOMAVIRUS LIFE CYCLE

Initial infection by PyV is asymptomatic and produces a viremia that allows the virus to infect specific tissues where it becomes latent. It is not clear if this is true latency, with no replication and limited to no viral gene expression, or if the infection is persistent with low levels of replication. Reactivation of viral replication can occur with no symptoms in healthy individuals. PyV infections are kept in check by the immune system, as PyV-associated disease or tumors that develop in the natural host occur with high incidence in immunocompromised individuals.(Kean and Garcea, 2009)

A productive PyV infection begins with the attachment of the virion to specific cell surface receptors followed by internalization through endocytosis. The virion is transported to the nucleus where the genome is released. The genome of PyV is a circular dsDNA of about 5 kilo-base pairs wrapped around cellular histones to create a mini chromosome (Yaniv, 2009). A non-coding region physically separates and controls the expression of the early and late transcriptional regions (Figure 1A). The non-coding region contains promoters for transcription of viral genes, a bi-directional enhancer, the origin of replication, and the viral packaging signals. Host RNA polymerase II transcribes PyV early mRNAs. Several early transcripts can be produced by alternative splicing, but all PyVs have been shown to make a large T antigen (LT) and small T antigen (sT) (Figure 1B). PyVs infect non-dividing, differentiated cells, so the early LT and sT proteins bind to cellular proteins to push the cell into S phase when cellular proteins

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for DNA replication, chromatin assembly, and nucleotide metabolism are present. LT is also required in all PyVs to initiate viral genome replication, to act as a helicase during replication (as a LT hexamer), and recruit host DNA replication proteins to the viral genome (Figure 2). Transition of transcription of the early region to the late region is dependent on at least two factors: 1) LTs ability to stimulate late region transcription (Keller and Alwine, 1984), and 2) the replication-dependent titration of cellular proteins that inhibit late region transcription (Liu and Carmichael, 1993; Wiley et al., 1993). The late region produces the viral capsid proteins allowing new PyV progeny virions to be assembled, which are eventually released from the cell.

For PyVs, tumorigenesis does not benefit the virus and is the result of an infection that does not end in production of virus progeny. Tumorigenesis, therefore, is an accident, brought on by the continued expression of polyomavirus T antigen proteins that were meant to reprogram the cell for virus production. Accordingly, expression of the T antigen proteins independent of the virus also induces tumorigenesis in animal models and transformation of cells (Grossi et al., 1982; Jat and Sharp, 1986; Saenz Robles and Pipas, 2009).
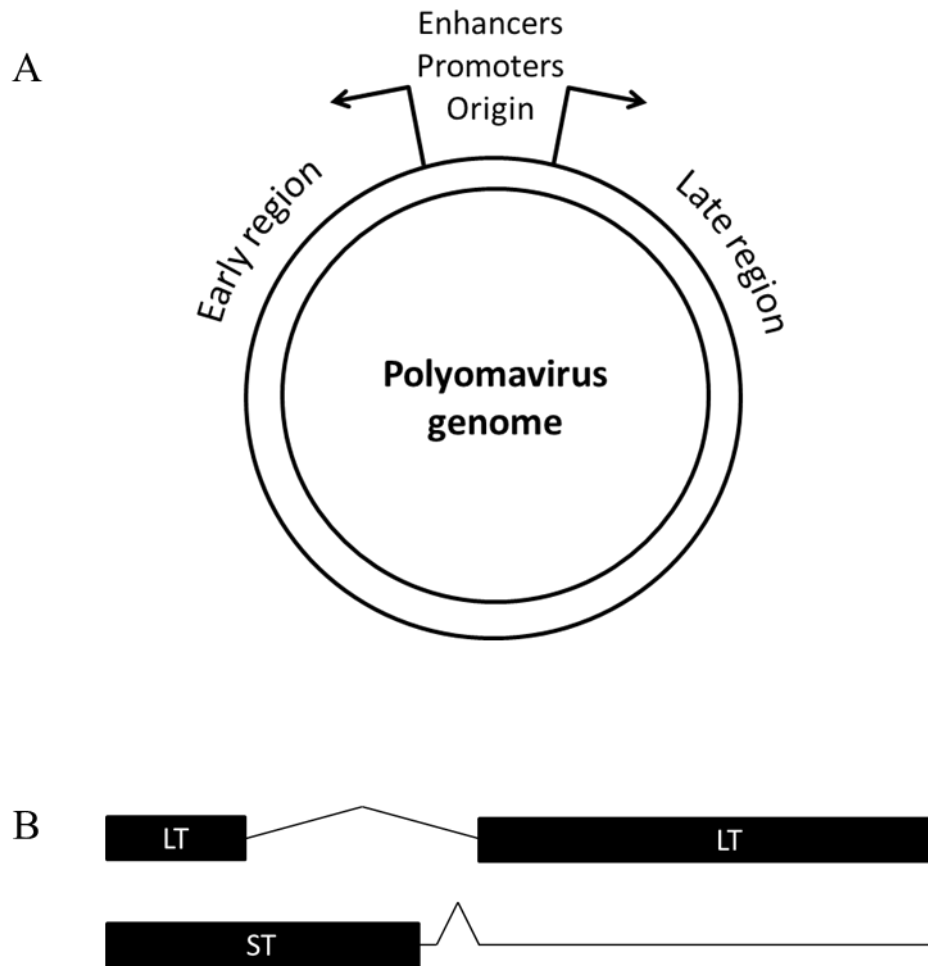
Figure 1. Common features of polyomavirus genomes.

A) PyV genomes contain a non-coding region, early transcriptional unit, and late transcriptional unit. The non-coding region contains bi-directional promoters for both early and late region transcription, enhancer elements, and the viral origin of replication. B) All polyomaviruses produce a large T antigen (LT) and small T antigen (sT) transcript by alternative splicing of the early region pre-mRNA. LT is created by removal of an intron near the 5' end. sT reads through the 5' splice site for the LT intron and uses an alternative 5' splice site within the LT intron to remove a smaller intron. The small T stop codon is found before the small T intron or directly after the sT intron. Black bars represent exons, carets symbolize introns, and black lines signify non-translated regions of the transcript.
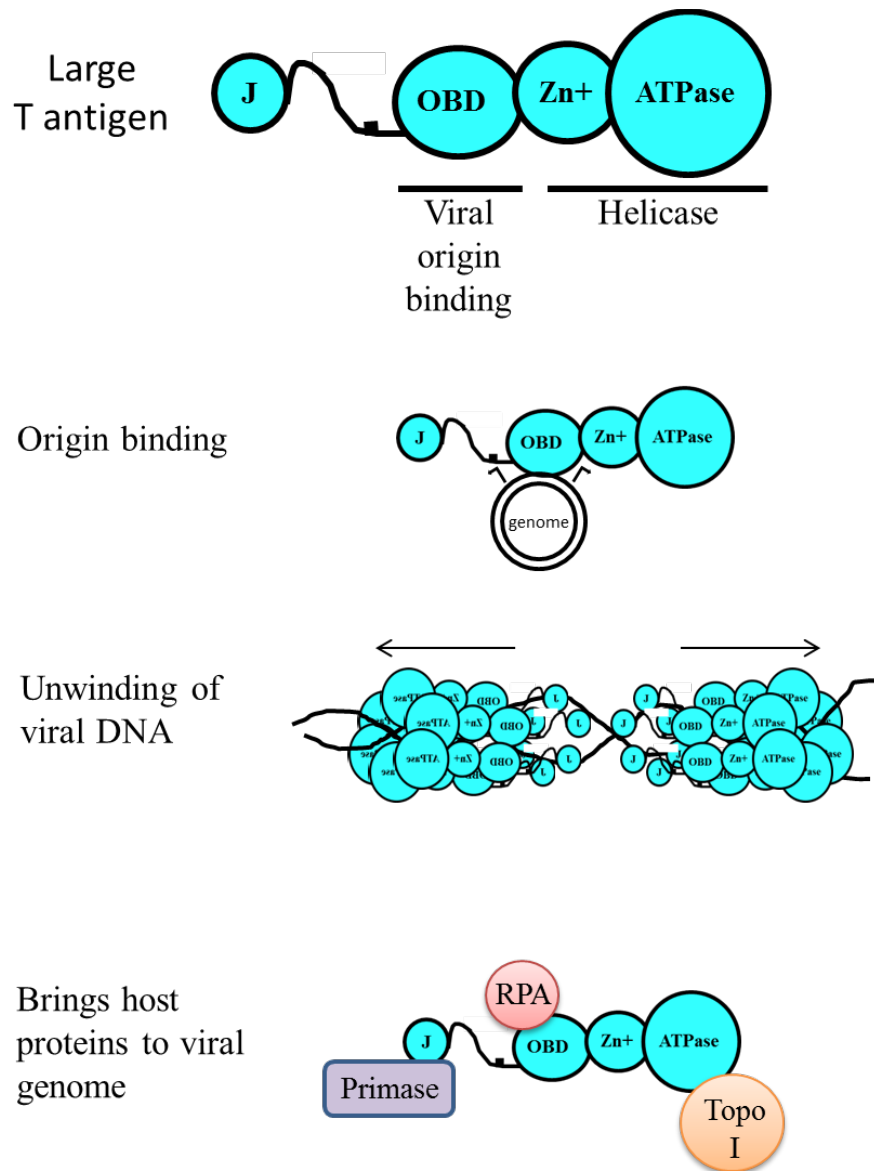
Figure 2. Functions of polyomavirus large T antigen in replication of the viral genome.

Polyomavirus large T antigens have a conserved domain structure. The origin binding domain recognizes and binds to sequences within the non-coding region of PyV genomes. This facilitates formation of two LT hexamers on the viral DNA facing in opposing directions. The ATPase domain and zinc-binding domains are required to form the helicase function that allows unwinding of the viral DNA during replication. Each hexamer is a separate helicase. The LT protein also recruits host replication factors to the viral origin which form the replisome. Cellular proteins that interact directly with SV40 LT are shown.

1.3 THE CONVERGENCE OF POLYOMAVIRUS T ANTIGENS AND MECHANISMS OF CANCER

A malignant tumor is a complicated thing. Many auxiliary mutations have already occurred at this point because several of the cellular systems that maintain the integrity of the genome have been compromised. Therefore, although there is much to be learned from large scale tumor sequencing projects like The Cancer Genome Atlas, this data is generally not useful to understand the essential mutations at the initial stages of development since samples are generally taken from late stage tumors. In addition, the critical altered pathways within the cell that are required to maintain the cancerous state cannot be readily deduced from the complicated picture of a fully developed tumor. Introducing individual mutations found in cells from these tumors into a cell culture system to understand the effects of each is tedious. PyV T antigen induced transformation and tumorigenesis offers a controlled way of taking a normal cell and making it cancerous.

PyV T antigens reprogram cells by binding to proteins that are key regulators of cellular signaling networks. Many of these signaling networks are also perturbed in cancer cells. In fact, several of the outcomes of reprogramming of cells in cancer and by PyV T antigens overlap (Figure 3), and many cellular proteins directly affected by PyV T antigens are found to be mutated or otherwise altered in cancers. For example, many PyV LT proteins inhibit the functions of the tumor suppressors pRB and p53, which are also frequently mutated in cancers (reviewed in Di Fiore et al., 2013; Olivier et al., 2004). The result of inhibition of these proteins leads to proliferative signaling and evasion of cell death in T antigen expressing cells and cancer cells.

The PyV T antigens reveal different combinations of pathways that, when disturbed, lead to transformation. As discussed in more detail in sections 1.5 and 1.6, the T antigens of the two most well studied polyomaviruses, SV40 and Murine PyV, interact with different sets of cellular pathways to illicit transformation and tumorigenesis. And, it is possible the T antigens of newly discovered polyomaviruses will promote transformation in ways that have not been seen previously. Discovering the mechanisms of these polyomavirus T antigens will give us a better understanding of the many ways a single cell can transform to a cancerous state.

1.3.1   How does transformation relate to tumorigenesis?

Transformation, in this dissertation, refers to the ability of a cell in culture to grow and proliferate in conditions where it otherwise would not. Several *in vitro* assays have been used to determine transformation of cultured cells by PyV T antigens. Each is affiliated with a distinct characteristic of growth that is not found in "normal" cells, but can be seen in cells isolated from tumors or expressing different oncogenes. While some of these assays create conditions that may be similar to some tumors, it is not always clear how these *in vitro* situations directly relate to the *in vivo* environment. Yet, transformation assays may be used as predictors of tumorigenic capability as discussed at the end of this section. Furthermore, it appears that these assays are distinct in that the ability to transform in one assay does not necessarily mean that transformation will occur by another assay.

Cells taken directly from the tissue of an organism generally have a limited lifespan in culture. After a given number of population doublings these "primary" cells will go into a crisis where they cease proliferation and begin to die. After expression of a mutated cellular protein or viral transforming protein, the lifespan of the cells can be tested by serial passaging in culture.

Cells that can be continually passaged without ever reaching crisis are termed **immortal**. Sometimes the cells are greatly delayed in the time to crisis and the mutation or viral protein expressed is said to have conferred an **extension of life** to the cells. Due to the length of time required to perform the transformation assays described below, immortalization or extension of life is a common prerequisite.

Dense focus formation assays look for transformation in the form of cells that continue to grow even after a monolayer of cells has formed on a tissue culture plate, resulting in dense clusters of cells, or foci. This transformation phenotype is termed loss of contact inhibited growth. Tumor cells can also acquire the ability to ignore normal inhibitory growth signals, allowing them to grow to high numbers.

Another form of transformation involves testing the ability of cells to grow in a nutrient-limited environment. This is most often achieved by limiting the amount of serum in culturing medium; therefore the assay is generally referred to as **growth in low serum**. Nutrient deprivation is also seen in stages of some tumors before angiogenesis creates new blood vessels (bringing fresh supplies of nutrients). Therefore cells of early stage tumors must evolve to grow in the nutrient limited environment.

Most cells in culture require attachment to a solid surface (i.e. a plastic tissue culture dish) to proliferate. When these cells are suspended they will not proliferate, but can still survive; transformed cells, on the other hand, will continue to proliferate in suspension to form colonies. Suspension is commonly achieved by mixing normal growth medium with a low percentage of agar. This transformation assay is termed the **soft agar** or **anchorage independence** assay.

Finally, cells that achieve transformation by one of the assays previously described are tested for the **ability to form tumors in nude mice**. Transformed or non-transformed cells are

injected into immunocompromised mice and tumor development is monitored over several weeks or months; fully transformed cells will form tumors within a few weeks, while non-transformed or partially transformed cells will not form tumors.

Although these assays attempt to simulate "body-like" conditions, the growth and manipulation of cells in a petri dish fundamentally alters the properties of that cell. Therefore, it cannot be said with complete certainty that transformation resulting from manipulation of a cell in culture is directly related to what happens to form a tumor cell. For a further discussion of the "cellular uncertainty principle" see (Ahuja et al., 2005). Nevertheless, transformation of cells in culture has been shown to be a good predictor of tumorigenic capability. Two observations support this statement. First, cells that were transformed *in vitro* also formed tumors in multiple publications that tested cells expressing T antigen for transformation with either the dense focus or soft agar assays and formation of tumors in nude mice (Hahn et al., 2002; Nguyen et al., 2014; Schuurman et al., 1992; Soejima et al., 2003; Yeh et al., 2004). Second, cell culture transformation has been used for decades to determine the carcinogenic nature of chemical compounds with a good rate of success (Combes et al., 1999; Creton et al., 2012). Thus, *in vitro* transformation remains an useful way to study the molecular disturbances that create a cancer cell.
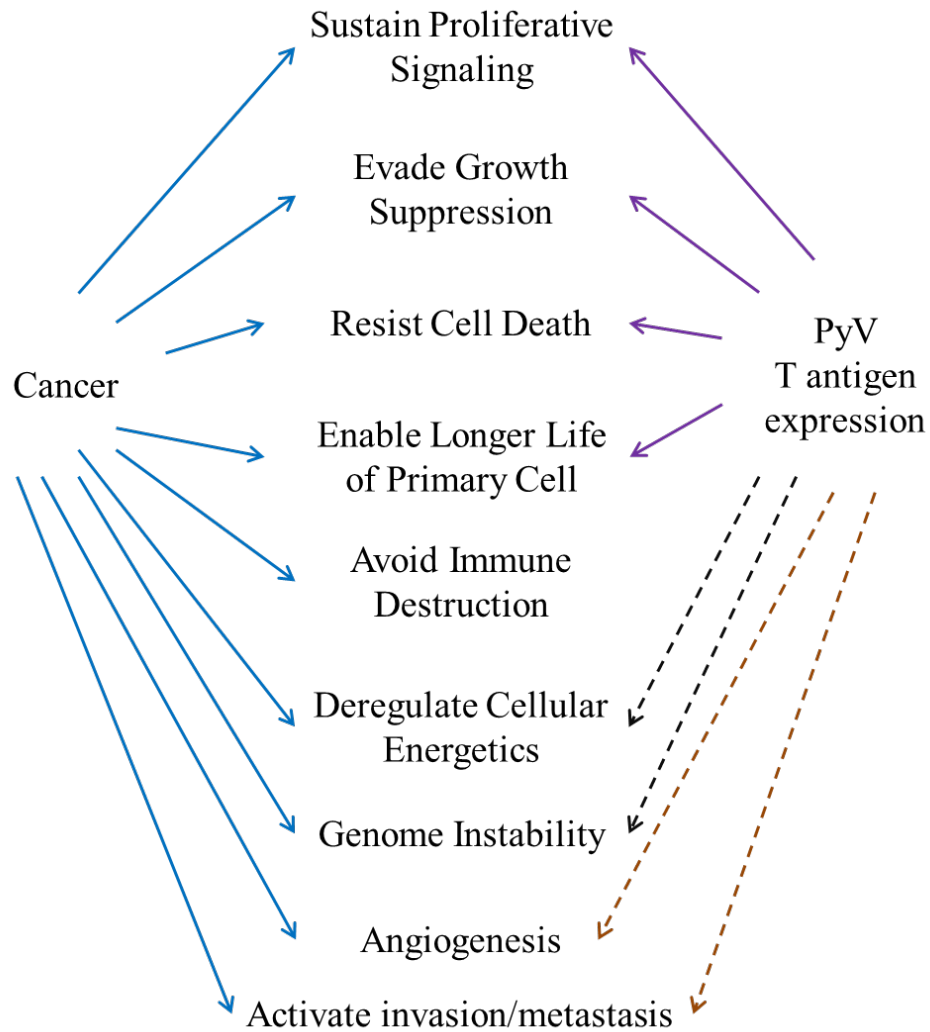
Figure 3. Cancer and PyV T antigen expression reprogram cells resulting in similar outcomes.

Decades of study of cancer and tumorigenesis revealed several defining features that are accumulated in cells of tumors (blue arrows). T antigens of polyomaviruses reprogram cells to proliferate and avoid death signals during infection and transformation similar to cancer cells (purple arrows). T antigen transformed cells in culture and from tumors also display genome instability and deregulation of cellular energetics (black dashed arrows). T antigen induced tumors also require angiogenesis and can metastasize in some models (brown dashed-arrows).

1.4   HUMAN POLYOMAVIRUSES

Thirteen polyomaviruses have been identified in humans, eleven of which have been discovered in the past seven years. Studies indicate that initial infection with PyVs occurs early in life, and that seroprevalence of human PyVs in the adult population can be from 35% to 92% (reviewed in DeCaprio and Garcea, 2013). Overall, the genomes of human PyVs are similar to the canonical primate SV40 polyomavirus in that they all appear to code for at least the large and small T antigen proteins in the early region and three capsid proteins in the late region. Several of the human PyVs however, are dissimilar in sequence and protein expression compared to both SV40 and other human PyVs. In particular, the LT proteins of the 13 human PyVs differ enough from each other (Figure 4) to suggest that their ability or mechanisms to induce tumors may also vary. Investigation of each human PyV's transformation potential in cell culture could lead to new insights into pathways that are involved in transformation of the normal cell to a cancerous cell.

BKV and JCV were the first human PyVs identified both in the early 1970s. For complete reviews of BKV and JCV disease and epidemiology see (Hirsch et al., 2013; Rinaldo et al., 2013). BKV was isolated from the urine of a patient who had undergone transplantation surgery several months prior (Gardner et al., 1971). BKV causes disease in 1-15% of transplant patients mainly stemming from viral replication in the kidneys or bladder. The two most common diseases are polyomavirus-associated nephropathy (PVAN) in kidney transplant patients and polyomavirus-associated hemorrhagic cystitis in stem cell transplant patients. The recommended treatment is to simply stop immunosuppressive drugs to allow the immune system to combat the virus; however, this could also lead to rejection of the transplant. JCV, on the other hand, was first found in the brain of a patient with Hodgkin's disease and progressive multifocal leukoencephalopathy (PML) (Padgett et al., 1971). Since then, it has been shown that JCV

reactivation is generally found in central nervous system tissues and the kidney. JCV has been linked to PML in patients with various immune system dysfunctions (e.g. AIDS, immune suppressive drugs due to transplant or rheumatoid arthritis). JCV can also be a causative agent of PVAN, but it is less common than BKV associated PVAN. Safe and effective antiviral therapies for illness caused by both viruses are lacking.

BKV and JCV viral DNA and, in some cases, LT protein have been detected in many human tumors, leading to the question of whether these viruses are involved in human cancer (Coelho et al., 2010; Hachana et al., 2012; Moens et al., 2014). The answer is not clear cut since BKV and JCV DNAs can be detected in both tumor and non-tumor samples in some studies, and because of the high seroprevalence and detection of these two viruses in healthy people (reviewed in Dalianis and Hirsch, 2013). One emerging hypothesis is that human PyVs can act as co-factors in human tumor progression as compared to a direct cause and effect role seen with JCV and PML. Moens et al. have proposed that human PyVs may act as co-factors to an additional viral infection in the same cell/tissue, for example Epstein-Barr herpesvirus or human papillomavirus infections, and they speculate that the products of the two viruses could work together to promote oncogenic transformation. Alternatively, Coelho et al. argue that JCV viral proteins act as cofactors for colorectal cancer by facilitating and/or enhancing key mutations of cellular proteins to promote tumorigenesis at different stages of tumor progression.

The human Merkel cell polyomavirus (MCPyV) was first identified *in silico* in 2008 using the digital transcriptome subtraction technique from a database of normal and cancerous tissues (Feng et al., 2008). MCPyV DNA has since been found in 80-97% of all Merkel cell carcinomas, and several lines of evidence now indicate that MCPyV is the first human oncogenic polyomavirus (reviewed in Spurgeon and Lambert, 2013). Merkel cell carcinoma (MCC) is a

rare skin disease and old age and immune system compromise are both risk factors, suggesting that immune surveillance is important in keeping MCPyV in check. The MCPyV early region produces at least four T antigens, and it is quite divergent from SV40, BKV, or JCV. Furthermore, the early regions of MCPyV found in MCC are mutated in a way that prevents expression of the full length LT and other T antigen proteins (Cheng et al., 2013; Shuda et al., 2008). This immediately suggests a difference in transformation strategy compared to other well characterized PyVs. Indeed, several reports on the transforming abilities of the four T antigens make it quite clear that this virus is unique (reviewed in Spurgeon and Lambert, 2013; Stakaitytė et al., 2014).

Tricodysplasia spinulosa-associated polyomavirus (TSPyV) causes a skin disease of the same name in severely immunocompromised hosts (van der Meijden et al., 2010). TSPyV is rarely detected in the skin of healthy people but seroprevalence is high suggesting that the skin may not be the main reservoir for the virus. Viral load is high in diseased tissue, and instead of tissue destruction like with BKV and JCV disease, TSPyV replication produces hyper proliferation of specific hair follicle cells which culminate in spiny follicular papules on the face, neck, and trunk of the body. Currently, no studies have looked at expression of the TSPyV early region or individual T antigen proteins in cell culture to ascertain the transforming potentials. (Kazem et al., 2013)

The first new human polyomaviruses to be discovered in over 30 years since the isolation of BKV and JCV were the Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV) (Allander et al., 2007; Gaynor et al., 2007). Both viruses were identified using high-throughput sequencing methods on DNA from respiratory samples, and have since been detected in respiratory tract, stool, and blood samples from every continent

(Babakir-Mina et al., 2013). There has been no clear evidence yet to associate WU and KI PyV infection with disease in healthy or immune-compromised people. By sequence, WU and KI PyVs are more closely related to each other than BKV, JCV, or SV40 (Figure 4 and (Gaynor et al., 2007)). The transforming properties of the T antigens from WU and KI have not been investigated; although, one study showed association of tagged WUPyV T antigen proteins with cellular pRb and p53 by tandem affinity purification coupled with mass spectrometry (TAP-MS) (Rozenblatt-Rosen et al., 2012).

Human polyomaviruses 6 and 7 (HuPyV6, HuPyV7) were isolated from skin swabs of healthy patients (Schowalter et al., 2010), and appear to create a new phylogenetic branch of the polyomavirus tree as do WU and KI PyVs (Figure 4 and (Schowalter et al., 2010)). They have not been associated with any disease to date, but appear to have a high seroprevalence. The T antigens of these viruses have not been investigated for their transforming capabilities, but one study found that HuPyV6 T antigens associate with p53 and pRb by TAP-MS, while HuPyV7 only showed an association with pRb (Rozenblatt-Rosen et al., 2012).

Several other polyomaviruses have been found in various human samples, none of which have yet been shown to directly cause a human pathology. The New Jersey polyomavirus (NJPyV) complete genome was uncovered by high-throughput sequencing from a skin lesion of a transplant patient, and it is the latest polyomavirus discovery (Mishra et al., 2014). Human polyomavirus 12 (HuPyV12) was isolated from resected liver tissue and subsequently detected in intestinal tissue samples as well as one stool sample (Korup et al., 2013). Preliminary seroprevalence studies showed 15-33% of adults tested were positive for HuPyV12 antibodies (Korup et al., 2013). Human polyomavirus 9 (HuPyV9) was discovered by high throughput sequencing and pieced together by nested PCR of multiple blood and urine samples (Scuda et al.,

2011). This virus is most closely related to a primate polyomavirus, lymphotropic papovavirus (LPV), which is known to have a tropism for B lymphocytes and is a potent transformer in animal models and cells culture. HuPyV9 T antigens have yet to be assessed for transformation potential. Malawi polyomavirus (MWPyV, other isolates are known as Mexico polyomavirus (MXPyV) and human polyomavirus 10 (HuPyV10)) and Saint Louis polyomavirus (STLPyV) were isolated from human stool samples (Buck et al., 2012; Lim et al., 2013; Siebrasse et al., 2012; Yu et al., 2012). A small study showed that 23-75% of people tested positive for reaction to MWPyV depending on age (Berrios et al., 2014). The same study also performed preliminary experiments that suggest association of MWPyV T antigens with cellular proteins similar to SV40 T antigens, but unlike SV40 T antigens, those of MWPyV did not transform human cells in culture.
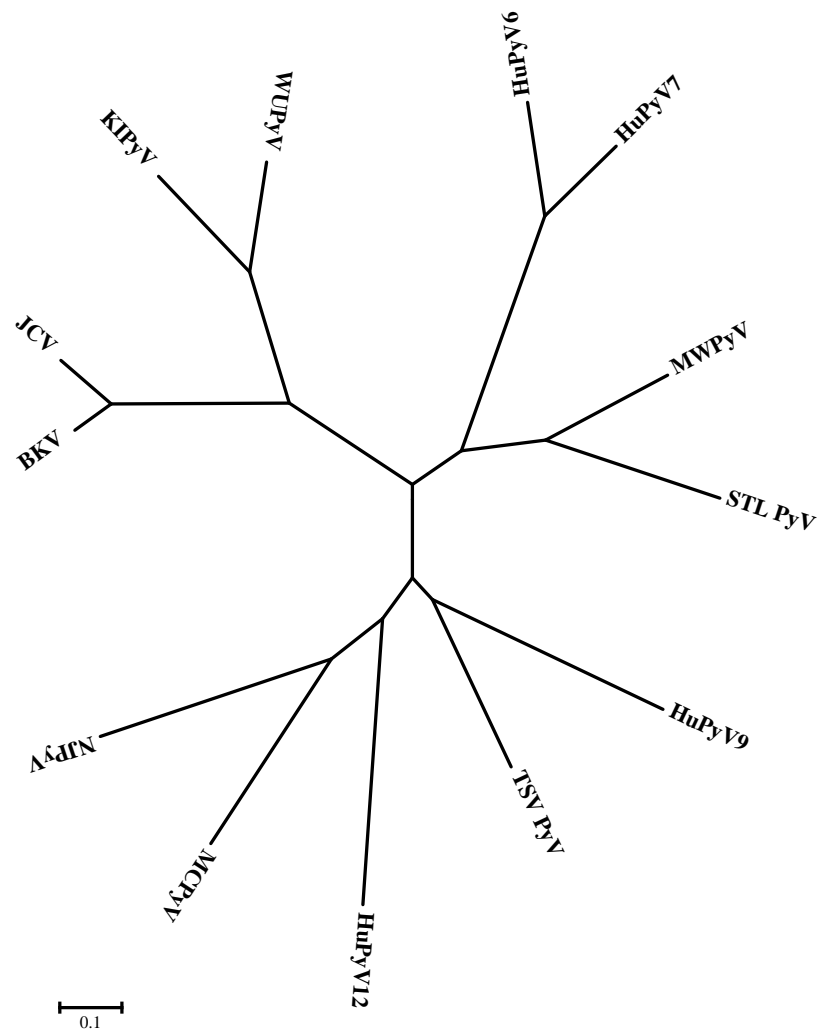
Figure 4. Evolutionary tree of human polyomavirus large T antigens.

Human PyVs LT sequences are diverse, which may suggest unique ways of inducing transformation. An alignment of all 13 human PyV large T antigens was performed using Muscle. The alignment was used to build a maximum likelihood phylogenetic tree with default parameters in the MEGA 6 software package.

1.5   THE SV40 LARGE T ANTIGEN MODEL OF TRANSFORMATION

The SV40 polyomavirus, which naturally infects species of Asian macaques, was discovered as a contaminant of primate cell lines used to create the polio vaccine (Sweet and Hilleman, 1960). This virus produces three T antigen species: LT, sT, and 17kT (Figure 5). The LT of SV40 is sufficient to induce full transformation in most established cells and tumors in transgenic models, while some systems require both the LT and sT (e.g. normal human cells). In general, two human PyVs, BKV and JCV, follow suit with both the structure and mechanisms of transformation seen with SV40.

The SV40 LT is a 708 amino acid phosphoprotein with distinct structural domains (Figure 6). The J domain is a conserved DNAJ co-chaperone, containing the required HPD motif for binding cellular chaperone Hsc70 (Srinivasan et al., 1997). The J domain is connected to the origin binding domain (OBD) by a flexible string of amino acids, called the N-terminal linker region. The N-terminal linker serves as a hub for binding of several cellular proteins to LT. The origin binding domain is followed by the zinc and AAA^+ ATPase domains. These two domains are required for the helicase function of all LT proteins. In addition, LT binds to p53 at the surface of the ATPase domain (Lilyestrom et al., 2006). Following the ATPase domain is an unstructured stretch of amino acids known as the variable region and host range region (VHR). In SV40, this region has mainly been associated with replication. Only three regions of SV40 LT have been identified by genetic studies as essential for transformation: the J domain, the N-terminal linker region, and the p53-binding region on the ATPase domain.
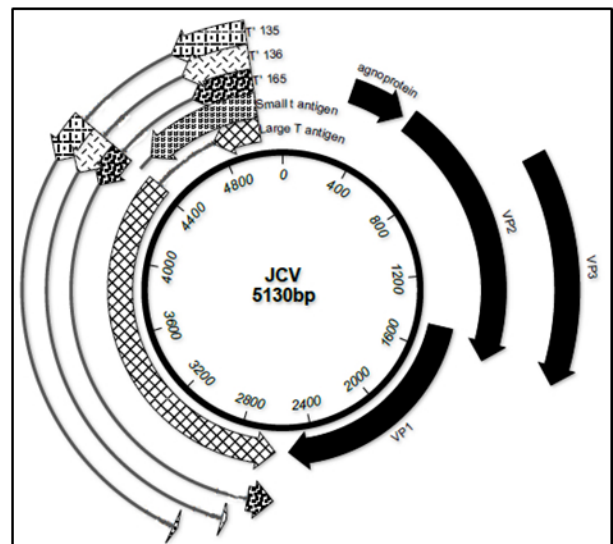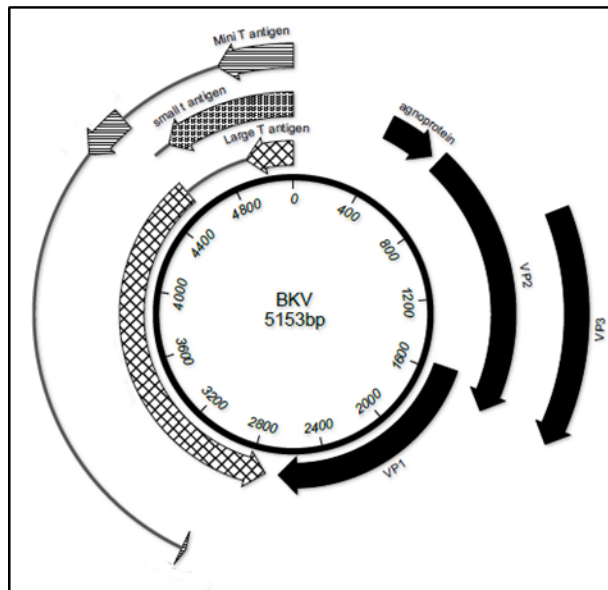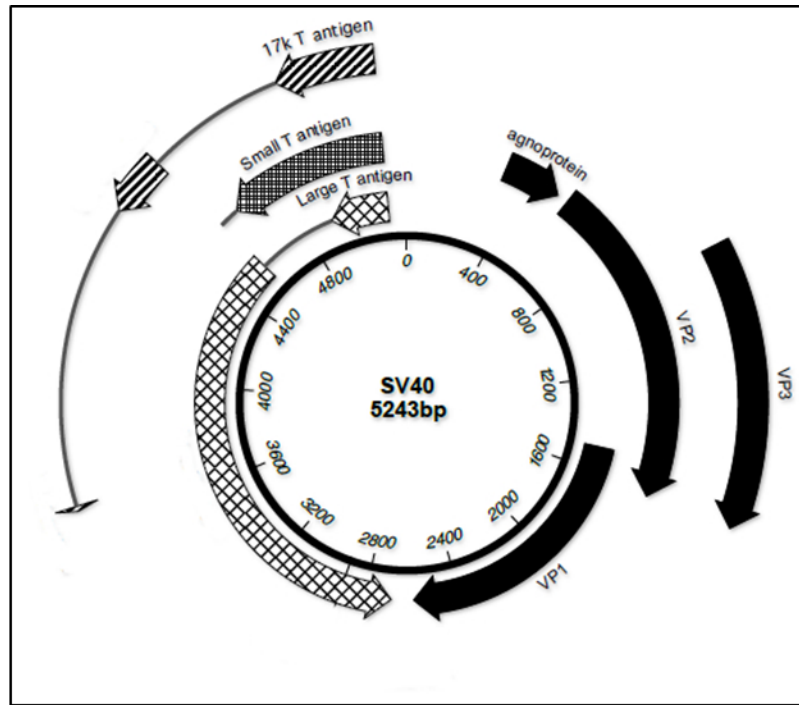
Figure 5. Genome organization and transcript map of large T antigen transforming viruses.

SV40, BKV, and JCV express similar large T antigen, small T antigen, and late region proteins (VP1, VP2, VP3, agno). Alternative splicing patterns of the early region T antigens are shown. Thick patterned arrows represent exons, while thin black lines represent introns.

1.5.1 Interaction with pRB family proteins

Together the LT J domain and LxCxE motif within the N-terminal linker region induce cell proliferation by inactivating inhibitory pRB-E2F complexes (Figure 6). The pRB family of proteins includes pRB, p107, and p130. These three proteins coordinate to regulate the transition from the G_1 to S phase of the cell cycle by directly binding and inhibiting the E2F transcription factor proteins. pRB specifically binds to activator E2Fs 1, 2, and 3, while p107 and p130 bind to repressor E2Fs 4 and 5. Release of the E2Fs occurs when pRB proteins are phosphorylated by cyclin dependent kinases and results in transcription of S-phase genes and, ultimately, DNA synthesis.

SV40, JCV, and BKV LTs bind to all three pRB proteins through an LxCxE motif located in the N-terminal linker region (Chellappan et al., 1992; Dyson et al., 1990; Harris et al., 1996; Tavis et al., 1994; Zalvide and DeCaprio, 1995). The requirement for the LxCxE motif in transformation by SV40 LT is well-documented by mutagenesis studies (Chen and Paucha, 1990; Srinivasan et al., 1997; Zalvide and DeCaprio, 1995). The importance of this motif is also shown by the fact that the same motif is required for other viral and cellular proteins to bind to pRB proteins (Chellappan et al., 1992; Singh et al., 2005).

SV40 LT specifically binds pRB in the under-phosphorylated forms thereby preventing its action during the G_0/G_1 phase of the cell cycle and allowing G_1 to S transition (Ludlow et al., 1989; Ludlow et al., 1990). Interaction with LT causes dephosphorylation of p130 and p107 (Lin and DeCaprio, 2003; Stubdal et al., 1996) and potentially p130 degradation (Stubdal et al., 1997). The binding of pRB proteins to the LT LxCxE is not sufficient to activate E2F-mediated transcription; this function also requires the J domain of LT (Pipas et al., 1983; Srinivasan et al., 1997; Zalvide et al., 1998). Hsc70 binding at the J domain and subsequent chaperone activity has

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been shown to specifically dissociate p107/E2F4 and p130/E2F4, but not pRB/E2F complexes (Sullivan et al., 2004). In line with this finding, double knockout p107 -/- ; p130 -/- MEFS that expressed SV40 LT with an LxCxE mutation were unable to grow in low serum and did not confer a growth advantage in normal serum, suggesting that pRB binding is required for these transformation phenotypes. But, when a J domain mutant of LT was expressed, both low and normal serum growth was restored (Stubdal et al., 1997). This suggests that either the J domain does not participate in dissociation of the pRB-E2F complex, or that dissociation of pRB/E2F is not required for transformation.

A chaperone model of the J domain-dependent dissociation of p130/E2F and p107/E2F complexes has been proposed (reviewed in Sullivan and Pipas, 2002). It begins with LT binding to p130/p107 through the LxCxE motif and Hsc70 through the HPD motif within the J domain. Hsc70 uses the energy gained from hydrolyzing ATP to dissociate E2F from p130/p107. This frees the E2F from the complex. The dissociation removes repressive complexes from E2F regulated cell cycle genes, which allows the activator E2Fs to bind and stimulate transcription (Figure 6). In accordance, cells that express SV40 LT show activation of a host of E2F regulated genes involved in cell cycling, DNA replication, and chromatin modification (Cantalupo et al., 2009).

1.5.2   Inhibition of p53

p53 is arguably the most well-studied tumor suppressor protein. It is a transcription factor that senses several kinds of cell abnormalities. In response, p53 activates genes like p21 that mediate growth arrest to allow the cell time to repair, or, when circumstances are dire, it activates cell death mechanisms through transcription of several well-known apoptosis associated genes,

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including Bax, Noxa, and Puma (Brady et al., 2011). SV40 large T antigen activates p53 through at least two mechanisms. First, dysregulation of the pRB family activates p53 most likely by an E2F-dependent mechanism (Polager and Ginsberg, 2009). Second, SV40 LT expression results in activation of DNA damaging sensors that, in turn, activate p53. This mechanism can occur though both Bub1-dependent and -independent mechanisms (Boichuk et al., 2010; Hein et al., 2009).

Although p53 is activated in LT cells, as evidenced by multiple post-translational modifications (Borger and DeCaprio, 2006), it is prevented from transactivating p53-secific promoters (Jiang et al., 1993; Segawa et al., 1993) because LT binds to the DNA binding domain of p53 (Lilyestrom et al., 2006). Thus, p53 cannot induce genes that would lead to apoptosis, senescence, or growth arrest in cells with LT. In contrast to other DNA tumor viruses, which remove p53 from the cell by inducing its degradation, SV40 LT stabilizes p53, and therefore p53 is readily detected by Western blot. Both BKV and JCV LT bind and inhibit p53 transactivation in a similar way (Bollag et al., 1989; Krynska et al., 1997; Nakshatri et al., 1988)

Two patches of residues on the surface of the ATPase domain of SV40 LT mediate binding to p53 – these are within a.a. 351-450 and 533-626. Mutation of these residues prohibits growth to high density in normal and low serum conditions, dense focus formation, and anchorage independence (Ahuja et al., 2009; Peden et al., 1989). However, extension of life and rescue of proliferation of serum-deprived primary cells occurs in the absence of p53 binding (Tevethia et al., 1998; Tevethia et al., 1988; Thompson et al., 1990), showing that p53 inhibition is not required for some aspects of cellular transformation.

LT has also been shown to be associated to CBP/p300 transcriptional co-activators through its binding to p53. This association results in acetylation of lysine 697 within the host

range region of SV40 LT (Poulin et al., 2004) and increased acetylation at specific residues on cellular histones (Saenz Robles et al., 2013). It is not clear if CBP and p300 are required to complex with SV40 LT and p53 to transform cells.

Although LT binding to p53 is undoubtedly important for transformation of cells, this may not be the whole story. Expression of a fragment of SV40 LT containing only the J domain and N-terminal linker was able to prevent p53 transactivation of a CAT reporter after UV stimulation, suggesting this fragment is capable of regulating p53-mediated transcription independently (Rushton et al., 1997). Also, inhibition of p53 through expression of dominant-negative p53 was unable to transform cells in the presence of a similar N-terminal fragment (Sachsenmeier and Pipas, 2001), which points to additional roles of functional p53 in transformation by SV40 LT.

1.5.3 Other proteins that affect transformation by SV40 LT: Cul7 and Bub1

Cul7 is a scaffold protein associated with the Cullin-RING E3 ubiquitin ligase complex. Genetic studies in mice have shown that loss of Cul7 results in severe pre- and post-natal retardation (Cheng et al., 2009). SV40 LT was shown to bind to Cul7 by immunoprecipitation from cellular lysates (Ali et al., 2004; Kohrman and Imperiale, 1992). Mutations within SV40 LT revealed a loosely defined set of amino acids that span the J domain and N-terminal linker region that contribute to Cul7 binding (aa. 69-83, 98, and 101; (Ali et al., 2004; Kasper et al., 2005)). The F98A mutant of SV40 LT that does not immunoprecipitate with Cul7 is impaired in inducing anchorage independent and low serum growth in primary murine embryo fibroblasts (Ali et al., 2004). A full understanding how this interaction affects transformation is lacking; however, a

recent publication suggests IRS1, a component of the insulin growth factor receptor pathway, as a target of Cul7 inhibition by SV40 LT (Hartmann et al., 2014).

Bub1 interaction with SV40 LT was discovered through a yeast-two-hybrid screen, and subsequently verified by co-immunoprecipitation from cell lysates. Deletion of amino acids 89-97 or mutation of individual tryptophans within this region (which are conserved in several other PyV LTs (Figure 10)) prevent association of Bub1 and LT. Immortalization of rat embryo fibroblasts by SV40 LT is not affected when Bub1 binding is prevented; however, dense focus formation of an established rat cell line may be inhibited (Cotsiki et al., 2004).

Bub1 is a kinase that participates in the spindle assembly checkpoint of mitosis (Williams et al., 2007). This checkpoint is activated during anaphase if kinetochores are not properly attaching chromosomes to spindle microtubules. In addition, Bub1 may have other roles in DNA repair and replication (Hu et al., 2013). Therefore, it is not surprising that Bub1 binding to SV40 LT significantly increases the incidence of tetraploidy in cultured cells (Hein et al., 2009), as well as several other hallmarks of chromosomal instability (Hu et al., 2013). Of note, Hanahan and Weinberg discuss genome instability as an enabling characteristic of cancer, allowing genetic mutations to accumulate more readily (Hanahan and Weinberg, 2011). Although it is not clear how Bub1 interaction with SV40 LT contributes to the immediate transformation of cells in vitro, it can easily be envisioned that the chromosomal instability resulting from this interaction plays a part in LT-induced tumorigenesis.

1.5.4 SV40 small T antigen contributes to cellular transformation

SV40 sT is identical to LT for the first 82 amino acids, which comprise the J domain. The J domain is functional, as it can stimulate the ATPase activity of Hsc70 in vitro (Srinivasan et al.,

1997); however, SV40 sT does not contain an LxCxE motif and cannot bind to pRB proteins. The remaining amino acids are unique to small T. This unique region contains two cysteine clusters (CxCxxC) that confer stability to the small T proteins through binding of two zinc ions, and it binds to the cellular protein phosphatase 2A complex (PP2A) through Cys-97, Pro-101, Cys-103, and residues 110-119 (Das and Imperiale, 2009). BKV and JCV sT proteins also bind to PP2A, but they also have LxCxE motifs in the unique region of small T that can bind to pRB proteins (Bollag et al., 2010); however the contribution to transformation of pRB binding by JCV or BKV sT is not known.

SV40 sT is capable of cooperating with LT to transform cells. It can enhance transformation when levels of LT are too low to fully transform cells (Bikel et al., 1987). In addition, normal human cells are fully transformed only when both LT and sT are combined with activated Ras and hTERT (Hahn et al., 2002). The binding to PP2A is arguably the most important role of sT in transformation, since mutations that prevent PP2A binding abolish its ability to complement LT (Hahn et al., 2002).

PP2A is comprised of 3 subunits: A, B, and C. In mammals there are 2 isoforms each of the core components of the complex, regulatory A and catalytic C subunits (Aα, Aβ, Cα, Cβ). The B subunits are much more numerous and confer substrate specificity, subcellular localization, and regulation to the phosphatase. Specific combinations of the PP2A complex function in normal cells to regulate many pathways, including apoptosis and cell survival, cell cycle regulation, cell adhesion, the DNA damage response, and embryonic development (reviewed in Seshacharyulu et al., 2013).

SV40 sT specifically binds to complexes containing Aα subunits (Andrabi et al., 2011). Binding to the A subunit either displaces or prevents the B subunits from binding. This results in

inhibition of the phosphatase activity and, thus, dysregulation of many growth factor signaling pathways. A few downstream proteins that are activated as a result of PP2A inhibition by sT have been shown to play a role in transformation. In one system using normal human cells, the stabilization of c-myc induced by SV40 sT was required for anchorage independent transformation and tumor formation in immunocompromised mice (Yeh et al., 2004). In mouse cells and human fibroblasts, SV40 sT specifically activates AKT in a PP2A and PI3K dependent manner leading to transformation (Rodriguez-Viciana et al., 2006; Zhao et al., 2003).
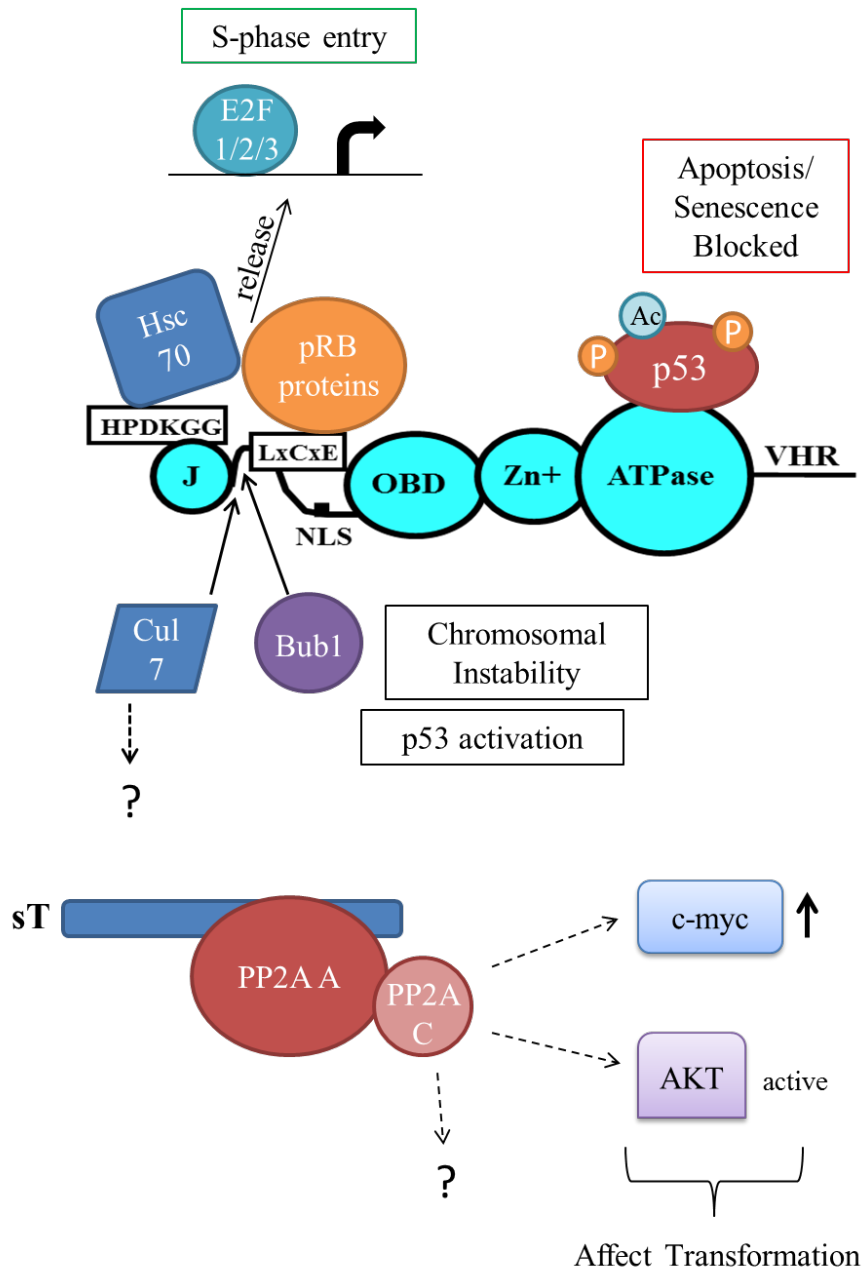
Figure 6. Cellular proteins that contribute to transformation by large T antigen and small T antigen of SV40.

The three transforming regions of SV40 LT are the J domain, the N-terminal linker region, and the ATPase domain. These regions bind the cellular proteins shown resulting in activation of cell proliferation and prevention of cell death mechanisms as described in the text. The SV40 small T antigen C terminus is required for PP2A binding. Binding to PP2A is necessary for sT to complement LT in transformation. As a result of PP2A binding to sT, the transcription factor c-myc is stabilized and AKT is activated. Dashed arrows indicated downstream effects, while solid arrows represent direct binding.

1.6   THE MURINE PYV MIDDLE T ANTIGEN MODEL OF TRANSFORMATION

The early region of MuPyV produces four T antigen species: LT, sT, middle T (mT), and tiny T (Figure 7). While LT and sT contribute to transformation, it is mT that is required for full transformation in culture and tumor formation. Hamster PyV is the only other PyV to produce and transform cells through the action of mT (Bastien and Feunteun, 1988; Goutebroze and Feunteun, 1992).

Middle T antigen is a membrane-associated phosphoprotein that interacts with several cellular proteins involved in critical signaling pathways to induce transformation. In cell culture, mT can transform established rodent cell lines on its own but primary cells require small T or large T to complement mT (Marti and Ballmer-Hofer, 1999; Rassoulzadegan et al., 1982; Treisman et al., 1981). Membrane association is critical for mT as deletion of the C-terminal hydrophobic region completely abolishes transforming activity (Novak and Griffin, 1981). Phosphorylation of specific tyrosine residues is also critical for middle T to bind to certain cellular partners, and mutations of these residues often alter transformation and tumorigenesis (reviewed in Fluck and Schaffhausen, 2009). A few of the cellular partners have been shown to be required for transformation by mT (summarized in Figure 8). While some of the interactions discussed below are needed universally for transformation by mT, others are only important in certain cell types or for tumor formation in animal models.

MuPyV mT exerts its transforming power by interaction with PP2A and phosphotyrosine kinases. Binding of proteins to middle T occurs in a sequential fashion where PP2A must bind first followed by a Src tyrosine kinase. c-Src phosphorylates mT at specific residues, which then become sites for binding to other cellular proteins. Mutations that prevent PP2A, c-Src binding, c-Src phosphorylation activity abolish transformation by mT (Carmichael et al., 1984; Guy et al.,

1994; Pallas et al., 1990; Walter et al., 1990). Phosphorylation at tyrosine 250 allows mT to bind to the ShcA family of proteins and leads to activation of MAPK pathway, although which downstream targets contribute to transformation is unknown (Dilworth et al., 1994; Ichaso and Dilworth, 2001). Overall, the signaling through Shc proteins activates cell proliferation.

Phosphorylation at tyrosine 315 mediates interaction with the p85 subunit of phosphatidylinositol 3-phosphate kinase (PI3K). Middle T that cannot bind PI3K is severely defective in transformation (Markland and Smith, 1987). PI3K is activated and produces the PIP3 as a result of association with mT. The PIP3 molecule targets several serine/threonine kinases leading to activation of Rac GTPase and AKT, which are expected to activate many downstream targets (reviewed in Fluck and Schaffhausen, 2009). Phosphorylation of tyrosine 322 allows mT interaction with phospholipase Cγ1 (PLCγ1), and mutation of this residue had an effect on growth in low serum of established cells (Su et al., 1995). PLCγ1 enzymatic activity is stimulated in response to mT binding, but the mechanism of transformation by this pathway is unknown (Su et al., 1995).

In comparison to SV40, roles of MuPyV LT and sT are very different. The contribution of each T antigen to transformation is summarized in Figure 8. MuPyV LT protein binds and disrupts pRB-E2F complexes (Sheng et al., 1997); however, this mechanism is only required for transformation in primary cells. This is in stark contrast to mutants of the LxCxE motif in SV40 LT which abolish interaction with pRB proteins and full transformation. Also, SV40 LT's direct inhibition of p53 is necessary for several transformation phenotypes, but none of the MuPyV T antigens directly inhibit p53. Even though it does not bind to p53, MuPyV LT alone can immortalize cells (Jat and Sharp, 1986; Rassoulzadegan et al., 1983). MuPyV sT complements mT by preventing inhibition of ARF-mediated activation of p53, which is induced by mT

expression in rat cells. The inhibition of the ARF pathway was dependent on sT binding to PP2A

(Moule et al., 2004).
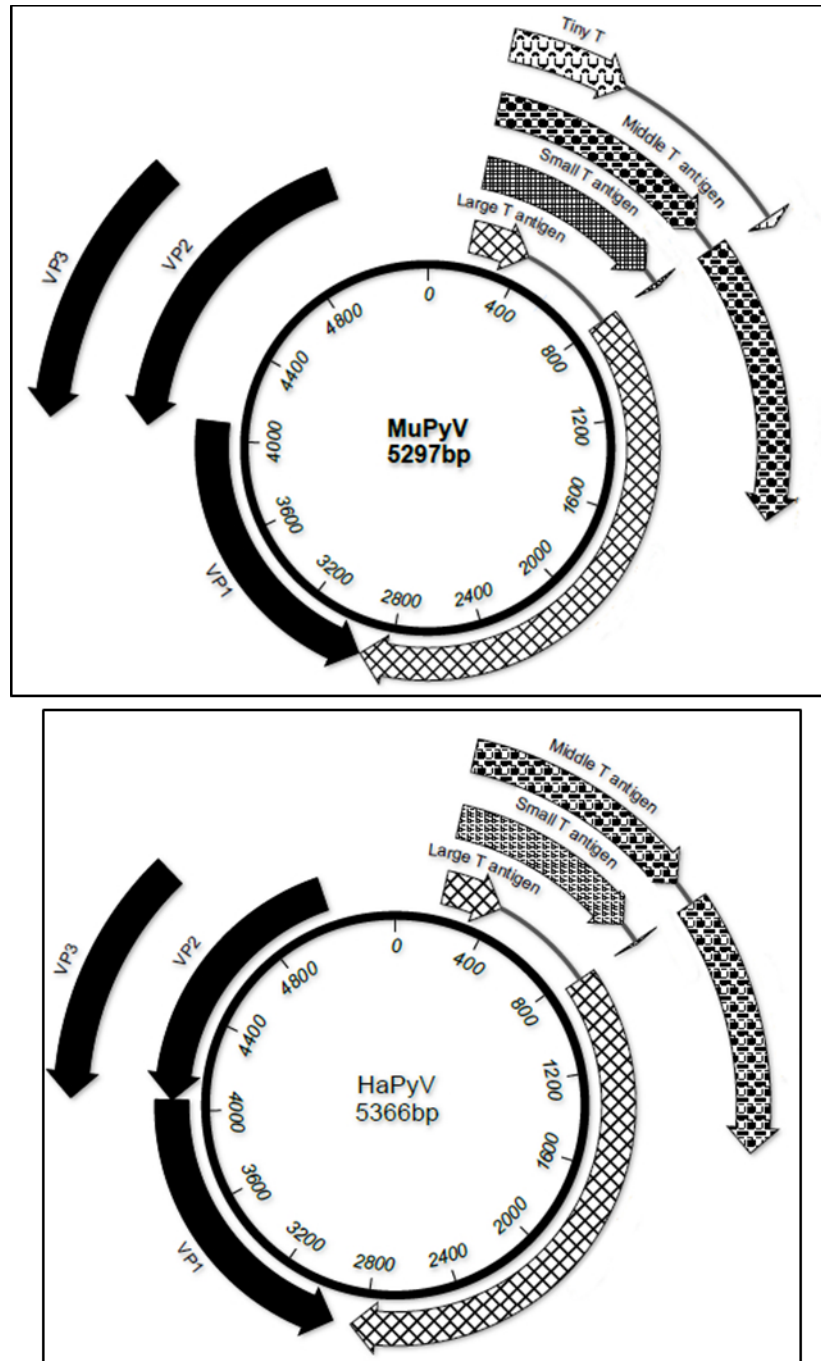
Figure 7. Genome organization and transcript map of middle T antigen transforming viruses.

Murine polyomavirus and hamster polyomavirus are the only two PyVs that have produce a middle T antigen protein. Both also produce a large and small T antigen, as well as capsid proteins VP1, VP2, and VP3. MuPyV also produces Tiny T. Alternative splicing patterns of the early region T antigens are shown. Thick patterned arrows represent exons, while thin black lines represent introns.
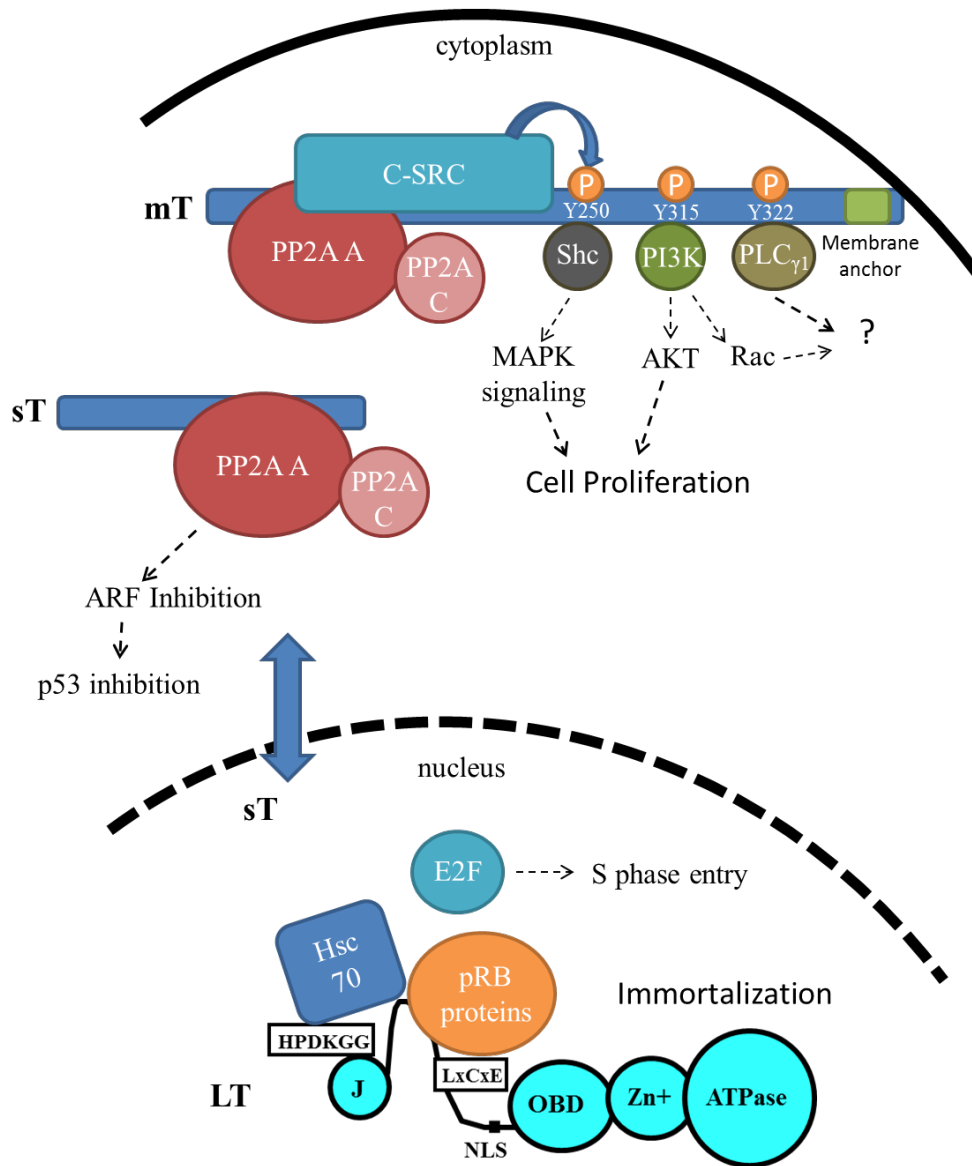
Figure 8. Murine PyV T antigens interfere with several cellular proteins to induce transformation.

Middle T antigen is required for transformation, while sT and LT play supporting roles. Each viral protein binds to one or more cellular proteins that results in modulation of signaling pathways as described in the text. mT is found associated to membranes mainly in the cytoplasm. Binding of PP2A is followed by association with c-src. The phosphotyrosine activity of s-src phosphorylates mT at specific Y residues. Phosphotyrosines on mT provide binding sites for mitogen-activated signal transduction proteins to bind. These proteins become activated; ultimately inducing cell proliferation and survival. Some mT binding proteins have been shown to have an effect on transformation, but the mechanism of action is unknown (?). sT complements mT transformation by inhibiting p53 activation normally mediated by ARF in mT expressing cells. LT can immortalize cells without mT or sT present. The only defined activity of MuPyV LT is the inactivation of pRB-E2F complexes by the Hsc70-J domain dependent mechanism described for SV40 LT. Dashed arrows indicated downstream effects.

1.7   BREAKING AWAY FROM THE MODEL

The study of transformation by other polyomaviruses has begun to uncover new models and discrepancies with existing models of transformation put forth by SV40 and MuPyV. One example is the Merkel cell PyV sT. In SV40 and MuPyV, sT does not fully transform cells independent of other T antigens. MCPyV sT, on the other hand, is fully capable of transforming rodent cells in culture to dense focus formation and anchorage independence. Furthermore, transformation by sT was independent of PP2A or Hsc70 binding (Shuda et al., 2011). Several lines of evidence indicate that MCPyV is the first human oncogenic polyomavirus (reviewed in Spurgeon and Lambert, 2013); therefore, understanding the unique mechanisms of transformation by these T antigens will give insight directly into a human cancer.

A standing observation that has yet to be fully explained is that of the reduced transformation ability of BKV and JCV T antigens when compared to SV40. The LTs of BKV and JCV are about 70% identical in amino acid sequence to SV40 LT, and they contain the same domain structure and important motifs (Pipas, 1992). They both bind pRBs and release E2Fs, and bind and inhibit p53 (Bollag et al., 1989; Harris et al., 1996; Harris et al., 1998; Tavis et al., 1994). The Frisque group and others have performed many comparisons of JCV, SV40, and hybrid SV40-JCV LTs in transformation assays that have highlighted this enigma. These studies showed that any time regions of the SV40 LT were swapped for JCV LT there was a reduction in transformation, and that JCV LT may form less stable complexes with p53 (Bollag et al., 1989; Haggerty et al., 1989; Sullivan et al., 2000; Tavis et al., 1994; Trowbridge and Frisque, 1993). Fewer comparisons of BKV and SV40 have been performed, but they all show less robust transformation (Bollag et al., 1989; Harris et al., 1996; Hayashi et al., 2001). It is possible that BKV and JCV LT do not bind as well to pRB or p53 proteins; however, the experiments required

to prove this have not been performed. Some of these reports cite a lower protein expression for either JCV or BKV LT as the reason for the inability to transform as well as SV40, however there has been no mechanism uncovered to explain why these LT protein levels would be lower or how less LT would cause the transformation differences.

A final issue that has yet to be resolved is how the LPV T antigens transform cells. Infection with LPV was shown to transform hamster embryo cells to growth in low serum, and to some extent, anchorage independence (Takemoto and Kanda, 1984). In addition transgenic mice expressing the LPV early region developed choroid plexus tumors and lymphomas, similar to SV40 (Chen et al., 1989). The overall genomic structure resembles that of SV40; however, the LPV LT protein shares little sequence identity with SV40 or MuPyV proteins (Figure 9). Unfortunately, the roles of the individual LPV T antigens in complex formation have not been investigated, and the few studies of the early region in transformation give mixed results about the LT's ability to bind p53 (Kang and Folk, 1992; Symonds et al., 1991). Interest in LPV has reemerged since a new human PyV, HuPyV9, with similarity to LPV was recently discovered (Scuda et al., 2011). Further investigation of LPV may give insight into the transforming potential and mechanisms by HuPyV9.

This thesis aims to unravel some the mysteries of BKV, JCV, and LPV transformation. Specifically the goal is to understand if domains of the LTs of these viruses affect transformation in the same way as SV40. First, the J domain and N-terminal linker regions of each virus are investigated for their ability to transform independent of the rest of the LT protein to determine if conservation of sequence relates to conservation of transformation function. Second, I take a closer look at the most divergent region of SV40 and JCV LT proteins, the variable linker and host range region, to see if it plays a role in the recorded discrepancy in transformation potency.

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Investigations of the T antigens of these viruses may give insight into new mechanisms of transformation in virus and non-virus associated cancers.

2.0   MATERIALS AND METHODS

2.1   CLONING OF T ANTIGENS INTO EXPRESSION PLASMIDS

The early regions (ER) from SV40, JCV, BKV, and LPV viral genomes (accession numbers listed in Table 1) were PCR amplified using specific primers (Table 2) and cloned into the pLenti6.3-TOPO vector (Invitrogen; SV40 and JCV only; used in section 4.0) or the pBabe-Puro retrovirus vector modified to work with the Gateway cloning system (Invitrogen; SV40, BKV, JCV, and LPV, used in section 3.0). pPyLT was received from Fried and contains the MuPyV LT cDNA. The LT cDNA was digested out of the original plasmid and ligated into the pLenti6.3 vector.

Truncations of viral ERs were produced by PCR. Primers were designed to amplify desired regions of each ER (Table 3). Then, each truncated ER fragment was cloned into the pLenti6.3-TOPO vector. An artificial Kozak sequence (ACC) was included before the start codon of all constructs to ensure efficient translation. The truncated constructs required the addition of a stop codon immediately after the sequence. The full length early region includes nucleotides 2691-5163 of SV40, 2603-5013 of JCV, 2722-5153 of BKV, and 2822-5270 of LPV. N-terminal fragments contain nucleotides 4410-5163 of SV40, 4259-5013 of JCV, 4396-5153 of BKV, 4316-5270 of LPV. Murine polyomavirus N200 fragment was made from pPyLT

mentioned above, therefore this construct contained nucleotides 175-411 joined to 797-2917. VHR-truncated early regions contain nucleotides 2943-5163 of SV40 and 2792-5013 of JCV.

2.2   CELL CULTURE, TRANSFECTIONS, AND TRANSDUCTIONS

Harvest of murine embryonic fibroblasts (MEFs) from FVB mice was performed as previously reported (Markovics et al., 2005). MEFs were maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO2. Where indicated, confluent MEFs were treated with a final concentration of 1 µg/ml of doxorubicin (Adriamycin, Sigma) in growth medium for 9 hours. The cells were then trypsinized and harvested for protein or RNA extraction as in sections 2.3 and 2.5.

MEF pools described in section 3.0 were created by transduction. Modified lentivirus vector particles harboring the various T antigen constructs were made according to the Invitrogen Virapower protocol. For pBabePuro constructs containing early region sequences, phoenix-eco cells in 10-cm tissue culture dishes were transfected with a mixture of 12 µg of pBabePuro-T DNA and 18 µl of Fugene6 or FugeneHD (Promega) in DMEM to a total volume of 600 µl. Mixing was performed according to the Fugene instructions. Virus-containing medium was collect 48-72 hrs later. The collection was spun at 2000xg for 15 min at 4 °C to remove cellular debris. Fresh, undiluted medium containing virus was placed directly onto MEFs and incubated for 24hrs in the presence of polybrene. Virus-medium was removed and replaced with fresh growth medium. MEFs were allowed to recover for 48 hrs before selection with 2 µg/ml Puromycin or 2 ug/ml Blasticidin. Cells were kept under selection until all mock-transfected MEFs died. All surviving cells from an individual transduction were pooled.

For pools described in section 4.0, T antigen plasmids were transfected into MEFs in 6-well culture plates using Fugene6 (Promega) following the manufacturer's protocol with minimal modifications. A ratio of 1 μg plasmid to 3 μl Fugene was used. Forty-eight hours after transfection, cells were transferred to 10-cm tissue culture dishes and allowed to recover overnight before starting selection with 2 μg/ml blasticidin (Invitrogen). Cells were kept under selection until all mock-transfected MEFs died. All surviving cells from an individual transfection were pooled, and three independent pools were generated for each plasmid construct. All experiments were performed with cells between passages 3 and 10.

2.3   WESTERN BLOT AND IMMUNOPRECIPITATION

All cells were collected at 2 days post-confluence and lysed with either RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Na Deoxycholate, 1% Triton X-100, 1 mM EDTA) for western blot analysis, or buffer containing 50 mM Hepes pH 7.9; 0.4M KCl; 0.5 mM EDTA; 0.1% NP40 and 10% glycerol for immunoprecipitation assays. Lysis and subsequent procedures were performed in the presence of protease and phosphatase inhibitors (1X Calbiochem protease inhibitor cocktail I, 1 mM pepstatin, 10 μM leupeptin, 1 mM Na3VO4, 1 mM NaF). Clarified lysates were collected and protein concentration was assessed by the Bio-Rad protein microassay.

For western blots, equal amounts of total protein were mixed with Laemmli buffer and heated at 95°C for 5 minutes to denature proteins. We routinely separated 15-30 μg of denatured protein by SDS-PAGE, then transferred proteins to PVDF, and performed western blot with indicated antibodies. T antigen specific antibodies are as follows: anti-JCV T antigen specific

monoclonals PAb 962 (Tevethia et al., 1992) and PAb 2003 (Bollag et al., 2000) (both received from Richard Frisque); anti-SV40 T antigen monoclonal antibodies PAb 416 and PAb 419 (Harlow et al., 1981); anti-MuPyV T antigen PN116 (Holman et al., 1994); anti-HuPyV10 received from Chris Buck, unpublished. Epitopes of each antibody and their reactivities to T antigens of different polyomaviruses are listed in Table 3.

The following antibodies were used to detect cellular proteins: p130 C-20 (SantaCruz sc-317x), p53 (PAb 421 (Harlow et al., 1981) or HRP-conjugated anti-p53 from Santa Cruz), S15 phospho-p53 (Cell Signaling 9284S), K379 acetyl-p53 (Cell Signaling 2570S), GAPDH (US Biological G8140-11).

For immunoprecipitation assays, 25 µl of Protein G Dynabeads magnetic particles (Life Sciences) were first incubated for an hour at 4 °C with 50 µl of anti-p53 PAb421 hybridoma supernatant, supplemented with PBS to achieve a final volume of 250 µl. Equivalent amounts of total protein extracts (50-100 µg) were then added to the different reactions and allowed to mix for 4 hours at 4 °C. Pellets containing the immunoprecipitated complexes were retrieved with a magnetic stand and washed four times at 4 °C with PBS plus protease inhibitors. Protein complexes were eluted from the magnetic beads by incubation with 20 µl 0.1 M glycine pH 2.8 for 10 minutes at room temperature, and were subsequently neutralized with 2 µl 1M Tris-HCl pH 8.0 and prepared for SDS-PAGE. T antigen and p53 proteins were detected by western blot as indicated.

2.4   TRANSFORMATION ASSAYS

2.4.1   Immortalization

Immortalization involves extending the lifespan of primary cells in culture. Primary cells are those that are taken directly from normal tissue and selected for growth in culture. They have a limited capacity for proliferation, sometimes lasting only a few passages before growth arresting. Mutations or viral gene products can be expressed and tested for the ability to prolong the life expectancy of primary cells.

Pools of MEFs that expressed a T antigen construct were passaged consecutively at either a 1:10 or 1:5 dilution. Normal MEFs were passaged at the same time, but these cells cease to proliferate between passages 4 and 6. MEFs expressing T antigen that survived 10 or more passages were considered immortal.

2.4.2   MEF survival assay

MEFs were plated in a 96-well plate at 1000 cells per well in a total volume of 100μl and incubated overnight at 37 °C. The cells were then transduced with 100μl of the appropriate retrovirus or lentivirus vector along with 12 ug/ml Polybrene (Invitrogen). Mock transduced cells were treated with Polybrene only, and all subsequent steps were performed the same as virus treated cells. Twenty-four hours later the virus was removed, replaced with normal growth medium, and the cells were allowed to recover for another 48 hrs. Cells from each well were trypsinized and transferred to individual 10cm tissue culture dishes with 10ml of normal growth medium. Cells were fed every four days. The plates were fixed and stained when dense colonies

were observed or after 18 days in culture. Cells were fixed with a solution of 10% methanol and 10% acetic acid in H_2O for 20 min. Colonies were visualized by staining with 5 ml of 0.4% crystal violet in 20% ethanol for 1-2 hrs then washed with tap water at least twice to remove excess stain.

2.4.3   Growth in low serum

3x104 cells were seeded onto 6-well tissue culture plates in DMEM + 10% FBS. To produce low serum conditions, the medium was replaced six hours later with DMEM + 1% FBS, after washing the cells once with PBS. Cells for both low and normal (10%) serum assays were incubated overnight before counting the first time point. This was considered time zero. Cells from two wells were counted with a hemacytometer and averaged every day (normal growth) or every other day (low serum growth) for up to 12 days.

The average number of cells counted (y-axis) at each time point (x-axis) was plotted on a graph and the doubling time was calculated using two time points within the exponential growth phase according to the equation:

$$\text{Doubling time} = T \ln2 \,/\, \ln(X2/X1)$$

where T is the incubation time in hours, X2 is the number of cells from the later time point, and X1 is the number of cells from the earlier time point. Doubling time was divided by 24 to convert the value into doublings per day.

For experiments in Section 3.0, pools expressing T antigen N-terminal fragments or normal MEFs were tested 2-4 times. Each independent experiment was plotted on a graph of $\log_2$ of the cell number vs. time.

For experiments in Section 4.0, each pool expressing the same early region construct was considered a biological replicate. Wild-type MEFs were independently tested twice, constituting technical replicates. The doubling times from all replicates were averaged and the standard deviation was calculated using the Excel function "STDEV.S". The standard error of the mean was derived by dividing the standard deviation by the square root of the number of replicates.

2.4.4 Dense focus formation

A total of 3×10^5 normal MEFs were plated onto a 10cm tissue culture dish and briefly incubated (less than 2 hours) while T antigen expressing MEFs were trypsinized and counted. One thousand T antigen-MEFs were then added to the normal MEFs, and gentle rocking was used to get an even distribution across the dish. Cells were incubated for up to 16 days, then fixed with a solution of 10% methanol and 10% acetic acid in H_2O for 20 min. Dense foci were visualized by staining with ~5 ml of 0.4% crystal violet in 20% ethanol for 1-2 hrs then washed with tap water at least twice to remove excess stain. Duplicate plates were prepared for each cell type per experiment. At least two experiments were performed.

2.4.5 Anchorage independence assay

1.6×10^4 cells were suspended in 0.3% agarose (Invitrogen Ultrapure) in growth medium on top of a 0.5% agarose medium base layer in 35-mm tissue culture dishes, as previously reported (Sun

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and Taneja, 2007). All experiments were performed in duplicate. Colony formation was observed after 21 days of incubation at 37 °C.

For experiments in Section 3.0, photographs of colonies or individual cells were taken at the same magnification. For experiments in Section 4.0, the area of all single and multi- cell events was measured. The area cut-off value chosen (1165 μm2) to define a colony allowed for 1% or less anchorage independent (AI) colonies in the control MEF populations. The number of colonies from each plate was divided by the total number of cellular events to obtain the percentage of anchorage independent colonies formed. Each pool was tested twice and the percentages of AI colonies from both assays were averaged. Pools expressing the same early region construct were considered biological replicates and the percentages of AI colonies from all three pools were averaged. One of the JCV.ER-expressing pools (pool C) was deemed an outlier as it did not accurately represent the behavior of JCV early region as seen in pools A and B, or as previously reported (Bollag et al., 1989; Hayashi et al., 2001; Trowbridge and Frisque, 1993). It was, therefore, excluded from the analysis and the percentages of AI colonies from the remaining two JCV.ER-expressing pools were averaged. The standard error of the mean was calculated as for the low serum growth assay.

2.5   REVERSE-TRANSCRIPTION AND REAL-TIME PCR

Standard techniques were used to extract total RNA from cells (Qiagen RNeasy Kit including the on-column DNA digestion with the Qiagen RNAse-free DNase set), and to synthesize cDNA (Saenz-Robles et al., 2007). The type and level of transcript species produced by different T

antigen constructs were monitored with reverse-transcription PCR. Levels of E2F-regulated genes were evaluated using either reverse-transcription or real-time PCR methods.

Primers used for all RT-PCRs are listed in Table 4. Amplification of all possible T antigen specific cDNAs by reverse transcription PCR was performed using primers A and B (SV40), E and B (JCV), or H and B (BKV) from 1 μg of total cDNA with the Roche Expand Long Template PCR system, using buffer 1 with the following cycling parameters: 94°C for 3 min, then 30 cycles of 94°C 30 sec, 54°C (SV40 & BKV) or 55°C (JCV) for 30 sec, 68°C 3 min, a final extension was performed at 68°C for 10 min. Specific E2F-target gene cDNAs or LT and sT cDNAs (primers: SV40 C+D, JCV F+G) were amplified from equal amounts of total cellular cDNA by PCR using GoTaq master mix (Promega) and the corresponding primers as previously reported (Rathi et al., 2009). Annealing temperatures were as follows: Pcna 55°C, Dhfr 55°C, Mcm3 55°C, Cdc6 55°C, Rpl5 56°C, T antigen (C+D) and (F+G) 55°C. PCR products were separated on a 2% agarose gel in 1x Tris-acetate-EDTA and stained with ethidium bromide. Images were captured with a Fujifilm LAS-3000 imager.

Real time PCR was performed on 1 μg of total cDNA was mixed with gene specific primers (Table 4) and SYBR Green real-time PCR master mix. Amplification was performed on an Applied Biosystems 7300 cycler using the default conditions suggested by the manufacturer.

Table 1. Polyomavirus sequences in this study

Virus	Strain	NCBI Accession #
SV40	776	NC_001669
BKV	BKV-D	JF894228.1
JCV	Mad1	NC_001699
LPV	pL02	M30540.1
MuPyV	PYA3	NC_001515

Table 2. Primers for PCR cloning of T antigen early regions and mutants

SV40.ER	F	ACCATGGATAAAGTTTTAAACAGAGA
	R	TTATGTTTCAGGTTCAGGGG
SV40.ERΔVHR	F	ACCATGGATAAAGTTTTAAACAGAGAGG
	R	CTAAACTCCAATTCCCATAGC
SV.N136	F	ACCATGGATAAAGTTTTAAACAGAGA
	R	CTACTTGGGGTCTTCTACCTTTCTCTT
BKV.ER	F	ACCATGGATAAAGTTCTTAACAGGGAA
	R	TTATTTTGGGGGTGGTGTTT
BK.N138	F	ACCATGGATAAAGTTCTTAACAGGGAA
	R	CTATTTAGGGTCTTCTACCTTTCTTTTT
JCV.ER	F	ACCATGGACAAAGTGCTGAATAGGG
	R	TTATTTTGGGGGAGGGGTCT
JCV.ERΔVHR	F	ACCATGGACAAAGTGCTGAATAGGG
	R	CTAAATGGGTCTCCCCATACCA
JC.N137	F	ACCATGGACAAAGTGCTGAATAGGG
	R	CTATTTAGGGTCTTCTACCTTTTTT
LPV.ER	F	ACCATGGACCAAACGCTGTCTAAG
	R	TTACATTTGTTCTTCAATTACAATTCC
LP.N200	F	ACCATGGACCAAACGCTGTCTAAG
	R	CTAATCTGGCTTCTTTTCTTCAGTTTT
Mu.N290	F	ACCATGGATAGAGTTCTGAGCAGAG
	R	CTAACTAGGAGCAGGGTCCTC

Table 3. T antigen monoclonal mouse antibody epitopes and reactivity.

			416	419	962	2003	116	Xt7
		Immunogen	SV40	SV40	JCV	JCV	MuPyV	HuPyV10
		LT Epitopes	N-term linker	J domain	J domain	J domain	J domain	J domain
Reactivity	SV40	LT	+	+	+	-	-	+
		sT	-	+	-	-	-	+
		17k T	+	+		-	-	+
	JCV	LT	+	-	+	+	-	
		sT	-	-	+	+	-	
		T' 135	+	-	+	+	-	
		T' 136	+	-	+	+	-	
		T' 165	+	-	+	+	-	
	BKV	LT	+	-	+	+	-	
		sT	-	-	-	+	-	
		mini T	+	-	-	+	-	
	LPV	LT	-	-	-	-	-	+
		sT	-	-	-	-	-	+
		putative T'	-	-	-	-	-	+
	MuPyV	LT	-	-			+	
		sT	-	-			+	
		mT	-	-			+	
		tiny T	-	-			+	

+, T antigen species is recognized. - , T antigen not recognized. Some antibodies were not tested for reactivity to certain T antigen proteins and are represented by a blank space. Some of the reactivity information listed is taken from previous publications. SV40 (Bollag et al., 2000) . JCV (Bollag et al., 2000). MuPyV (Holman et al., 1994).

Table 4. Primers for reverse-transcription and real-time PCR

T Antigen	A	ACCATGGATAAAGTTTTAAACAGAGAGG
	B	ACCGAGGAGAGGGTTAGGGAT
	C	CTGACTTTGGAGGCTTCTGG
	D	AAGGAAAGTCCTTGGGGTCT
	E	ACCATGGACAAAGTGCTGAATAGGG
	F	GCCTGATTTTGGTACATGGAA
	G	ACAGCTTGACTGAGGAATGC
	H	ACCATGGATAAAGTTCTTAACAGGGAA
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
Rpl5	F	CCAAACGATTCCCTGGTTATGAC
	R	GACGATTCCACCTCTTCTTCTTCAC

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3.0 LARGE T ANTIGEN FRAGMENTS CONTAINING THE J DOMAIN AND LINKER REGION FROM SEVERAL POLYOMAVIRUSES INCREASE THE LIFESPAN OF MEFS, BUT VARY IN TRANSFORMATION

3.1 INTRODUCTION

SV40 large T antigen has at least three distinct elements that contribute to transformation of cultured cells: the J domain; the LxCxE motif; and the p53 binding region (Zhu et al., 1992). Truncation analysis has revealed that fragments of SV40 LT that express the J domain and various lengths of the linker region containing the LxCxE motif allow certain transformation phenotypes independently of the p53 binding C-terminus region in rodent cells. An SV40 LT fragment containing amino acids 1-147 (T147) extended the life of primary mouse cells in culture, but could not immortalize (Tevethia et al., 1998). T147 is also sufficient to cooperate with oncogenic Ras to transform primary rat cells (Beachy et al., 2002; Cavender et al., 1995). Transformation of established cell lines as determined by dense focus formation occurred with similar LT fragments, although the efficiency of transformation was lower than full length LT (Sachsenmeier and Pipas, 2001; Srinivasan et al., 1989). Finally, anchorage independence was *not* achieved by N-terminal fragments in primary mouse cells (Thompson et al., 1990).

The transformation phenotypes described above are consistent with the N-terminal fragments' ability to inhibit the pRB family of proteins to induce cell proliferation, and inability to control growth suppression eventually brought on by p53. Although many large T antigens

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have been shown to bind pRB through the LxCXE motif (Dyson et al., 1990) and contain a J domain, it remained to be seen if comparable short N-terminal fragments of other transforming polyomavirus LTs behaved similarly to SV40.

A phylogenetic tree made from an alignment of the large T antigen (LT) protein from five transforming polyomaviruses gives an idea of the extent of variation in sequence of the LT (Figure 9 A). SV40, BKV and JCV LTs are very closely related, while LPV and Murine LTs are more distantly related from each other and from the SV40/BKV/JCV cluster. This clustering also closely mirrors the transforming properties of the LTs (Figure 9 B). SV40, BKV, and JCV LTs are capable of transforming rodent cells on their own, and they bind to both pRB and p53. It has been shown previously that MuPyV inhibits pRB proteins, but does not bind to p53, which contributes to its inability to fully transform cultured cells. Large T antigen of LPV has been shown to bind to pRB; however, there are mixed reports about its ability to bind to and inhibit p53 (Kang and Folk, 1992; Symonds et al., 1991).

One of the regions of LPV and MuPyV that is least conserved with SV40 LT is the linker region between the J domain and OBD (Figure 9 C), which is known to bind many cellular proteins in SV40 LT. Despite the overall sequence disparity, contained within all of the N-terminal linker regions are motifs for binding pRB (LxCxE), Hsc70 (HPDKGG), and nuclear localization (NLS) (Figure 10). In addition, SV40, BKV, JCV, and LPV have a motif shown in SV40 to be required for binding to the cellular protein Bub1 (WxxWW) (Cotsiki et al., 2004); however, this interaction has not been tested in other T antigens. SV40 N-terminal linker region can also bind to Cul7 (Figure 10). The binding sequence found in SV40 LT is not well conserved in the other polyomaviruses, suggesting that Cul7 binding may not occur with other LTs. Although the Bub1 and Cul7 interactions with full length SV40 LT contribute to transformation

(Hartmann et al., 2014; Kasper et al., 2005), the significance of these interactions with the SV40 N-terminal fragment have not been established. Finally, the linker of LPV and MuPyV is longer than that of SV40, BKV, or JCV (Figure 10), insinuating that more cellular proteins could bind to this region and perhaps effect transformation.

The presence of pRB family binding and inhibitory elements suggests that transformation phenotypes that require only this activity of LT would be conserved for each of the T antigens. However, for LPV and MuPyV, the variation in sequence of the J domain and linker region and in the length of the linker region compared to SV40, might allow for other interactions that affect transformation. To test this hypothesis, both the early regions and N-terminal fragments of LT (J domain and linker region only) for each virus were expressed in primary mouse cells and compared in parallel for the ability to transform in several assays. Each construct was also tested for its ability to affect the pRB pathway.

A)

Large T antigen

B)

Chaperone and pRB binding | Helicase and p53 binding

SV40 LT

C)

	SV40	BKV	JCV	LPV	MuPyV
AA Length	708	695	688	697	782
% Identity to SV40					
J domain	100	82	82	38	34
J domain + linker	100	79	81	32	18
OBD	100	83	80	40	45
Helicase	100	77	75	47	48
VHR	100	30	39	-	-
% Identity to MuPyV					
J domain	34	33	35	38	100
J domain + linker	18	18	19	24	100
OBD	45	42	41	48	100
Helicase	48	47	46	50	100

Chaperone and pRB binding | Helicase

MuPyV LT

Figure 9. Comparison of sequence and structure of polyomavirus large T antigen proteins.

A) SV40, BKV, JCV, LPV, and MuPyV LT proteins were aligned and a phylogenetic tree was created using the maximum likelihood method. The tree shows 3 main branches. SV40, BKV, and JCV are very closely related. Murine PyV LT is the most distantly related to SV40 LT, while LPV LT makes up a third branch that is distantly related to both SV40 and MuPyV LTs. **B)** SV40 and MuPyV large T antigen domain structure. Both LTs share a similar distinct domain structure and contain motifs needed to bind and inhibit the pRB family of proteins (HPDKGG and LxCxE). Several important differences are highlighted. The linker region between the J domain and OBD is longer in MuPyV LT. SV40 LT contains a C-terminal tail known as the variable linker and host range region (VHR), and it can bind to the p53 tumor suppressor through its ATPase domain. **C)** Each domain of LT was aligned to either SV40 or MuPyV LT and a percent identity of amino acids was calculated.

```
                                                         HPDKGG
Mu.N290    1  MDRVLSRADKERLLELLKLPRQLWGDFGRMQQAYKQQSLLLHPDKGGSHALMQELNSLWG    60
LP.N200    1  MDQTLSKEERNELMDLLQITRAAWGNLSMMKKAYKNVSKLYHPDKGGDSAKMQRLNELFQ    60
SV.N136    1  MDKVLNREESLQLMDLLGLERSAWGNIPLMRKAYLKKCKEFHPDKGGDEEKMKKMNTLYK    60
BK.N138    1  MDKVLNREESMELMDLLGLERAAWGNLPLMRKAYLRKCKEFHPDKGGDEDKMKRMNTLYK    60
JC.N137    1  MDKVLNREESMELMDLLGLDRSAWGNIPVMRKAYLKKCKELHPDKGGDEDKMKRMNFLYK    60
              **..*..  :     *::** : *   **::   *.:** .  .   ******.   *: :* *:
                                                                      WxxWW
Mu.N290   61  TFKTEVYNLRMNLGGTGFQGSPPRTAERGTEESGHSPLHDDYWSFS----YGSKYFTREW   116
LP.N200   61  RVQVTLMEIRSQCGSSSSQGYFSEDFYFGPTTFQYSPMDRD-AVREDLPNPGEGSWGKWW   119
SV.N136   61  KMEDGV------------------------KYAHQPDFGGFWDATEIPTYGTDEWEQWW    95
BK.N138   61  KMEQDV------------------------KVAHQPDFGT-WSSSEVPTYGTEEWESWW    94
JC.N137   61  KMEQGV------------------------KVAHQPDFGT-WNSSEVPTYGTDEWESWW    94
              .:  :                              :.*          *     :    *
                                              LxCxE
Mu.N290  117  NDF-FRKWDPSYQSPPKTAESSEQPDLFCYEEPLLSPNPSSPTDTPAHTAGRRRNPCVAE   175
LP.N200  120  REFVNRQCC--------------DDLFCSET----------------------------   136
SV.N136   96  NAF-NE------------------ENLFCSEE----------------------------   108
BK.N138   95  SSF-NEKWD-------------EDLFCHED----------------------------   110
JC.N137   95  NTF-NEKWD-------------EDLFCHEE----------------------------   110
                *                       :*** *

Mu.N290  176  PDDSISPDPPRTPVSRKRPRPAGATGGGGGGVHANGGSVFGHPTGGTSTPAHPPPYHSQG   235
LP.N200  137  -----------------------------------MSSSSDEDTPPAAQPPPPPAPS   158
SV.N136  109  -----------------------------------MPSSDDEATADSQHSTP-----   125
BK.N138  111  -----------------------------------MFASDEEATADSQHSTP-----   127
JC.N137  111  -----------------------------------MFASDDENTG-SQHSTP-----   126
                                                  :  .   *   . :..*
                                                            NLS
Mu.N290  236  GSESMGGSDSSGFAEGSFRSDPRCESENESYSQSCSQSSFNATPPKKAR-EDPAPS   290
LP.N200  159  PEEE-------------DEIEFVEETPSSCDGSCSQSSYTCTPPKRKKTEEKKPD   200
SV.N136  126  -----------------------------------------PKKKRKVEDPK--   136
BK.N138  128  -----------------------------------------PKKKRKVEDPK--   138
JC.N137  127  -----------------------------------------PKKKKKVEDPK--   137
                                                         * *. . *:
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Figure 10. Sequence analysis of N-terminal fragments containing the J domain and linker region of five T antigen proteins.

Alignment of N-terminal fragment sequences was performed with MUSCLE. Amino acids are colored according to the following chemical properties: red are small and/or hydrophobic, blue are acidic residues, pink are basic residues, and green includes hydrophilic residues and glycine. Yellow highlight regions are well known cellular protein binding motifs. Vertical black lines denote the boundary between the J domain and linker region. Regions highlighted in blue are important for SV40 binding to Cul7.

3.2.1   Construction and expression of T antigen early regions and fragments containing the J domain and linker regions.

Both the early region (ER) and fragments containing the J domain and linker region of each polyomavirus were cloned into retrovirus or lentivirus vectors. Early regions have the potential to express all alternatively spliced forms of T antigen, including the LT and sT proteins. In the case of MuPyV, the LT cDNA was cloned instead of the entire early region. This construct cannot produce the middle T or small T, and it acts as a negative control for transformation assays. The N-terminal fragments contained the entire amino acid sequence from the initial methionine to the last amino acid before the OBD. Abbreviations for N-terminal fragments are as follows: polyomavirus initials.N(# of amino acids from LT). For example, the SV40 N terminal fragment is SV.N136, since the J domain and linker region comprise the first 136 main acids of SV40 LT.

Primary murine embryonic fibroblasts were transduced with one of the viral vectors and selected for integration with the appropriate antibiotic. For early region constructs, two independent pools were created but only one pool for each construct is presented, however similar results were seen with both pools. Only one pool for each N-terminal fragment was created.

Expression of T antigen proteins was evaluated by western blot in pooled cells that survived antibiotic selection (Figure 11). Due to the variance in amino acid sequence, antibodies created against four polyomavirus T antigens were used to detect the different T antigen proteins. All of the antibodies used have epitopes within the J domain or linker region, thus they should be

able to detect full length and N-terminal fragments (Table 3). It was not known if any of the antibodies would cross react with LPV T antigens.

The SV40 antibodies PAb416 and PAb419 (Table 3) successfully identified LT expression in MEFs with SV40.ER and JCV.ER constructs and the LT N-terminal fragment in MEFs with SV.N136, BK.N138, and JC.N137 constructs (Figure 11, top left). Small T antigen was not seen in either SV40.ER or SV.N136 MEFs (data not shown). PAb 416/419 did not cross-react with LPV or MuPyV T antigens.

Since the SV40-specific antibodies did not detect BKV LT in BKV.ER samples nor did they have the ability to cross-react with the small T antigens of BKV and JCV, we employed a set of JCV-specific monolconals, 962 and 2003 (Table 3). These antibodies did not identify proteins from either BKV.ER or BK.N138 samples; however, they did detect JCV T antigen proteins (Figure 11, top right). With these antibodies, it was clear that the JCV.ER MEFs were expressing LT protein as well as other smaller protein/s between 20 and 25 kD. At this size, this band could represent JCV sT (~ 21 kD), T'136 (~ 20 kD), or T'165 (~ 25 kD) proteins. A third JCV T' protein, T'135 (~ 17 kD), was not detected. PAb 962 + 2003 also confirmed expression of the JCV LT N-terminal fragment.

Murine PyV-specific antibody, PAb 116, showed expression of MuPyV LT as well as the N-terminal fragment in MEFs (Figure 11, bottom left). This antibody did not cross-react with BKV or LPV T antigens (data not shown). An antibody directed against HuPyV10 (a.k.a. MWPyV), Xt7, was able to detect purified LPV T antigen protein (data not shown). Furthermore, the LPV LT and N-terminal fragment from MEF cell lysates were recognized by this antibody (Figure 11, bottom middle). A doublet appeared in the LP.N200 sample. I hypothesize that this

doublet represents post-translationally modified forms of the LT N-terminal fragment since they are so close in size.

BKV T antigen protein was not observed in BKV.ER MEFs. There were at least two possibilities for this: 1) BKV transcript was not produced due to some unknown reason, or 2) BKV protein was being expressed but at very low levels that could not be seen by western blot. To address the first possibility, reverse-transcription PCR was performed on total RNA isolated from BKV.ER MEFs using primers that would allow for detection of all alternatively spliced transcripts (Figure 12). Three main transcripts were detected: LT, mini T, and a transcript that did not correspond to the size of any known T antigen mRNAs. Interestingly, LT transcript was in the lowest abundance, while the mystery transcript was the highest. Based on the size of the mystery transcript, this transcript is predicted to be spliced at the second intron but not the first intron (see diagram in Figure 12). Detection of LT transcript implies a post-transcriptional step that is effecting the expression of BKV LT protein.
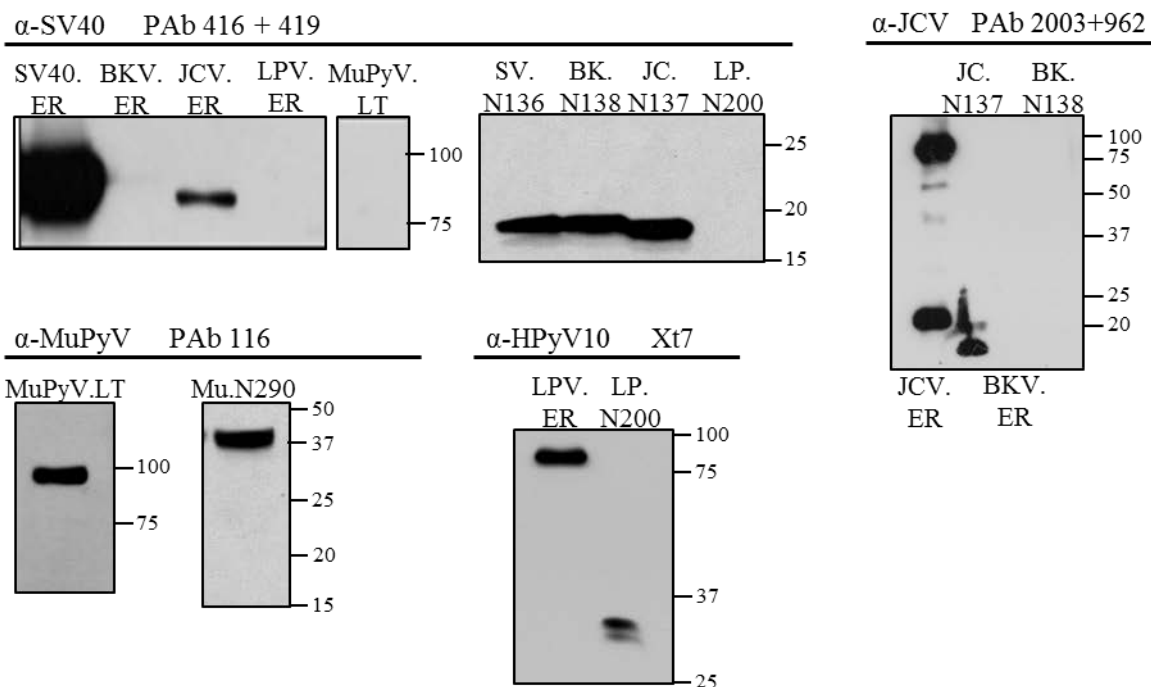
Figure 11. T antigen protein expression in MEFs.

Proteins from pools with T antigen early region or N-terminal fragment constructs were separated by SDS-PAGE and visualized by western blot. Several antibodies directed against T antigen proteins from different polyomaviruses were used to detect T antigen protein expression. A molecular size marker in kiloDaltons (kD) is indicated to the right of each blot. Large T antigens run between 100 and 75 kD. N-terminal fragments of the T antigens run between 15 and 37 kD. In JCV.ER samples, PAb 962+2003 detected a smaller T antigen between 20 and 25 kD that could be sT or the T'136 protein. T antigen protein was not seen by western blot in BKV.ER MEFs.
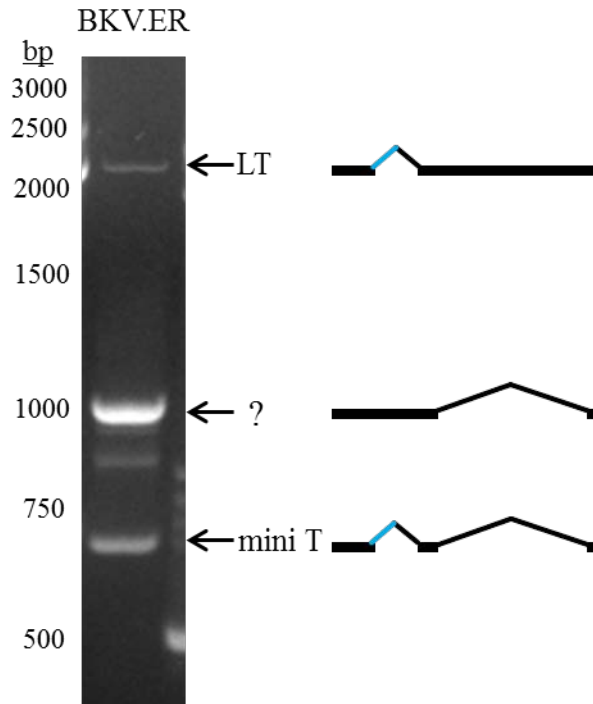
Figure 12. BKV early region transcript expression in MEFs.

T antigen mRNAs were amplified by RT-PCR. Primers surrounding the entire early region were used to capture all alternatively spliced transcripts. Bands corresponding to the large T antigen (LT) and mini T proteins are indicated. A diagram of the mRNAs for each band is shown at the right: horizontal lines indicate exons, while carets represent introns that have been removed by splicing. The band at approximately 1000 bp does not correspond to a known BKV T antigen product. A possible transcript based on the size of the band is shown.

3.2.2 N-terminal fragments of five different T antigens are sufficient to extend the life of MEFs

Once expression of T antigen was established, we set out to determine if the early regions and N-terminal fragments could extend the life of MEFs similar to SV40.N136. Two experimental approaches were utilized. In the first, the MEF pools described above were subjected to consecutive passaging in culture alongside normal MEFs. Table 5 lists the number of passages each cell pool survived. Normal MEFs were passaged five times before they became growth arrested and eventually began to die. All ER/LT expressing MEFs were passaged beyond 10 times with no signs of growth arrest. MEFs with the N-terminal fragments survived a consecutive 10 passages before the experiment was stopped.

The second method used is referred to as the MEF survival assay (diagrammed in Figure 13 A). In this assay, ER or N-terminal fragment constructs were introduced into a small number of MEFs by retrovirus or lentivirus transduction in 96-well plates. After a recovery period, transduced cells were transferred to a 10cm dish effectively creating an extremely low density environment. Colonies of surviving cells were visualized by staining with crystal violet. Within a few days, normal MEFs cease proliferation and take on a large flat morphology and staining is very light and sparse (Figure 13 B and C). Control vectors containing LacZ or Cre recombinase genes did not increase the ability of MEFs to survive, whereas all T antigen constructs allowed MEF colony formation (Table 6; Figure 13 B). It was clear that SV40.ER produced the densest and most numerous colonies of all constructs tested, while MuPyV.LT showed the weakest staining and least number of colonies over several experiments (Figure 13 B; Table 6). Although all N-terminal fragments permitted colony formation, there was an unmistakable decrease in the number of colonies produced compared to the full length counterparts (Table 6). Regardless of

the number of colonies, the morphology of the cells within colonies was clearly transformed compared to control MEFs (Figure 13 C).

Table 5. Extension of life of MEFs by

polyomavirus T antigen constructs

	Consecutive passages survived
SV40.ER	> 10[a]
BKV.ER	> 10[a]
JCV.ER	> 10[a]
LPV.ER	> 10[a]
MuPyV.LT	> 10[a]
SV.N136	10[b]
BK.N138	10[b]
JC.N137	10[b]
LP.N200	10[b]
Mu.N290	10[b]
MEF	5[c]

a Early region and MuPyV.LT expressing MEFs were carried beyond 10 passages.

b MEFs expressing N-terminal fragments were only carried for 10 consecutive passages and then the experiment was stopped.

c At 5 passages, normal MEFs became flat and enlarged and stopped proliferating.

A)

overnight · 48h · <18 days

Plate MEFs 1000 cells per 96 well

Lentivirus vector transduction 24h

Move each well to a 10cm dish

Fix, stain, and count colonies

B)

SV40.ER · BKV.ER · JCV.ER · LPV.ER · MuPyV.LT

SV.N136 · BK.N138 · JC.N137 · LP.N200 · Mu.N290

Mock · pBP-Cre · pL6.3-LacZ

C)

SV40.ER · BKV.ER · JCV.ER · LPV.ER · MuPyV.LT

SV.N136 · BK.N138 · JC.N137 · LP.N200 · Mu.N290

Mock · pBP-Cre · pL6.3-LacZ
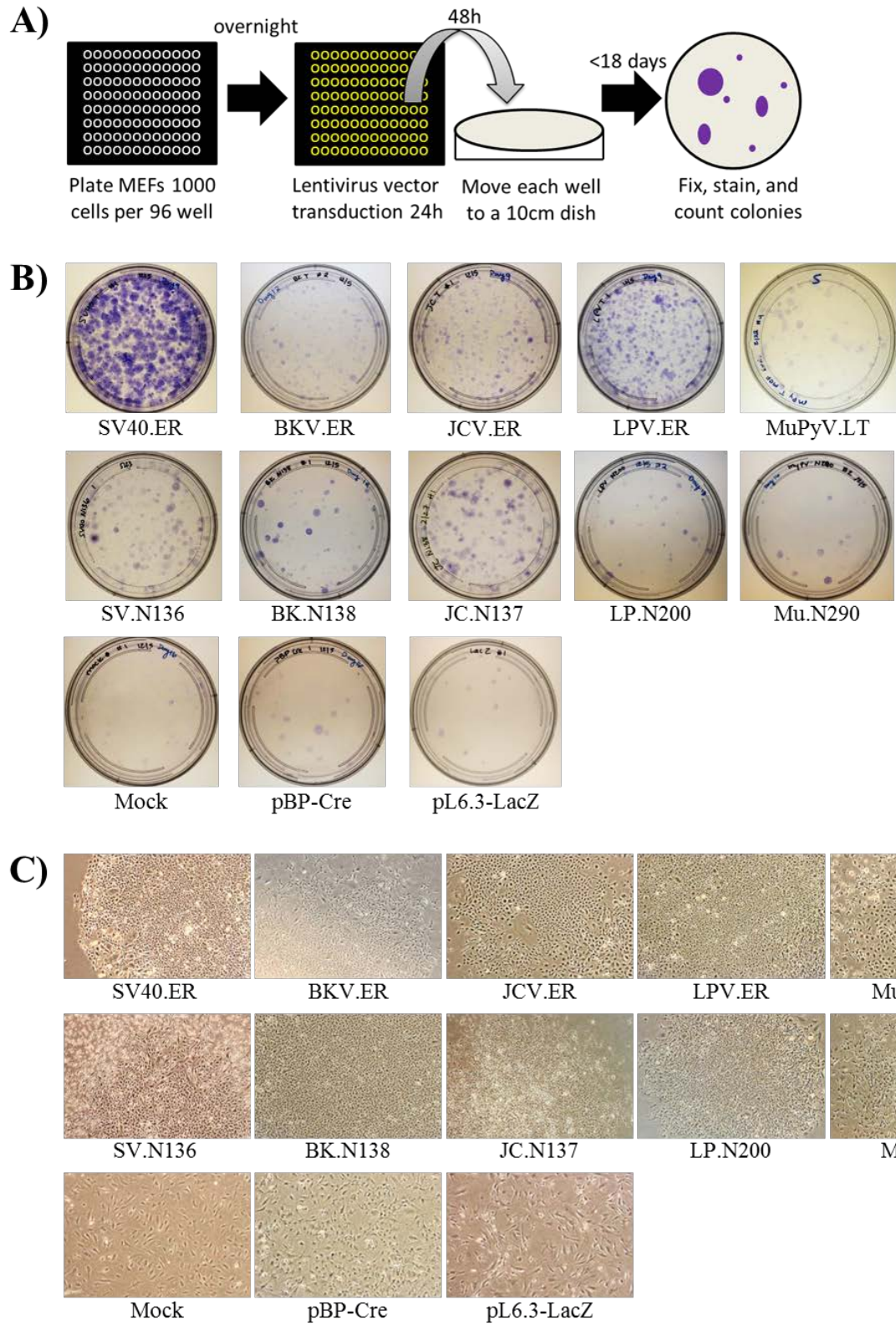
Figure 13. T antigen full length and N-terminus fragments extend the life of MEFs in the MEF survival assay.

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A) Steps in the MEF survival assay. One thousand normal MEFs are plated into each well of a 96 well plate and allowed to incubate overnight. These cells are then transduced with viral vectors containing one of the polyomavirus genes or gene fragments. Medium containing viral vectors is removed 24 hours later, and the cells are allowed to recover for 48 hours. Cells from one well are trypsinized and transferred to a 10cm tissue culture dish. The highly diluted cells are incubated for up to 18 days, at which time they are fixed and stained to visualize cell colonies. **B)** Colony formation produced by different T antigen constructs. Mock transduction and transduction with vectors containing control genes (LacZ, Cre) show very few light staining colonies, while T antigen expressing MEFs display several dark staining colonies. **C)** Images of cells from colonies of MEFs. Each image was taken at the same magnification with a camera attached to a bright field microscope. Note the distinct difference in morphology of cells transduced with T antigens compared to control plasmids or mock.

Table 6. Number of colonies produced in the MEF Survival Assay

Gene	Vector	Experiment					
		1	2	3	4	5	6
SV40.ER	pBabePuro	TMTC	TMTC	-	-	-	-
BKV.ER	pBabePuro	TMTC	TMTC, TMTC	-	-	-	-
JCV.ER	pBabePuro	TMTC	TMTC	-	-	-	35
LPV.ER	pBabePuro	TMTC	TMTC, TMTC	-	-	-	-
MuPyV.LT	pLenti6.3	25	2, 1	6, 22	-	-	-
SV.N136	pLenti6.3	-	-	-	-	0, 0, 0, 8	45, 36, 68
BK.N138	pLenti6.3	-	20, 20	-	54, 60	-	-
JC.N137	pLenti6.3	-	2, 3	-	65, 80	-	-
LP.N200	pLenti6.3	-	30, 25	-	37, 31	-	-
Mu.N290	pLenti6.3	-	4, 8	-	27, 9	-	-
Cre	pBabePuro	0	15	-	-	-	-
LacZ	pLenti6.3	0	0, 0	0, 0	0, 0, 0	-	-
Mock	-	0, 0	0, 0, 3, 1	0, 2, 0, 0	0, 9, 3	-	2

TMTC, too many to count.
(-), indicates that gene was not tested during the particular experiment.

3.2.3 Polyomavirus early regions induce transformation of MEFs

We investigated the ability of the T antigen constructs to stimulate three distinct transformation phenotypes in MEFs: growth in low serum, loss of contact-inhibited growth (dense focus assay), and anchorage independent growth (soft agar assay). SV40 early region has been shown to cause all three of these phenotypes in rodent cells. On the other hand, it has been well documented that MuPyV LT alone cannot induce loss of contact-inhibited growth or anchorage independent growth, but does allow growth of cells in reduced serum conditions (Rassoulzadegan et al., 1982). SV40.ER and MuPyV.LT MEFs behaved as expected in the low serum growth, dense focus formation, and soft agar assays (Figures 14, 15, and 16). JCV, BKV, and LPV early regions were also able to transform MEFs, similar to SV40.ER, in all assays.

3.2.4 Most N-terminal fragments of T antigen are sufficient for growth in low serum, but vary in dense focus formation

The N-terminal fragments showed variable abilities to transform cells in both low serum and dense focus assays. Cells expressing SV.N136, BK.N138, JC.N137, and LP.N200 went through at least two population doublings when cultured in low serum conditions, whereas normal MEFs could barely reach one population doubling (Figure 14). Also, under low serum conditions, the cells with N-terminal fragments showed a lower cell density at the plateau phase compared to the same cells in normal serum. This lower plateau was not seen in cells expressing the early region. During the exponential phase of growth, cells with SV.N136, BK.N138, JC.N137, or LP.N200 constructs grew at equivalent or slightly slower rates in low serum compared to growth in normal serum (Table 7).

Mu.N290 MEFs doubled only once and then began to decline while in low serum conditions, similar to normal MEFs (Figure 14; Table 7). This was initially unexpected since the MuPyV LT had been shown to allow growth in nutrient deprived conditions; however, the p130 protein levels (used as an indicator of T antigen interaction with pRB proteins) in Mu.N290 cells remained much higher than any of the other T-antigen cell pools (Figure 17 A). Thus, the pRB interaction with Mu.N290 may be impaired, but further tests are needed to confirm this hypothesis.

Normal MEFs will cease to proliferate when in a crowded environment, forming a monolayer of cells. This is known as contact-inhibition. Cells that loose this ability continue to grow vertically above the monolayer creating clusters of cells called dense foci. It has been shown previously that SV.N136 cannot induce loss of contact inhibition in primary-like rodent cells (Sachsenmeier and Pipas, 2001). Accordingly, we did not find any foci formed with SV.N136 MEFs (Figure 15). LP.N200 and Mu.N290 MEFs also failed to form dense foci. Interestingly, BK.N138 and JC.N137 MEFs formed foci; however, they were not as dense as the foci seen in BKV.ER or JCV.ER MEFs.

3.2.5 The N-terminal fragment of T antigens cannot induce anchorage independent growth in MEFs

Turning a normally adherent cell into one that can grow in an anchorage independent environment is considered one of the most stringent transformations. While the SV40, BKV, JCV, and LPV early regions were all able to form colonies while suspended in soft agar, this was not the case with any of the N-terminal fragments (Figure 16). MEFs expressing the N-terminal fragments remained as single cells for the entire three week incubation in soft agar.

66
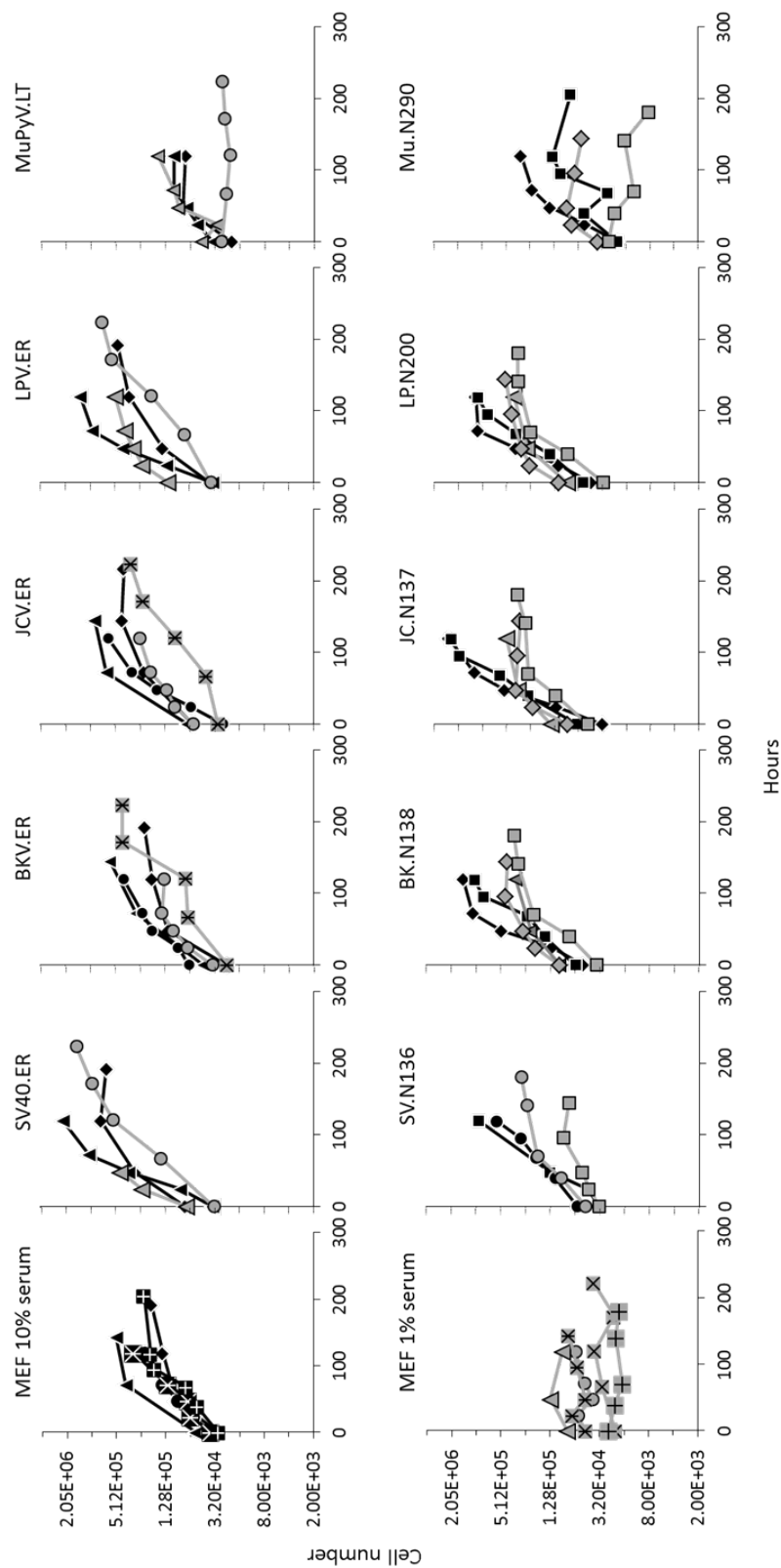
Figure 14. Most T antigen early regions and N-terminal fragments allow growth of MEFs in low serum.

MEFs with or without T antigen constructs were grown under 10% (black) or 1% (gray) serum conditions for up to 12 days. Cells were counted at least 3 times during the course of the experiment. Each set of data points represents an individual experiment, and within each graph, experiments performed on the same date have data points of the same shape. The y-axis is shown as $\log_2$.

Table 7. Doubling time of T antigen expressing MEFs in varying serum conditions.

	Serum	
	10%	1%
MEF	1.8 +/- 0.1	4.6 +/- 2.9
SV40.ER	1.0 +/- 0.3	1.3 +/- 0.1
SV.N136	1.4 +/- 0.3	2.2 +/- 0.7
BKV.ER	1.5 +/- 0.1	1.6 +/- 0.1
BK.N138	0.9 +/- 0.2	1.4 +/- 0.2
JCV.ER	1.2 +/- 0.2	1.8 +/- 0.3
JC.N137	0.7 +/- 0.1	1.2 +/- 0.2
LPV.ER	1.0 +/- 0.3	1.7 +/- 0.1
LP.N200	0.8 +/- 0.2	1.1 +/- 0.1
MuPyV.LT	2.2 +/- 0.2	3.3 +/- 0.7
Mu.N290	1.3 +/- 0.4	-0.6 +/- 2.2

Cells were grown as described in figure 14. Doubling times were calculated from data points within the exponential phase of growth. Doubling times from at least two experiments were averaged and the standard error of the mean was determined.
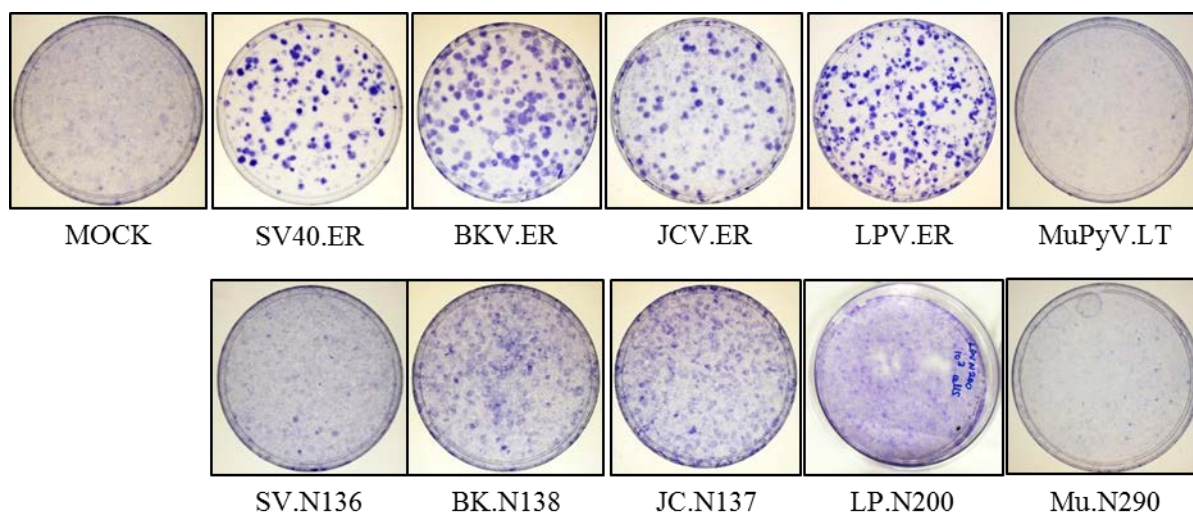
Figure 15. Dense focus formation by T antigen expressing MEFs.

One thousand T antigen expressing MEFs were seeded onto a monolayer of normal MEFs. After incubation for up to fifteen days, cells were fixed and stained to visualize foci. The mock is a MEF monolayer with no cells seeded that was stained after 15 days. Pictures are representative of results seen in at least two experiments.
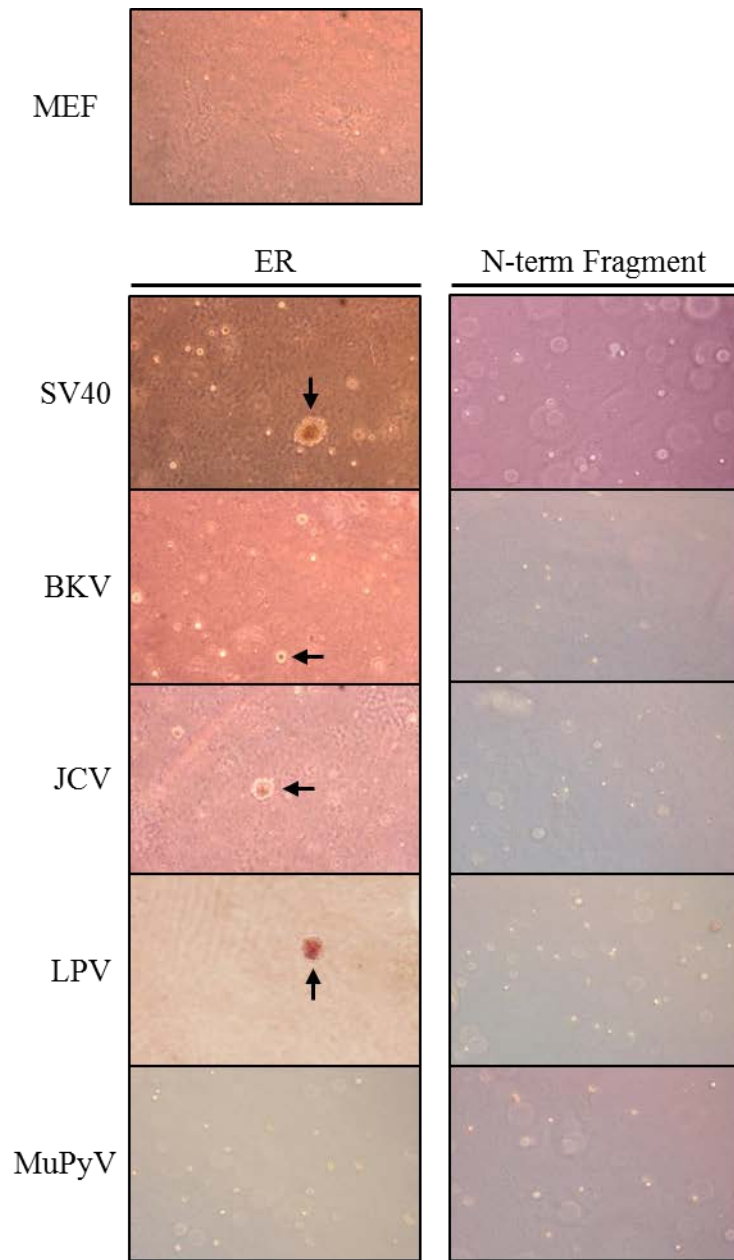
Figure 16. Anchorage independent transformation is lost in cells expressing T antigen N-terminal fragments.

Cells were suspended in soft agar medium and incubated for 21 days. Those that were able to proliferate formed anchorage independent colonies (black arrows). Cells that did not attain anchorage independent transformation remained as single cells.

3.2.6   T antigen N-terminal fragments show minimal activation of E2F-target genes even though interaction with pRB proteins are retained

Both early region and N-terminal fragments were assessed for the ability to interfere with the pRB pathway, since this function of T antigen is known to be required for transformation. We examined the pathway at two steps: 1) interference with p130 protein, and 2) activation of common E2F regulated genes.

p130 is a member of the pRB family of proteins that forms complexes with E2F4/5 transcription factors. The complex sits at the promoter of cell cycle genes inhibiting their expression. Large T antigen binds p130 through the LxCxE motif, and the breaking of the inhibitory complex requires the J domain. In addition, cells expressing SV40 LT display decreased levels of p130 protein (Stubdal et al., 1997). MEFs expressing the polyomavirus early regions and MuPyV LT showed decreased levels of p130 protein compared to normal MEFs, suggesting a functional interaction with LT (Figure 17 A). Cells expressing N-terminal fragments of all T antigens also had lower levels of p130; however, Mu.N290 MEFs did not show as great of a decrease as the other N-terminal fragments (Figure 17 A).

Large T antigen also disrupts a complex of pRB and E2F1/2/3. In this complex, pRB is preventing the activator E2Fs from binding to cell cycle gene promoters and triggering transcription. When LT breaks this complex, these E2Fs go on to activate expression of genes required to induce the S phase. We monitored the expression of a set of canonical E2F target genes in MEFs expressing the polyomavirus early region or N-terminal fragments by real-time PCR. ER and MuPyV.LT expressing MEFs induced transcription of E2F target genes at least 5-fold over the level in normal MEFs (Figure 17 B, top). It was expected that all of the N-terminal fragments would show similar induction levels of E2F target genes; however, only SV.N136 and

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JC.N137 cells showed at least a 3-fold induction of all genes tested (Figure 17 B, bottom). For BK.N138 cells, two genes showed a 2-fold increase and three others were at equivalent levels or only 1.5-fold higher than normal MEFs. In cells expressing LP.N200, only one gene tested was expressed above the 2-fold induction mark. Mu.N290 cells were not included in this experiment.
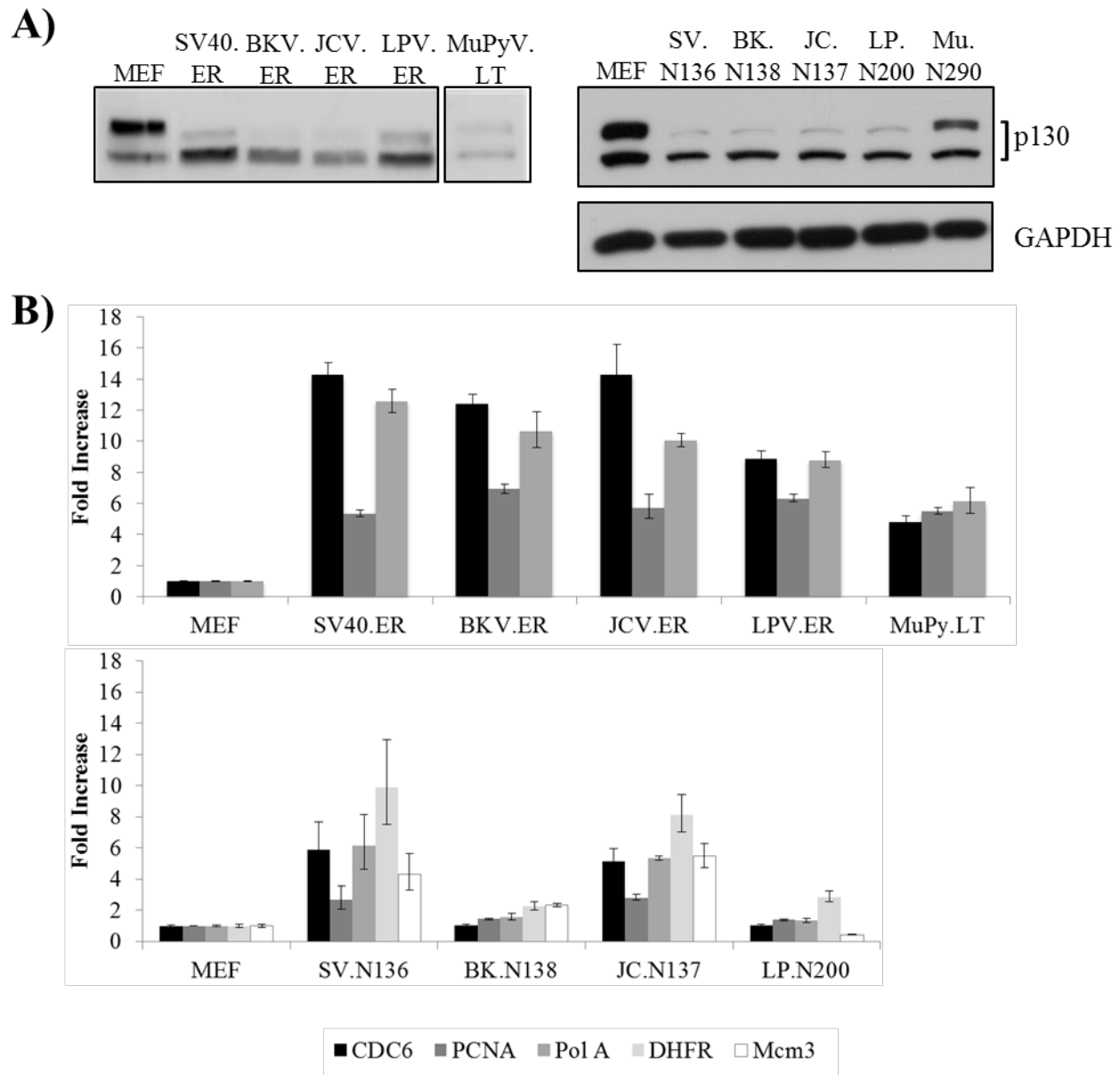
Figure 17. Downstream effects of binding to pRB family proteins are altered in cells expressing N-terminal fragments of T antigen.

A) Steady-state levels of p130 protein determined by western blot. **B)** Expression of several common E2F controlled genes. Real-time PCR using primers specific for the indicated cellular genes was performed on total cDNA from the specified cells. Fold increase of transcript level over normal MEFs is shown.

3.3 DISCUSSION

To understand if similarities or differences in amino acid sequence of the J domain and linker regions of the LTs of transforming polyomaviruses correlated with differences in transformation phenotype, four polyomavirus full length early regions and five N-terminal fragments were compared in several transformation assays. Murine PyV LT served as a negative control, since it is known that the middle T is required for full transformation. As suggested by previous reports using immortal and primary rodent cells, we found that all early regions and MuPyV LT could extend the life of primary MEFs in two different assays (Bollag et al., 1989; Cavender et al., 1995; Rassoulzadegan et al., 1983; Symonds et al., 1991). Furthermore, SV40, BKV, JCV, and LPV early regions efficiently transformed the cells in all assays, as expected.

Large T antigen protein was expressed in all early region expressing MEFs, except for BKV. Yet, BKV LT cDNA was seen by RT-PCR. The plasmid used for transduction was verified by sequencing and no mutations were found (data not shown). It is possible that the large T antigen transcript is not translated for unknown reasons. A more likely explanation, however, is that the level of BKV LT protein is too low for detection by Western blot. Other reports have also seen very low expression of BKV LT (Bollag et al., 1989; Harris et al., 1996). Preliminary experiments where BKV.ER MEFs were treated with MG132 did not show an increase in BKV LT protein expression, which suggests that LT is not degraded by the proteasome (data not shown). Still, other mechanisms of protein degradation that are known to regulate the level of cellular proteins (*e.g.* autophagy) cannot be ruled out. Further experimentation would be needed to determine the reason for the inability to detect BKV LT protein. There is most likely some LT present, as the ability to transform was retained in two independent cell pools made with the same BKV.ER construct.

A few common features of transformation by the N-terminal fragments were revealed in this study, but this data should be considered carefully since only one cell pool for each N-terminal fragment was tested. First, each T antigen N-terminal fragment increased the lifespan of primary MEFs; however, the lower number of surviving colonies for SV.N136, BK.N138, JC.N137, and LP.N200 in the MEF survival assay suggests that they were less efficient than the early regions. This result shows that while the J domain and linker region are sufficient, an activity in the C-terminus of the LTs enhances the cell's ability to survive the stress of the MEF survival assay. For SV40, BKV, and JCV, this activity is most likely p53 inhibition. Indeed, for SV40, it has been shown that both the N- and C-terminus of LT are required to immortalize MEFs *in cis* or *in trans* (Tevethia et al., 1998). This may be the case for LPV as well, since I have seen p53 stabilization by western blot in MEFs expressing LPV.ER similar to JCV.ER (data not shown), but this does not determine whether LPV is directly binding to p53.

Second, none of these fragments were capable of inducing anchorage independent growth of MEFs. This confirms that, alone, LT binding to pRBs and other activities of the J domain and linker regions are not sufficient for anchorage independent growth.

Finally, when other transformation phenotypes were achieved with N-terminal fragments, there appeared to be some deficit compared to the early regions. This was apparent in the low serum growth assay. SV.N136, BK.N138, JC.N137, and LP.N200 allowed growth in low serum; however, the saturation density of these cells was noticeably reduced in low serum compared to cells expressing the early region counterparts. Also, in the focus formation assay, BK.N138 and JC.N137 MEFs formed less dense and less well-defined foci than the early region expressing cells.

Quite unexpected was the observation that BK.N138 and JC.N137 both produced dense foci. Based on overall amino acid conservation, BK.N138 and JC.N137 were predicted to behave similar to SV.N136, which does not form dense foci. Focus formation with BK.N138 and JC.N137 was observed in repeated assays. The foci were not as dense as the early region expressing cells, which could indicate an alternate mechanism for achieving loss of contact inhibition as compared to the full length early region. On the other hand, activities of the missing BKV and JCV LT domains may contribute to the robust phenotype seen when the entire early region is expressed.

It will be interesting to further explore the mechanisms by which BK.N138 and JC.N137 are inducing dense foci. A swap of the SV40 linker region with either the BKV or JCV linker would address the question of which domain is responsible for dense focus formation. In addition, the ability of BK.N138 and JC.N137 to form dense foci suggests that the features in common with SV40.N136 (pRB, Bub1 motifs) are not solely responsible, and that an unidentified activity of BK and JC in the J domain and linker regions contributes to loss of contact inhibition. A scan of the protein sequence alignment of BKV and JCV LT J domain and linker regions (Figure 18) reveals several individual amino acids that are similar in BKV and JCV, but differ in SV40. Sequential and combinatorial mutations of these amino acids in BKV and JCV to that of SV40 or another amino acid could reveal which differences are important for the dense focus formation by the N-terminus fragment. BKV and JCV also contain an insertion of the same three amino acids (KWD) in the linker region immediately preceding the LxCxE motif, which is not found in the SV40 linker region. Although I could not find information

regarding any known functions for the KWD sequence, it stands out as a potential motif and should be further studied to determine its role in BKV and JCV LT transformation.

Another puzzling result was that even though it appeared that SV.N136, BK.N138, JC.N137, and LP.N200 were able to bind and affect the pRB proteins equivalent to their early region counterparts (as seen by a reduction of p130 steady-state levels compared to normal MEFs), E2F-regulated gene expression was minimal or not increased at all. While all of the early regions and MuPyV LT were able to induce the transcription of a few E2F target genes to at least 5-fold over normal MEF levels, only SV.N136 and JC.N137 showed an induction of the same genes. BKV and LPV N-terminal fragments did not induce these E2F target genes, and other genes tested only showed a 2-fold induction if at all. Several possibilities could contribute to this result. One scenario is that for BKV, LPV, and JCV, T antigens other than LT may be required to inhibit all pRB family proteins. T' proteins from JCV show different affinities for binding pRB, p107, or p130 (Bollag et al., 2000) – this could also be the case for BKV and LPV. Another possibility is that the steady state level of p130 protein is not an ideal marker to show the integrity of pRB binding, and that perhaps pRB and p107 binding by N-terminal fragments is not as efficient as with the full length LT. This could result in less E2F release and therefore less E2F target gene activation. Finally, gene expression is regulated by several different protein factors, such as chromatin remodeling proteins or multiple transcription factors (reviewed in Lemon and Tjian, 2000).Thus, it is possible that, in addition to activation of the E2F transcription factors, the full length LT activates multiple transcriptional regulation proteins that act on the E2F target genes that were tested, while the N-terminal fragments only activate a subset of these factors leading to less transcription of this set of genes. It should be noted that the

expression of the E2F targets that were measured did not affect the ability of the N-terminal fragments to extend the life of MEFs or allow proliferation under low serum conditions.

The N-terminal fragment of LPV with approximately 30% identity to SV40 conferred the same transformation capabilities as SV40. The major role of this region for SV40 is to bind to pRB and Hsc70 which together release the E2Fs and induce cell proliferation. This result suggests that these same functions are performed by the LPV N-terminal fragment. Although there still may be unique cellular proteins that interact with this region of LPV, they clearly do not have an effect when the N-terminus fragment is expressed out of context of the full length LT. To address the possibility that unique portions of the LPV LT linker region may still contribute to transformation within the full length LT, mutations or small deletions of the region should be performed. There are no obvious motifs or binding sites within this region that are not also found in SV40 LT (Figure 10 and 18). Therefore, future studies should begin with a more in depth analysis of the linker region with programs that predict protein binding sites or motifs to identify a hierarchy of sequences to target for mutagenesis.

Previous studies did not test the LPV LT and sT proteins for their individual abilities to transform cells. The experiments with LPV.ER in this section also did not separate the LT and sT. Further studies must investigate the contributions to transformation of cells for each protein individually. LPV small T antigen shares only 35% identity with SV40 sT and even less with MuPyV sT. However, the motifs required for binding PP2A are present indicating that LPV sT should bind to the cellular protein complex. The *effect* of binding to PP2A is more difficult to predict since the study of SV40 and MuPyV sT proteins, which both are required to bind to

PP2A for transformation, show completely different phenotypes in cells when expressed without the companion LT (Andrabi et al., 2011).

Finally, the question of whether or not LPV LT directly binds to p53 must be addressed. These experiments could not be performed at the start of this work because at the time we were not in possession of antibodies that could recognize LPV LT protein. The Xt7 antibody was obtained at the end of this thesis work. Immunoprecipitation (IP) conditions with Xt7 must be worked out to begin to determine if p53 and LPV LT associate. Though, IP experiments should be regarded with caution. Recently it was shown that full length MCPyV LT precipitated with p53, but did not bind directly as determined by FACS-FRET analysis (Borchert et al., 2014). It is clear that LPV early region possess potent transforming potential but the details of how the T antigen proteins transform remains to be determined.

Multiple sequence alignment of polyomavirus large T antigen N‑terminal (J‑domain) regions for SV40, BKV, JCV, LPV and MuPyV. Dots (·) indicate identity to SV40, dashes (–) indicate gaps. Blue boxes mark conserved motifs; red underlines mark individual highlighted residues.

```
Block 1
SV40   M D K V L N R E E S L Q L M D L L G L E R S A W G N I P L M R K A Y L K K C K E
BKV    · · · · · · · · M E · · · · · · · · · · · · · A · · · · · · · · · · · R · · · ·
JCV    · · Q T · S K · M E · D · · · · · · · · · L · · A · · I T · · · · K · · N V S · ·
LPV    · · · R · · · A D K E R · L E · Q I T · A · · L S M · · · K · · K Q Q S L L · ·
MuPyV  · · · S · · · · · · · · · · · · · · · · · · · · D F G R · Q L · Q Q · · · · · ·

Block 2
SV40   [F H P D K G G] D E E · · · · · · · · · K M K K M N T L Y K K M E D G V K
BKV    · · · · · · · · D · · · · D · · R · Q · D · · · · · · · · · · · · · · · · ·
JCV    L · · · · · · · D · · · · D · · R · Q · · · · · · · · · · · · · · · · · · ·
LPV    Y · · · · · · · S A · Q R L · F Q R V Q V T L M E I R S Q C G S S S Q G · ·
MuPyV  L · · · · · · · S H A L · Q E L · S W G T F K T E Y N L R M N L G G T G F Q̲ G

Block 3
SV40   – – – – – Y A H Q P D F G G F W D A T E I̲ P T Y G T D E [W E Q W]
BKV    – – – – – · V · · · · · · · – T – S S S · V̲ · E · · · · · · S
JCV    – – – – – · V · · · · · · · – T – N S S · V̲ · · · · · · · · S
LPV    Y F̲ S̲ E D F Y F G P T T F Q Y S · D A V R E D L P N P – · E G S · G K
MuPyV  S P P R T A E R G T E E S G · L H D D Y · S F S Y G S K · F · R ·

Block 4
SV40   N A F N E – – E [N L F C S E] M P S S D D
BKV    · S S · · – – – · · · · D · – · H D F A · E
JCV    · · T · · – – – · · · · D · – · H · F A ·
LPV    · R E · V N – – · – R · C C D – · – T – M · S – – S S D E D T
MuPyV  · R K W D P S Y Q S P P K T A E S S E Q P D · Y · P L L · P N P S S P T D T

Block 5
SV40   – – – – – – – – – – – – – – – – – – – –
BKV    – – – – – – – – – – – – – – – – – – – –
JCV    – – – – – – – – – – – – – – – – – – – –
LPV    – – P P A A Q – – – P P P P P A P S P E E – – –
MuPyV  P A H T A G R R N P C V A E P D D S I S P D P P R T P V S R K R P R P A G A T
```

Polyomavirus large T antigen sequence alignment (SV40, BKV, JCV, LPV, MuPyV). Residues that match SV40 are shown as dots (·); gaps are shown as dashes (–). The green dashed line highlights a region in LPV/MuPyV of block 3.

Block 1
```
SV40   --------------------------------
BKV    --------------------------------
JCV    --------------------------------
LPV    --------------------------------
MuPyV  GGGGVHANGGSVFGHPTGGTSPPHYSQGSESM
```

Block 2
```
SV40   --------------------------------
BKV    --------------------------------
JCV    ----------------C------G---N----
LPV    -EDEIEFVEETPSS--C--DG--SCSQSYTCA
MuPyV  GGSDSSGFAEGSFRSDPRCESENESYSQSCSQSFNA
```

Block 3
```
SV40   KKRKVE-DPKDFPSELLSFLSHAVFSNRTLACFAIYTTK
BKV    ·····K··········D·HQ··············V····
JCV    ·····K··-····VD·HA··············V·····
LPV    ·R··KTEKK···D··VC·Y···IY·K··IY···LL··SL
MuPyV  ·-····ARED PA·S·····TGY···········S···
```
(green-highlighted region in LPV/MuPyV: "…DPK…D…S…")

Block 4
```
SV40   EKAALLYKKIME-KYSVTFISRHNSYN-----HNILFFLTP
BKV    ····QI····L···I·········M·····CAG-I---
JCV    ····QI····L···GFGG·········G·····----
LPV    ·····RQ··TVEKS·IV·D···KASFSYQDEEGE---GCL-
MuPyV  ·····CKQ·DT·G--RPE·KCLVHYE--·EGGM---
```

Block 5
```
SV40   HRHRVSAINNYAQKLCTFSFLICKGVNKEYLMYSALTRDP
BKV    ····FC·····L··········V·R·L·KT·C·Q·
JCV    ····C·····F·C·········A··RLEL·SKPR
LPV    ·VKHFCVSQ·IH···SV·····A·TPMEC·QVV
MuPyV  ·VK·CS·M·SV··········A··T····AA·
```

Protein sequence alignment of polyomavirus large T antigens (SV40, BKV, JCV, LPV, MuPyV). Residues are shown relative to SV40; dots (·) indicate identity to SV40 and dashes (−) indicate alignment gaps. The figure is printed rotated; sequences below are read N→C. Coloured markers in the original: a blue dashed line (block 1), a pink dashed line (block 3), and an orange box around the SV40 "D" (block 5).

Block 1
```
SV40   F S V I E E S L P G G L K E H D F N P E E A E E T K Q V S W K L V T E Y A M E T
BKV    Y H T · V · · · I Q · · E · · S · · · · · P · · K Q · · · · · L · I · · · · V L ·
JCV    Y A V · · · F · I Q · · E · · · · · · · · P · · K · · · · A · Q · · · · F N H L ·
LPV    · K L L · − V S M F E · · Q − · K · Q − C N · · A · · K Q E I C N F · N · A L N
MuPyV  · Q L · T · H Q F E T − · · P − · D · Q · A D · · P − A D F · I M A D F · L N · R
```

Block 2
```
SV40   K C D D V L L L L G M Y L E F F Q Y S F E M C L K C I K K − E Q P S H Y K Y H E
BKV    · · · · · · · F · · · · E · · N V E K · · · − · Q · · D · Y · F N H ·
JCV    · · · · F · · · M · · · E N P Q Q K · · · − · E · D · · F N H ·
LPV    N I S · · · · · I I D · P G K · · G E · − · · · H K F N · ·
MuPyV  N L · P · · · Y · A K E V P S I · · S · E T R L Q I W · R
```

Block 3
```
SV40   K H Y A N A A I F A D S K N Q K T I C Q Q A V D T V L A K K R V D S L Q L T R E
BKV    · F · · I · · E · · · · · · · · · · S · T · H M · ·
JCV    · Y · · Q · · · S · · S · · Q · A · Q · I H M · ·
LPV    A · H · · C L · E · R A · · · · · · L K L V E C S I
MuPyV  · · · A E · D L · N C · A · · · · G · R L K L V E C S ·
```

Block 4
```
SV40   Q M L T N R F N D L L D R M D I M F G S T G S A D I E E W M A G V A W L H C L L
BKV    E · · V E · H I · K · · · · · A H N · · V L · Q Y · · I · ·
JCV    E · · E · H I · K · · · L I A H N · · V L · Q Y · · · T I ·
LPV    E · · L Q V F · E · D F L − · H − I E · E I E · L R · · Y T I Q
MuPyV  L · · K E · Q Q S · L K E L − · − − · − S · A L L Y L · · · · ·
```

Block 5
```
SV40   P K M D S V V Y D F L K C M V Y N I P K K R Y W L F K G P I D S G K T T L A A A
BKV    · · · · I F H · F · V · R · · · · · · · · · · G
JCV    · · Q · T I H I L · · V · · I · · · Y T Y Q ·
LPV    D N C W D · F Q N M V Q L I T T S Q R · N V · R N I · · ·
MuPyV  E D F P Q T L F K M · L L T E V · R · N I · · R · V N G
```

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Protein sequence alignment (read vertically in the original; reconstructed here as horizontal rows). Dots (·) indicate residues identical to the SV40 reference; letters indicate substitutions. Orange boxes and purple underlines mark highlighted residues.

```
Block 1
SV40   L L E L C G G K A L N V N L P L D R L N F E L G V A I D Q F L V V F E D V K G T
BKV    · · · D · · · · · · · · · · · M E · · T · · · · · · · · · Y M · · · · · · Q
JCV    · · · D · · · S · · · · M · · · E · · · · · · · G · · · L C · · · L L D · Q
LPV    F M D F F D · · I · C · A · K · · · · · · · C · · · V · · · L L D ·
MuPyV  · I S · · L · · · S · C · A · K · A · · · · Q · · · · · · · · · · C ·

Block 2
SV40   G G E S R D L P S G Q G I N N L D N L R D Y L D G S V K V N L E K K H L N K R T
BKV    · · A · · · K · · H · · · · S · · · · · · · · · · · · · · · · H · · ·
JCV    · · A · · · · H · · · · C · · · · · R · · · · · · · · · · · · R Q V S
LPV    I T L N K H · Q P · · · T I · · · · H · · · · · · · · · · · · · · ·
MuPyV  I A L N K Q · Q P · M · V A · · · · · · · · · · · · · · · · S

Block 3
SV40   Q I F P P G I V T M N E Y S V P K T L Q A R F V K Q I D F R P K D Y L K H C L E
BKV    · · · · L · · · · · · · · · · · · · · · P · · · · R · · · D · · · · I · R K S Q
JCV    · · V · · R · · · · · · · · · · · · · · · R · · · R · · · D · · · A A · R K S S
LPV    · · · · · L L · R I G V · · · · · · · · · · · · · · · T P K V · Q S ·
MuPyV  · · · L L · Q V W · · · · · · · · · · · A · · · H M V L · T C P H A Q S ·

Block 4
SV40   R S E F L L E K R I I Q S G I A L L L M L I W Y R P V A E F A Q S I [Q] S R I V E
BKV    N · · · · · · · · · · · · L · M T · · · · · · · F · D · · T D · · H · Q
JCV    C · Y · · · · · · · · · L · M T · · · · · · · F · D · · A A · E · R K S S
LPV    K · D V V A K – · · · · Y T I · · · V · L · · · N · D S · T P K V E K V Q
MuPyV  K C · Q R E · · · · D T · A · L · · · · · · · · · N F T S D V D P D · G L V K ·

Block 5
SV40   W K [E] R L [D] K E F S L S V Y [Q] K M K F N V A M G I G̲V̲ L D W L R N S D D D E D
BKV    · · · · · · S · I · M Y T F S R · Y · I C · · · K̲C̲I̲ · I T – – – – E – – –
JCV    · · · · L · I · M Y T F S T A · G · · · · · R̲P̲I̲ · F P – – – – E – – –
LPV    · · T · E · Y V · I T Q F G N I Q Q · I I D · · · – – – – – – – – – –
MuPyV  V R D Q F A S · C · Y · L F C D I L C · Q E · · · – – – – – – – –
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SV40    S Q E N A D K N E D G G E K N M E D S G H E T G I D S Q S Q G S F Q A P Q S S Q
BKV     . E . E - - - - - - - T . . . G S S T E . . . C . S . V S D T . A
JCV     . E . E - - - - - - - A . . . G S S T E . . . C F S . V S E A . G
LPV     - - - - - - - - - - - K P L . G I V . E E . M - - - - - - - - - -
MuPyV   - - - - - - - - - - - D P L K D I C E Y . - - - - - - - - - -

SV40    S V H D H N Q P - - - - Y H I C R G F T C F K K P P T P P P E P E T
BKV     P A E . S Q R S D P H S Q E L . K . . R . K . . . - - K - - -
JCV     - - A . T Q E N - - - . . C T F . . . Q . . K . - - K - -
LPV     - - - - - - - - - - - - - - - - - - - - - - - - - -
MuPyV   - - - - - - - - - - - - - - - - - - - - - - - - - -
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Figure 18. Alignment of the full length large T antigen proteins from five PyVs.

The alignment shows the amino acids and gaps as compared to SV40 LT. Gaps are represented by dashes (-). When an amino acid is the same as that found in SV40 LT it is represented by a dot (.). Any amino acids that differ from that found in SV40 LT are shown. The domain boundaries are indicated by vertical lines. From the first amino acid to the red line is the J domain. From the red line to the green line is the linker region. From the green line to the blue line is the origin binding domain. From the blue line to the pink line is the zinc binding domain. From the pink to purple line is the helicase domain. Beyond the purple line lies the variable region and host range region in SV40, BKV, and JCV, while only a few amino acids are present after the helicase in LPV and MuPyV. Blue boxes highlight some motifs discussed in this paper that are required for cellular proteins to bind to SV40 LT. Amino acids in orange boxes have been shown to create direct binds with amino acids in p53 and are required for the SV40 LT-p53 interaction (Lilyestrom et al., 2006).

4.0 REMOVAL OF A SMALL C-TERMINAL REGION OF JCV AND SV40 LARGE T ANTIGENS HAS DIFFERENTIAL EFFECTS ON TRANSFORMATION

This work has resulted in a publication:

Seneca, N.T., Saenz Robles, M.T., Pipas, J.M., 2014. Removal of a small C-terminal region of JCV and SV40 large T antigens has differential effects on transformation. Virology 468-470C, 47-56.

4.1 INTRODUCTION

JCV and SV40 have been shown to cause tumors in rodent animal models and transformation of cells in culture, and both LTs are necessary and sometimes sufficient to induce tumorigenesis by targeting both pRB and p53 (Frisque et al., 2006; Pipas, 2009). In spite of this similarity, SV40 T antigen is more efficient than JCV T antigen in side-by-side comparisons of transformation ability (Bollag et al., 1989; Haggerty et al., 1989; Hayashi et al., 2001; Tavis et al., 1994; Trowbridge and Frisque, 1993). The reason for the difference has yet to be fully defined. A comparison of the amino acid sequences reveals that JCV LT is about 70% identical to SV40 LT, and that they are up to 80% identical in specific shared functional domains (Figure 19 A). Interestingly, the short C-terminal section immediately following the ATPase domain, known in SV40 LT as the variable linker and host range region (VHR), shows the least similarity between both LTs (Figure 19 A,C).

Previous studies have indicated that the SV40 LT VHR contributes to viral productive infection by allowing viral growth in different host cells (Cole and Stacy, 1987; Pipas, 1985; Tornow and Cole, 1983), and is required to permit growth of adenovirus in normally restrictive cells (Cole et al., 1979). While early studies suggested that the SV40 VHR is not required for immortalization or dense focus formation in rodent cells (Pipas et al., 1983; Tevethia et al., 1988), it remained to be seen whether this region contributes to other transformation phenotypes.

SA12, BKV and JCV polyomavirus LTs also have a VHR, but their roles in viral infection or transformation have not been well studied. Though these 3 VHR sequences are highly conserved, there is little conservation with the SV40 VHR except for a stretch of 18 amino acids within the host range region. Within this homologous sequence there are conserved residues that have been shown in SV40 to be acetylated and that bind cellular proteins (Figure 19 C) (Fine et al., 2012; Poulin and DeCaprio, 2006; Poulin et al., 2004; Welcker and Clurman, 2005). Little examination of the JCV LT VHR region has been performed. Only one report has identified a cellular protein, the SCF ubiquitin ligase component β-TrCP1/2, which binds to the variable linker of JCV LT (Reviriego-Mendoza and Frisque, 2011). The β-TrCP1/2 binding motif is fully conserved in BKV and SA12. None of these interactions has been tested for the ability to affect transformation.

I hypothesized that the JCV LT VHR does not have the same biological functions as SV40 LT VHR due to its sequence disparity, and that removal of the JCV VHR would allow it to transform cells to the same extent as SV40 LT. This chapter describes the effects on cellular transformation produced by removing the VHR from JCV and SV40 T antigens.
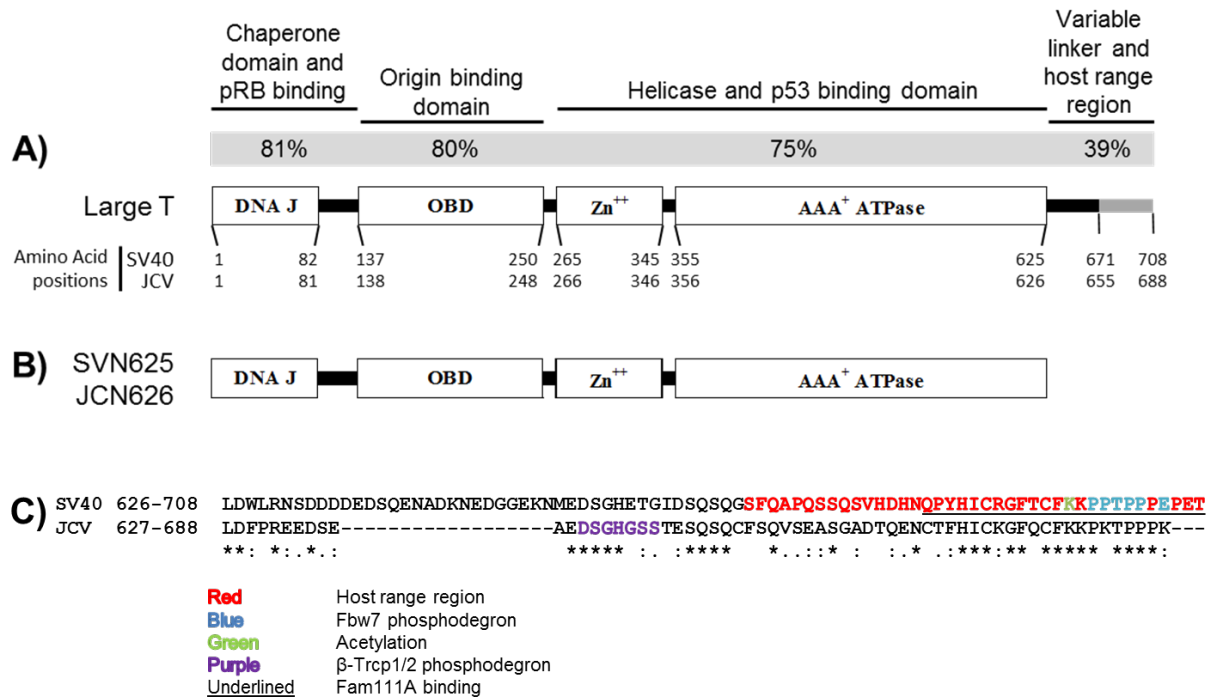
Figure 19. Structure and similarity of large T antigens.

A) Structure of the large T antigen protein. Functional domains (rectangles) within large T are depicted. The functions and percent amino acid identity (gray bar) of each domain within SV40 and JCV large T are indicated above the diagram. At the N-terminus are the DNA J domain and linker region, which are both required to fully inhibit pRB activity. The origin binding domain is required for the viral genome replication. The zinc binding domain and the ATPase domain together contribute to the helicase activity of large T. Cellular p53 binds large T through surface residues of the ATPase domain. The variable linker region and host range region are poorly characterized in comparison to the other regions of large T antigen. **B)** VHR truncations of large T antigen. The variable linker and host range region was removed from each TAg gene. The VHR truncations express amino acids 1-625 of SV40 large T (SVN625) or amino acids 1-626 of JCV large T (JCN626). **C)** Protein sequence comparison of SV40 and JCV VHR regions. An alignment of amino acids was performed with LALIGN. The host range region of SV40 is highlighted in red letters, and was used to define the boundaries of the JCV host range region. Previously described motifs and post-translational modification sites are shown.

4.2   RESULTS

4.2.1   Establishment of a system to compare the early regions of SV40 and JCV

The full length (SV40.ER and JCV.ER) and VHR-truncated (SV40.ERΔVHR or JCV.ERΔVHR) early regions for both SV40 and JCV were cloned into the same expression vector. All four constructs were introduced into murine embryonic fibroblasts (MEFs), and pools of cells that survived selection were collected. We compared the ability of VHR truncated early regions to transform versus the full length counterparts. Three individual pools of cells were generated and tested for each construct to ensure that the observed phenotypes within an individual pool were not random.

The early regions of both SV40 and JCV are capable of producing multiple protein products through alternative splicing mechanisms (Figure 20 A) (Trowbridge and Frisque, 1995; Zerrahn et al., 1993). Truncation of the JCV early region removes the 3' splice acceptor sites for the T'165 and T'136 introns, ultimately preventing the corresponding proteins from being made. In addition, deletion of the VHR shortens the LT proteins to 625 and 626 aa in SV40 and JCV, respectively (Figure 19 B), causing a shift in apparent molecular size from ~94 to ~70 kD (Figure 20 B). Table 8 lists the expected protein products from each construct.

Table 8. Protein products potentially expressed from early region constructs.

Expected Protein Products	
SV40.ER	SV40.ERΔVHR
Large T	Large T (N625)
small T	small T
17kT	17kT
JCV.ER	JCV.ERΔVHR
Large T	Large T (N626)
small T	small T
T'165	T'135
T'136	
T'135	

Potential protein products from both SV40.ER and SV40.ERΔVHR constructs are equivalent. Truncation of the VHR in JCV.ER removes two 3' splice sites, which prevents expression of the T'165 and T'136 proteins.
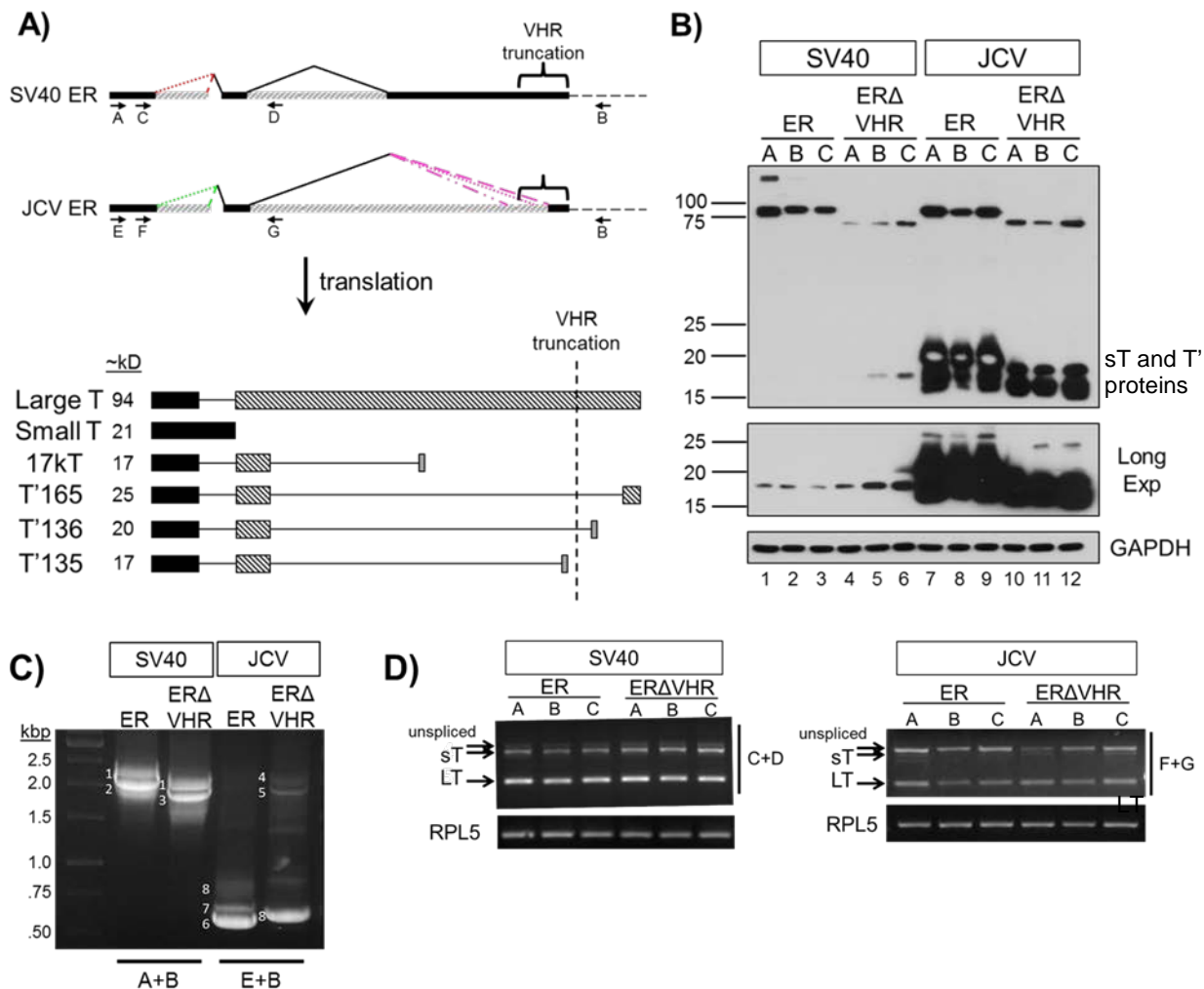
Figure 20. Expression of T antigens from full length or VHR-truncated SV40 and JCV early region in MEFs.

A) Transcripts and proteins encoded by the SV40 and JCV viral early region (ER). Both the SV40 and JCV ER produce multiple T antigen transcripts by alternative splicing (top panel). Translation of these transcripts results in a large T (LT), small T (sT), and one or more smaller T proteins (lower panel). Bars of the same color or pattern represent an expressed region of the protein within the same reading frame, while continuous lines represent regions that are removed due to alternative splicing of the early region pre-mRNA. Approximate sizes of the different T antigen proteins are listed. Removal of the VHR region in JCV prevents the expression of T'165 and T'136. Both SV40 and JCV VHR truncations produce a smaller LT protein. **B)** T antigen protein expression in MEFs from full length or VHR-truncated SV40 and JCV ER. Three individual pools are shown (labeled A, B, and C) for each construct. Equivalent amounts of whole cell lysates were separated by SDS-PAGE, transferred to PVDF, and blotted for T antigens with a mixture of SV40 and JCV specific antibodies (materials and methods). GAPDH, loading control. **C)** T antigen transcripts amplified by RT-PCR using primers that surround the entire open reading frame (A and B or E and B, indicated by arrows in panel A). Each band corresponds to a different T antigen transcript: 1) SV40 sT, 2) SV40 LT, 3) SVN625, 4) JCV sT, 5) JCN626, 6) JCV T'165, 7) JCV T'136, 8) JCV T'135. **D)** RT-PCR specific for LT and sT transcripts (using primers C and D or F and G, indicated by arrows in panel A). Rpl5 was used as a cDNA loading control.

4.2.2 Removal of the VHR alters the pattern of SV40 and JCV early region protein expression

We first determined the protein and messenger RNA expression of T antigen products in each pool of cells by western blot and RT-PCR, respectively. In SV40.ER expressing cells LT was the dominant product identified at both the protein and mRNA levels (Figure 20 B, lanes 1-3; Figure 20 C and D, SV40.ER lanes). Small T protein was only detected after a long exposure (Figure 20 B lanes 1-3), and the level of sT mRNA was lower than LT mRNA (Figure 20 D, SV40.ER lanes). LT protein expression was reduced when the VHR was removed from the SV40 early region, while sT protein expression increased (Figure 20 B lanes 4-6). The altered levels of LT and sT protein could not be attributed to changes in mRNA transcripts since there was no detectable difference in LT or sT transcripts between cell pools expressing SV40.ER and SV40.ERΔVHR (Figure 20 C and D, compare SV40.ER and SV40.ERΔVHR lanes). In preliminary experiments, we observed only a slight decrease in the half-life between full length and VHR truncated- LT protein (data not shown). This suggests that the stability of the protein was not significantly altered and should not account for the drastic decrease of SV40 LT protein in SV40.ERΔVHR cells.

We were unable to detect the SV40 17kT protein. This is not surprising, since the level of 17kT transcript was either extremely low or undetected in multiple experiments performed with all SV40-expressing pools (Figure 20 C and data not shown).

Unlike with SV40, LT was not the dominant protein seen in cells expressing the full length JCV early region. Rather, smaller T antigen proteins (sT, T'135, T'136, T'165) were expressed in the highest abundance (Figure 20 B, lanes 7-9), and this was reflected in the mRNA expression. The T' transcripts were so abundant that we could not detect LT or sT transcripts

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using a set of primers that amplify all T antigen transcripts (Figure 20 A, primers E and B; Figure 20 C, JCV ER lane). The inability to detect JCV LT and sT transcript was mostly likely because the T' transcripts absorbed most of the primers during PCR amplification. To address this issue, a set of primers designed to amplify only LT, sT, or unspliced transcripts was used for RT-PCR (Figure 20 A, primers F and G), and both LT and sT transcripts were detected in the JCV.ER samples (Figure 20 D, JCV ER lanes). Removal of the VHR from JCV early region resulted in a decrease in LT protein (Figure 20 B, lanes 10-12) but, there was no significant change in the level of LT mRNA (Figure 20 D, compare JCV.ER to JCV.ERΔVHR lanes). Similar to JC.ER pools, the total level of smaller T antigens was higher than LT in JCV.ERΔVHR-expressing cells for both protein and mRNA (Figure 20 B, lanes 10-12; Figure 20 C, JCV ERΔVHR lane).

The identity of smaller T antigen proteins expressed from JCV.ER and JCV.ERΔVHR was difficult to discern by migration in SDS-PAGE. Instead, cDNA size analysis and sequencing revealed that JCV.ER pools expressed sT and all three T' transcripts, while JCV.ERΔVHR pools produced only sT and T'135 transcripts due to the deletion (Figure 20 C,D and data not shown). Under our experimental conditions, the level of LT protein was similar between the SV40 and JCV ER-expressing cell pools (Figure 20 B, compare lanes 1-3 with lanes 7-9). However, expression of the VHR truncated JCV LT appeared to be higher than that observed for SV40 LTΔVHR in the corresponding pools (Figure 20 B, compare lanes 4-6 with lanes 10-12), and the expression of both truncated LT proteins was comparatively reduced to that of their full length counterparts (Figure 20 B).

4.2.3 Growth in low serum conferred by SV40 is hindered by removal of the VHR

MEFs respond to a reduction in serum levels in culture medium by ceasing proliferation and eventually dying (Figure 21 A,D and data not shown), but unrestricted growth in normal (10%) or low (1%) serum conditions takes place in the presence of some oncogenes, including SV40 LT. All T antigen-expressing MEFs grew similarly in 10% serum (Figure 21 B,C,D), and the expression of either SV40.ER or JCV.ER allowed proliferation of MEFs at a similar rate in 1% serum (Figure 21 B,C,D). Truncation of the VHR from the JCV early region did not significantly alter the rate of cell growth in low serum (Figure 21 C,D). On the other hand, SV40.ERΔVHR was unable to allow cell growth in low serum. These cells doubled only once during the low serum treatment, similar to wild-type MEFs, whereas all other T antigen-expressing cells doubled at least twice while cultured in low serum for the same amount of time (Figure 21 B,C). Furthermore, the doubling time for the SV40.ERΔVHR MEFs was greater than that of all of the other T antigen-expressing cells (Figure 21 D). We did not find any difference in the level of large T antigen expressed in cells cultured in low serum compared to those in normal serum for either SV40.FL or SV40.ERΔVHR cells (data not shown).

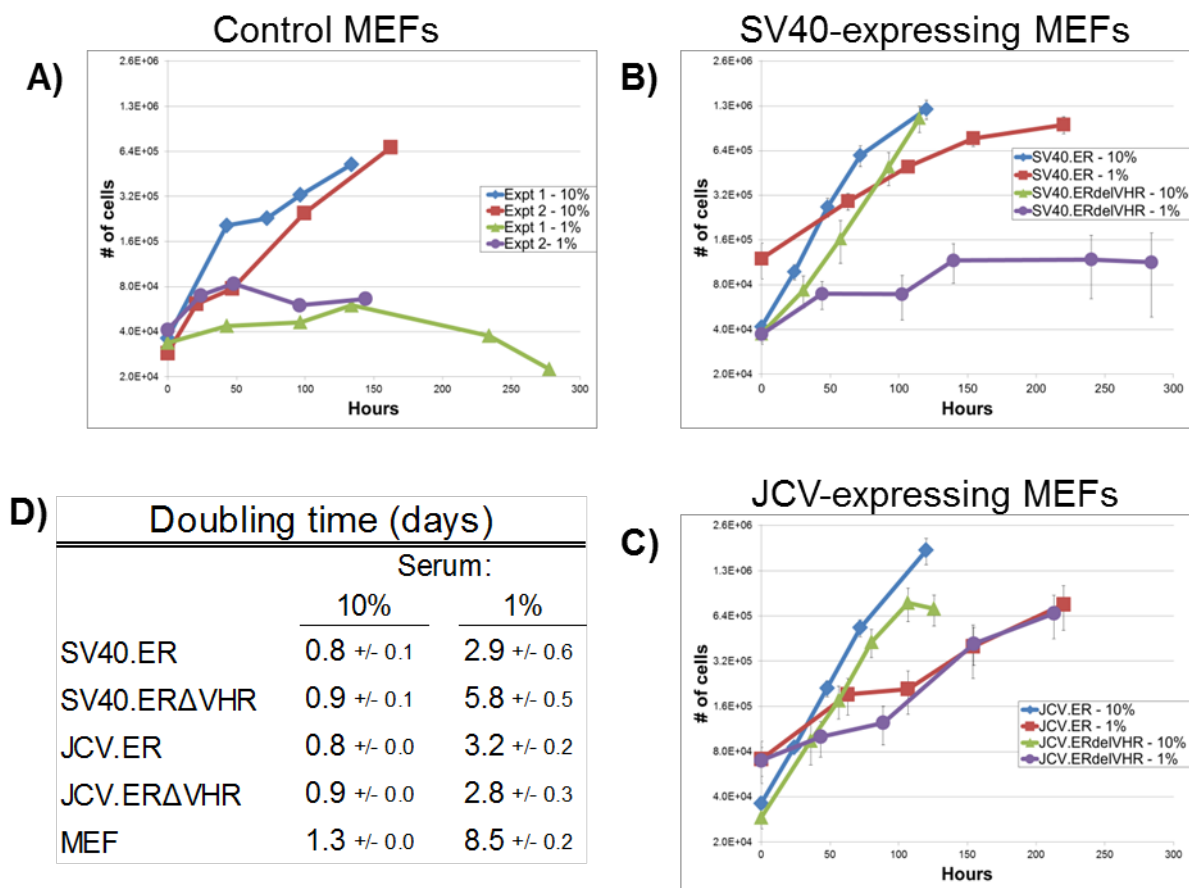**Figure 21. SV40.ERΔVHR-expressing MEFs show limited growth in low serum.**

Cells were grown in medium containing either 10% or 1% serum for up to 12 days. Duplicate wells were counted at regular intervals and averaged. Two experiments performed with wild type MEFs are shown separately (**A**). For SV40 (**B**) and JCV samples (**C**), each point on the graph represents the average number of cells from 3 different cell pools expressing the same early region construct. Error bars denote the standard error of the mean. The doubling time was calculated from time points within the exponential phase of growth and the results are summarized in (**D**). Each value represents the average doubling time from 3 cell pools expressing the same early region construct. MEF values are the average of 2 independent experiments. The standard error of the mean is indicated.

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4.2.4 Truncation of the JCV VHR region enhances anchorage independent transformation

Both SV40 and JCV early regions have independently been shown to induce anchorage independent growth. We analyzed the ability of full length and VHR-truncated viral early regions to induce this phenotype in MEFs. As expected, control MEF cells were unable to form a significant number of colonies when grown in agar suspension, but expression of all early region constructs resulted in formation of many colonies (Figure 22 A). In addition, the size of colonies formed in all early region expressing pools was significantly larger than colonies formed by control MEFs (Figure 22 B,C).

We found that SV40.ER pools generated three times more colonies than JCV.ER pools (Figure 22 A), and also showed a higher frequency of extremely large outlier colonies (Figure 22 B). However, the overall distribution of colony sizes was not significantly different between cells expressing these two constructs (Figure 22 C). This is consistent with previous reports using anchorage independence assays to compare SV40 and JCV T antigen transformation properties (Bollag et al., 1989; Hayashi et al., 2001; Trowbridge and Frisque, 1993).

MEFs expressing the SV40 early region with or without the VHR showed similar numbers and sizes of anchorage independent colonies (Figure 22 A,B,C). On the other hand, removal of the VHR from JCV early region induced an increase in the number of colonies by more than 3 fold compared to cells expressing full length JCV early region (Figure 22 A). Furthermore, the size for JCV.ERΔVHR colonies was significantly larger than that observed for JCV.ER colonies (Figure 22 B,C,D). This was reflected in both an overall increase in the number of large-sized colonies and in the high frequency of extremely large outlier colonies found in JCV.ERΔVHR cell pools (Figure 22 B).
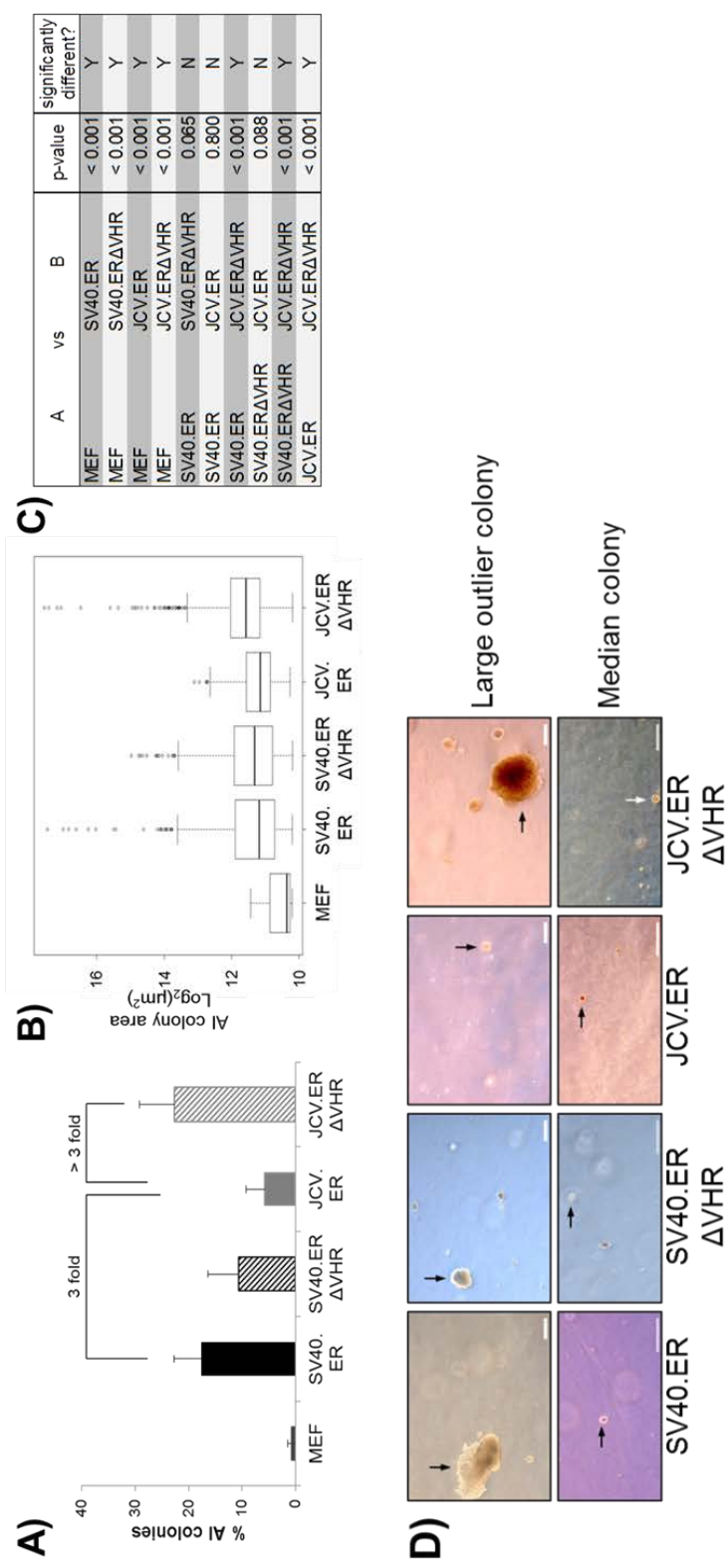
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Figure 22. Removal of the VHR in JCV TAg increases anchorage independent colony formation.

Cells expressing different T antigens were suspended in agar medium, allowed to grow for 21 days, and all single cell and multi-cell events were counted and measured as described in methods. **A**) The number of colonies was divided by the total number of events to produce the percent of AI colony formation for at least two pools expressing the same T antigen is shown (see text). Error bars represent 2 times the standard error of the mean. **B**) The sizes of AI colonies from all experiments of the indicated cell type were pooled and are shown in box-and-whisker format. The horizontal black line within the box represents the median colony size for each population. Extremely large individual outlier colonies are shown as open circles outside of the whiskers. **C**) All possible pairs of cell populations from (**B**) were tested to determine if the distribution of AI colony sizes between the two cell types was significantly different. The p-value from each test is displayed in the table. P-values less than 0.05 were considered significant. **D**) An image of the largest colony and a median-sized colony for each TAg-expressing population is presented. All images were taken at the same magnification. Images of median sized colonies were digitally magnified to better visualize the colony. Scale bar indicates 200 µm.

4.2.5   The VHR region is not required to interfere with the pRB pathway

We assessed the ability of ERΔVHR products to perturb the pRB and p53 pathways, as both must be inhibited for full LT-mediated transformation to occur. Two markers were used to evaluate the integrity of the pRB pathway. First, western blot analysis was employed to assess the steady-state levels of the cellular protein p130, a member of the pRB family of proteins, which is reduced upon binding to SV40 LT (Lin and DeCaprio, 2003; Stubdal et al., 1997; Stubdal et al., 1996). Levels of p130 in all ER- and ERΔVHR-expressing MEFs were decreased compared to control MEFs (Figure 23 A). Next, we examined the expression levels of E2F target genes, which are increased as a downstream effect of LT inhibition of pRB proteins (Cantalupo et al., 2009). RT-PCR showed an increase in the level of transcript of a set of canonical E2F-dependent genes in all cells expressing ER or ERΔVHR constructs when compared with non-proliferating MEFs (Figure 23 B). These combined results suggest that the pRB pathway is effectively inhibited in all SV40 and JCV ER-expressing cells, irrespective of VHR truncation or level of LT produced.
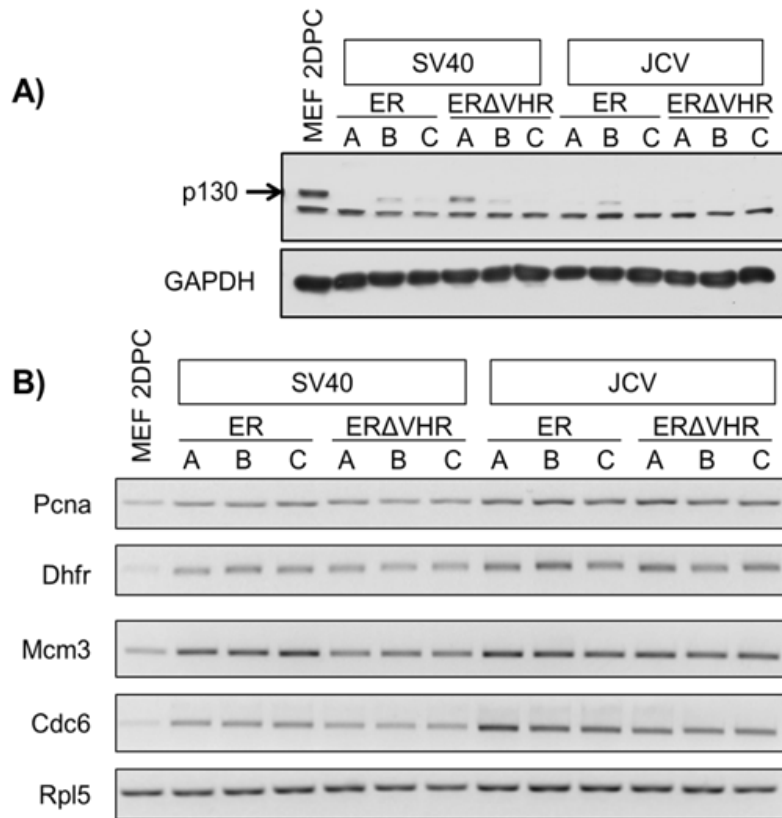
Figure 23. Removal of the VHR does not alter the ability of T antigens to disrupt the pRB pathway.

A) Steady state levels of p130 decrease in MEFs upon expression of either ER or ERΔVHR constructs, as monitored by Western blot. GAPDH was used as a loading control. **B)** E2F-target gene expression is increased in all T antigen-expressing MEFs. The expression of four E2F-regulated genes was assessed by RT-PCR of RNA from 2 days post-confluent cells. Pcna: proliferating cell nuclear antigen; Dhfr: dihydrofolate reductase; Mcm3: minichromosome maintenance complex component 3; Cdc6: cell division cycle 6. Rpl5 is an endogenous control.

4.2.6　p53 is bound and stabilized in all T antigen expressing MEFs

Inactivation of the p53 pathway by LT is critical to induce most transformation phenotypes. One of the consequences of LT expression is the accumulation of p53 protein to very high levels in the cell. However, this p53 product is not functional, as LT binds the DNA binding domain of p53 and so prevents it from activating genes whose expression would lead to cell death (Jiang et al., 1993; Lilyestrom et al., 2006; Segawa et al., 1993). All ER- and ERΔVHR-expressing MEFs showed increased levels of p53 when compared to wild-type MEFs and MEFs treated with doxorubicin, a DNA damaging agent and inducer of p53 (Figure 24) (Attardi et al., 2004; Lorenzo et al., 2002). Although lower levels of total p53 were observed in cells expressing SV40.ERΔVHR when compared with SV40.ER pools, this decrease is consistent with the corresponding decrease in the amount of LT (Figure 24, compare lanes 3-5 with 6-8). In contrast, there was an increase in total p53 levels in JCV.ERΔVHR pools in comparison to JCV.ER pools, but in this case the LT expression levels remained similar or were slightly decreased when the VHR truncated LT was expressed (Figure 24, compare lanes 9-11 to 12-14). Despite the observed differences regarding total p53 levels attained in these cells, the ability of large T antigen to bind p53 – with or without the VHR – was conserved in both SV40 and JCV as shown by immunoprecipitation (Figure 25).

In cells transformed by SV40 LT, the p53 protein bound by the large T antigen shows post-translational markers of activation (Borger and DeCaprio, 2006). We assessed the activation state of p53 in cells expressing the SV40 or JCV ER and ERΔVHR by examining K379-acetylation and S15-phosphorylation by western blot. Control MEFs were treated with doxorubicin as a positive control for p53 activation. Doxorubicin-treated cells showed a significant increase in both post-translational modifications of p53 when compared to untreated

MEFs (Figure 24, compare lanes 1 and 2). SV40.ER pools produced the highest levels of acetylated and phosphorylated p53 (Figure 24, lanes 3-5). Similar to the total levels of p53, the amount of post-translationally modified p53 was decreased when the VHR was removed from SV40 (Figure 24, lanes 6-8). JCV.ER-expressing cells displayed a very low level of K379 acetylation and S15 phosphorylation of p53 (Figure 24, lanes 9-11). Cells expressing the VHR truncated JCV early region showed increased levels of post-translationally modified p53 compared to full length JCV early region pools (Figure 24, compare lanes 9-11 and 12-14), although these levels did not reach those observed in SV40.ER pools (Figure 24, compare lanes 12-14 and 3-5). At present, it is unclear if variations in the p53 levels and modifications have a role in tumor induction by T antigens.
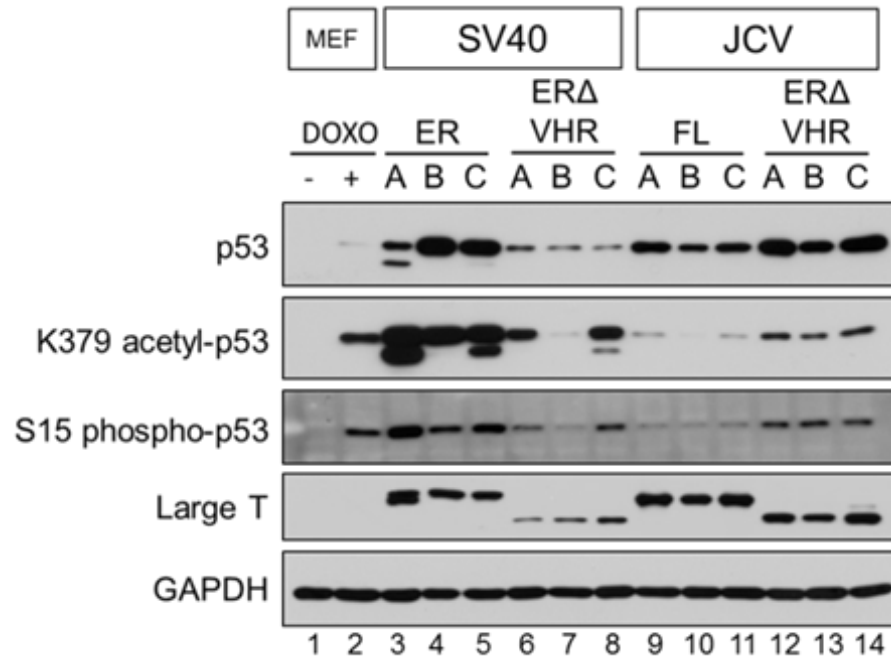
Figure 24. p53 is stabilized and shows different levels of activation in full length and VHR-truncated early region expressing MEFs.

Steady-state levels of total and post-translationally modified p53 protein were detected by western blot in T antigen expressing cells. Large T-antigen levels are included for comparison. MEFs treated with doxorubicin were used as a positive control for p53 stabilization and activation. GAPDH, loading control.
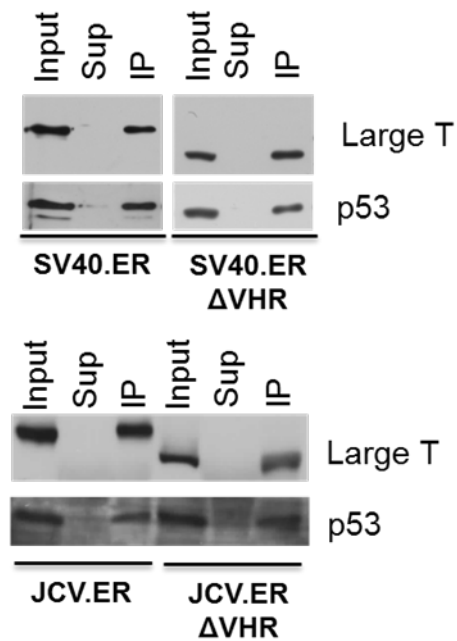
Figure 25. VHR truncated large

T antigens retain the ability to bind p53.

Protein extracts were immunoprecipitated with a p53-specific antibody (PAb 421, SV40.ER and SV40.ERΔVHR samples) or a T antigen specific antibody (PAb 416, JCV.ER and JCV.ERΔVHR samples). Co-precipitated proteins were resolved by SDS-PAGE. The presence of large T and p53 was monitored by western blot.

4.3   DISCUSSION

In order to explore contributions of the VHR to transformation, we examined the ability of SV40 and JCV full length LT and LT mutants lacking the VHR to alter the growth properties of primary MEFs. We took advantage of the co-linearity of SV40 and JCV LT amino acid sequences to design VHR truncations that would not interfere with the structure of other LT domains. The full length and truncated early regions were cloned into the same vector and expressed from the CMV promoter. T antigen-expressing MEFs were then characterized for the ability to grow in low serum and to form anchorage independent colonies.

Whereas normal MEFs proliferated slowly in 10% serum, were growth arrested in 1% serum, and could not form anchorage independent colonies, MEFs expressing the full length SV40 ER grew well at both serum concentrations and formed a significant number of AI colonies. Surprisingly, we found that cells expressing the SV40 VHR truncation failed to grow in 1% serum, but showed no change in the ability to induce anchorage independent transformation. This was unexpected since the expression of SV.N136 in MEFs was sufficient to allow growth in low serum (Figure 14). This result indicates a new level of complexity of the synergy between the different domains of the SV40 LT protein in cell culture transformation.

Some reports suggest that the degree of transformation by LT is dependent on the level of LT expression (Price et al., 1994; Sompayrac and Danna, 1992; Trowbridge and Frisque, 1993), and we observed a clear decrease in LT expression from the VHR-truncated SV40 ER, suggesting that this may contribute to the observed decrease of growth in low serum. This explanation is less likely, since anchorage independent growth was not affected. Another possibility is that a cellular protein that associates with the VHR region of SV40 LT contributes to the ability to grow in low serum. The variable region of SV40 LT shares little sequence

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conservation with that of JCV, increasing the likelihood that a unique cellular protein could bind SV40 but not JCV in this region.

Finally, the SV40 VHR mutant did not disrupt the interaction of LT with pRb proteins or binding to p53, which are required for LT to transform. However, it is possible that the VHR truncation alters the effect of LT on pRb or p53 in some way that our tests did not detect. One example could be that removal of the SV40 VHR disturbed CBP/p300 association with the LT-p53 complex, resulting in the transformation defect seen here.

In contrast to the SV40 VHR truncation, growth in low serum was not affected by removal of the JCV VHR region. Both mutant and full length JCV early regions grew at similar rates in 10% and 1% serum. Unexpectedly, VHR truncation of the JCV ER greatly enhanced the number and size of anchorage independent colonies compared to the full length JCV ER. JCV LT binding to the pRB and p53 proteins was not affected by removal of the VHR, indicating that the VHR mutant did not change one of these fundamental pathways required for transformation. Like SV40, LT expression decreased when JCV VHR was removed, but, unlike SV40, there was an increase of transformation, which challenges previous reports linking higher LT levels to increased transformation. However, due to the multiple T antigen proteins produced by the JCV early region constructs, it remains to be determined if the altered pattern of expression of JCV T antigens, the removal of the LT VHR region itself, or a combination of the two is responsible for the increased capacity to induce anchorage independent transformation.

Another possible factor contributing to the altered transformation induced by the SV40 and JCV truncated ERs is the level of p53 in the cells. For both truncated LTs, although the ability to bind p53 was not affected, the level of total, K379 acetylated, and S15 phosphorylated p53 changed depending on whether or not the VHR was present. The changes in total and
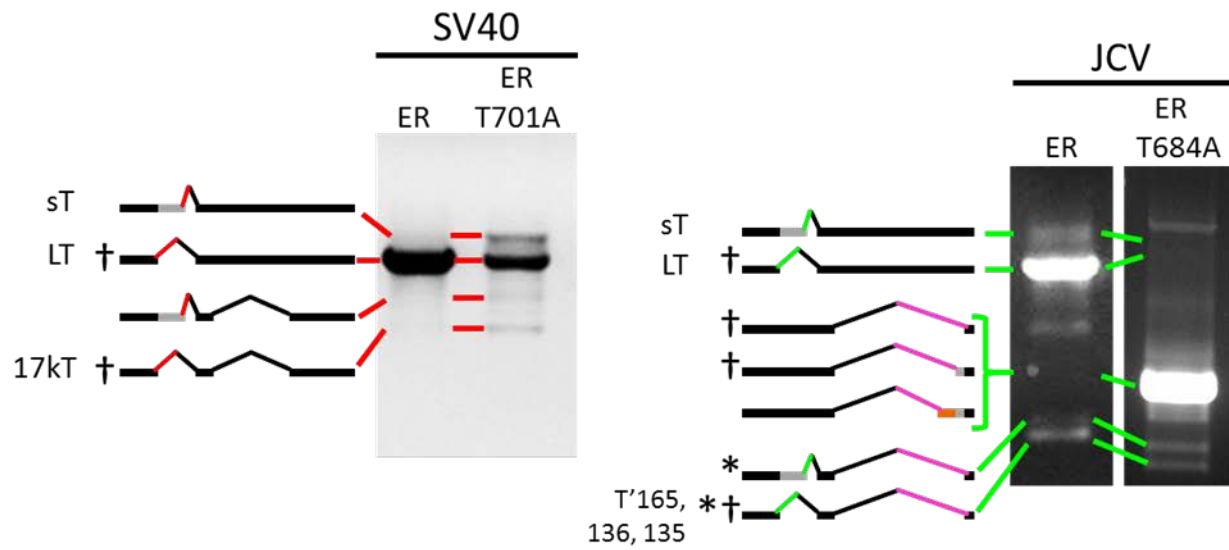
activated p53 levels correlated with the observed changes in transformation; growth in low serum was prevented and p53 levels were decreased when the VHR was deleted from the SV40 early region, and removal of the VHR from JCV early region resulted in increased anchorage independent transformation and increased p53 levels. It is unclear how the level of p53 in the cells would contribute to the ability to transform if the interaction of LT with p53 only confers a loss of p53 function.

Unfortunately, attempts to express either the SV40 or JCV VHR region alone or in combination with full length or truncated early regions in MEFs were not successful. Also, efforts to express and test individual JCV T antigens were complicated by the limited life span of MEFs. As such, complementation studies or independent characterization of the JCV T antigen proteins or of the VHR alone were unable to be pursued.
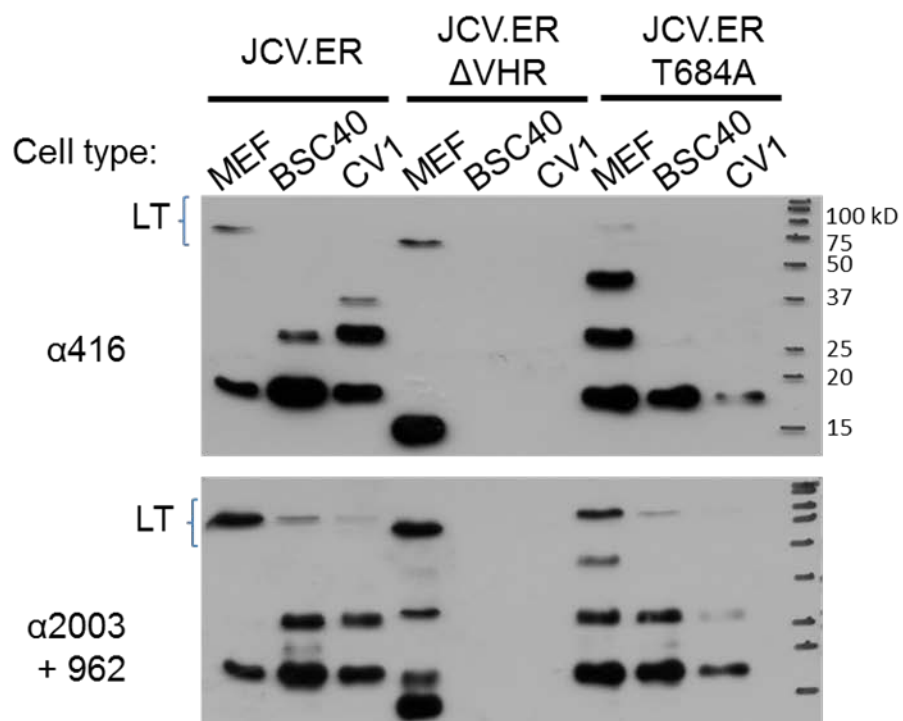
Further investigation of motifs within the VHR of both LTs was also hindered by unforeseen complications. The VHR region of SV40 has been shown to bind to the cellular protein Fbxw7, the specificity component of the SCF ubiquitin ligase complex, through a Cdc4 phosphodegron motif between amino acids 699-705 anchored by threonine 701 (Welcker and Clurman, 2005). The full significance of this interaction is not known, but it is thought that SV40 LT sequesters Fbxw7 from its normal cellular targets of degradation. Interestingly, JCV LT contains a partial Cdc4 phosphodegron motif, retaining the critical threonine residue and prolines at +1 and +2, but lacking a negatively charged residue at position +4 that was important for in vitro binding of SV40 LT to Fbxw7. Attempts to mutate the threonine to alanine or other residues resulted in a drastic change of splicing and protein expression for JCV ER (Figure 26 A and B); however, mutation of T701 to alanine did not significantly affect splicing or protein expression of SV40 ER (Figure 26 A and data not shown). The altered expression patterns in

cells expressing the JCV.ER point mutant could be due to the creation of a canonical splice acceptor site that does not occur with the same threonine point mutation in SV40 LT (Figure 26 C).

A)

B)

C)

JCV ER

ASF
SC35
ATGTTTCAAAAAACCAAAG**ACC**CCTCCCCCAAAATAA
SC35

JCV T684A

ASF
SC35
ATGTTTCAAAAAACCAAAG**GCC**CCTCCCCCAAAATAA
SRp40   SC35
ASF

SV40 ER

ASF
ASF   ASF
TAAAAAACCTCCC**ACA**CCTCCCCCTGAACCTGAAACATAA
ASF
SRp40

SV40 T701A

ASF   ASF
ASF   ASF
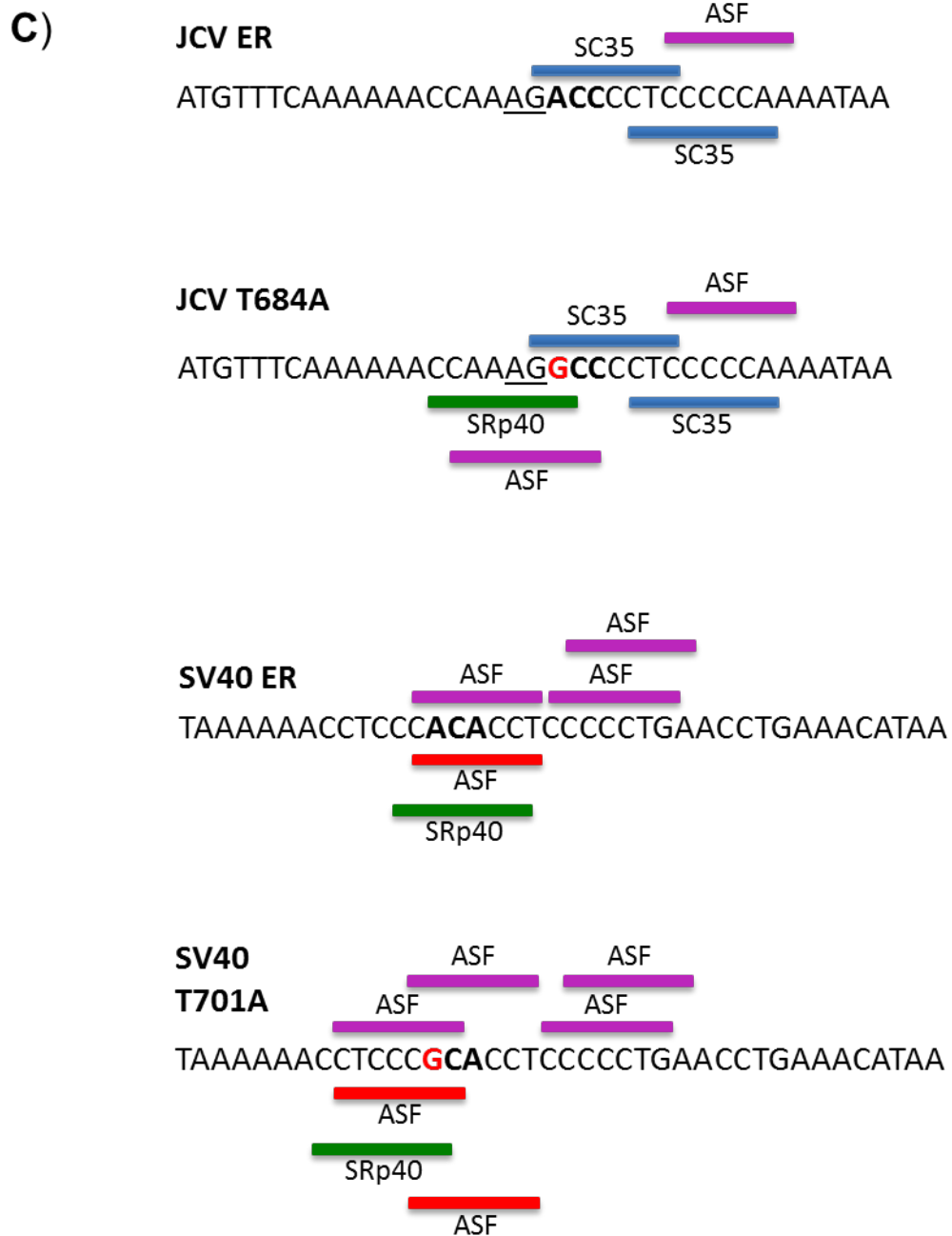TAAAAAACCTCCC**GCA**CCTCCCCCTGAACCTGAAACATAA
ASF
SRp40
ASF

Figure 26. Mutation of the threonine of the Fbw7 binding site within the JCV VHR alters splicing and protein expression of T antigens.

A) Transcripts from MEF pools expressing full length ER or a T to A mutation of the Fbw7 binding site of JCV or SV40 constructs were reverse transcribed and amplified as described in Figure 18C. Predicted alternatively spliced mRNA species that correspond to the cDNA bands are depicted on the left. Transcripts for known T antigen products are designated.

†, these mRNA species were verified by sequencing. *, due to limiting resolution, these JCV TAg bands could potentially include mRNA species with other 3' splice acceptor sites; however, only the one shown has been verified by sequencing. **B**) The JCV ER, ERΔVHR, or ER.T684A constructs were expressed in MEFs and two monkey cell lines, BSC40 and CV1, and protein expression of T antigens was observed by Western blot. The top panel shows a blot performed with anti-SV40 antibody, 416, and the bottom panel was blotted with anti-JCV antibodies, 962 + 2003. The location of LT is indicated. The proteins below 25 kD should include sT, T'165, T' 136, and T'135 proteins; however the resolution of this gel was not good enough o say which band is which. Proteins located between 50 and 25 kD are unique JCV T antigen species that have not been previously described. These westerns were performed by M. T. Saenz Robles. **C**) The last 37 nt of JCV and 40 nt of SV40 normal and mutated ERs and were analyzed in the ESEfinder program to identify any change in exonic splicing enhancer binding sites introduced by the point mutation. The threonine codon is highlighted in bold, and the point mutation used to change the codon to alanine is in red. A cryptic 3' splice acceptor site found in the JCV sequences is underlined. The point mutation in the JCV sequence introduces two new potential ESE binding sites and creates a common splice acceptor nt sequence AGG. The SV40 point mutation also adds more ESE binding sites, but no cryptic splicing acceptor sequences were found. ESE analysis was performed by Ping An.

A final perplexing result was the decrease in both SV40 and JCV LT protein expression when the VHR was removed. Why did these protein levels go down? LT mRNA analysis did not show a difference in LT transcript between full length and VHR truncations, indicating there was not a change in transcription of LT. I also performed preliminary experiments to determine if there was a decrease in protein stability when the VHR was removed (data not shown). Cyclohexamide chase showed no difference in half-life for JCV LT proteins, and only a slight decrease in half-life for SV40 LTΔVHR. These results did not convince me that stability was an issue. Several other mechanisms could regulate the level of protein including mRNA stability and shuttling out of the nucleus, frequency of translation of the LT transcript, or association to polysomes. These mechanisms will require further experimentation; however, at present, the results indicate that the VHR region is somehow linked to translation of the LT transcript.

In summary, this is the first evidence linking the VHR region to transformation by any polyomavirus T antigen. Overall, our results indicate that the VHR from each virus early region confers different functional capabilities in terms of transformation, and that different transformation phenotypes are probably induced by distinct molecular mechanisms. Given these results, it is possible that VHR or C-terminal tail regions found in polyomaviruses other than SV40 may contribute to transformation in different ways than SV40 LT.

5.0   FUTURE DIRECTIONS AND PERSPECTIVES

While we have learned much about polyomavirus T antigen induced transformation and tumorigenesis from the study of SV40 and MuPyV, the investigation of other PyVs has brought challenges to these established models. This thesis focused on two observations. The first unexplained observation is that BKV and JCV LTs do not transform with the same efficiency as SV40 LT, even though the amino acid sequences and mechanisms of transformation are highly similar. Second, the mechanisms of transformation of LPV are not well characterized and this has returned to the spotlight since the discovery of a very similar human virus, HuPyV 9. I took a structure-function approach to understand how individual domains of the LTs contribute to transformation by these viruses. Removal of specific domains of each LT revealed unique transformation properties for some polyomavirus proteins. Although fairly similar to SV40 in sequence, the short N-terminal fragments of BKV and JCV LT comprising the pRb binding domain and linker regions showed an additional transformation ability compared to an equivalent fragment for SV40 and LPV LTs. At the other end of the LT protein, removal of the variable linker and host range region from SV40 and JCV LTs induced changes in transformation by both proteins. These changes were opposing and specific to a particular assay. JCV LT VHR deletion allowed enhanced anchorage independent growth, while SV40 LT deletion prevented growth specifically in the low serum assay. The results from both studies highlighted many questions about the roles of individual T antigen proteins in transformation, the possibility of a p53 gain of

function activity when in complex with LT, and the ability of C-terminal tails to influence transformation potential. Below I discuss the future directions to address these questions.

5.1 UNCOVERING THE MECHANISMS OF ENHANCED TRANSFORMATION BY DELETION OF THE VARIABLE REGION AND HOST RANGE REGION FROM JCV EARLY REGION

While removal of the VHR region from both SV40 and JCV early regions resulted in interesting changes in transformation, the effects of truncation on the JCV early region should take priority in further studies for several reasons. First, JCV is a human virus that is directly involved in human disease and possibly cooperates in the development of some types of human cancer (as discussed in section 1.4). Thus, a better understanding of the regulation of transformation by JCV T antigens would directly benefit the human condition. Second, although truncations of JCV large T have not been observed in human samples, it is possible that a similar truncation could occur in an infected tissue and contribute to transformation. Third, one of the possible reasons for enhanced anchorage independence may lie in the altered expression of the JCV T' proteins. Many other human polyomaviruses produce several T antigens while SV40 does not. Therefore, understanding the role of JCV T' proteins in transformation may give insight into the other human polyomavirus T antigens. Finally, the more refined technique for assessing size and number of anchorage independent colonies developed in this work will allow further experiments to be monitored with precision, as compared to the low serum assay which is more susceptible to human error. Two possibilities should be experimentally explored to explain the enhanced

transformation capability of the VHR truncated JCV early region. These mechanisms are not mutually exclusive.

First, there is the possibility that the removal of the VHR region from the JCV LT protein removes a binding site for a cellular protein that, when bound to JCV LT, dampens its ability to induce anchorage independent growth. A more refined and targeted mutation strategy within the VHR region tested in the anchorage independence assay could help to identify any cellular protein binding sites that enhance transformation.

The VHR consists of two parts: the variable region and the host range region. The variable linker (variable in that this sequence differs the most between the four viruses that contain a VHR region) of JCV LT is 28 amino acids long and links the ATPase domain to the 35 amino acid host range region (Figure 19 C). The host range region (HR) was initially identified in SV40 LT as required for virus to form plaques in CV-1 cells, but not in BSC or Vero cells (Tornow and Cole 1983, 1987). In addition, removal of the SV40 HR prevents a phenotype known as the adenovirus helper function (Tornow and Cole 1983). At least one known cellular protein, Fam111A, binds to full length SV40 LT through the HR, and is required to allow infection of certain cell types and to maintain the adenovirus helper function (Fine et al., 2012). The normal cellular function of Fam111A is currently unknown. The binding site is fairly conserved in JCV HR region, but has not been tested (Figure 19 C). Another protein known to bind specifically to the JCV LT VR is β-Transducin-repeat-containing protein 1/2 (Reviriego-Mendoza and Frisque, 2011), which has not been tested for its potential to contribute to transformation by JCV LT.

To understand if the binding of these proteins to the JCV LT VHR contributes to transformation they should be targeted for point mutation. Alanine or oppositely charged residue

substitutions of these sequences alone and in combination should be sufficient to reveal any effect on transformation. Since the Fam111A binding site is found in both SV40 and JCV HR regions, I expect that mutation of this site should not enhance transformation of MEFs by JCV LT. Conversely, as β-Transducin-repeat-containing protein 1/2 only interacts with JCV VR, this is a good candidate for potentially contributing to alteration of transformation. If there is no effect on transformation by mutation of these sequences, then a more general approach can be taken to isolate regions that, when deleted or mutated, enhance transformation. For instance, the HR or VR region alone can be deleted, or alanine scanning of the residues of each region may be performed. Alanine scanning should be performed with care so not to drastically change RNA signals in the T antigen mRNA that could complicate results (see section 4.3 Discussion).

Second, the altered expression of JCV T antigen proteins could be responsible for the enhanced transformation of the JCV VHR truncation. The truncation was prevented from expressing T'165 and T'136, and the level of T'135 appeared to increase relative to the full length JCV early region (Figure 20 B and C). This could mean that T'165 and/or T'136 dampen the ability to transform cells to anchorage independent growth by JCV LT, or that T'135 overexpression enhances transformation by JCV LT. There is one piece of evidence that mildly supports this hypothesis. In one previously published study from the Frisque group, each T' protein was tested individually for its ability to cooperate with activated H-Ras to transform primary rat embryo fibroblasts (REFs) in a dense focus assay (Bollag et al., 2006). While all T' proteins were able to cooperate with Ras, a test of immortalization of cells from several foci resulted in only half of the T'165 + Ras cell lines becoming immortal. In contrast, practically all of the T'135 and T'136 + Ras cell lines immortalized. This may suggest a potential inhibitory effect on transformation by T'165. Additionally, the authors felt that T'135 had the most robust

effect in the Ras cooperation assay since there was a consistently higher number of foci formed compared to the other T' proteins. Unfortunately, a complete combinatorial analysis was not performed in this study.

The effects on transformation when different combinations of JCV T antigens are expressed must be assessed. This includes the full length and VHR truncated JCV LT proteins expressed in combination with each T' protein and sT. From a review of the JCV T antigen literature it is apparent that many studies use a JCV LT cDNA (which prevents sT expression but can still allow for T' protein expression) without checking for T' protein expression. Thus, a transcript that will only produce JCV LT must be created to gain a clear picture of how JCV LT behaves on its own in transformation studies. Of note, the Frisque group was successful in creating a LT only expression plasmid only after mutation of several cryptic splice sites that arose after mutation of known T' splice sites; however, this construct was not tested in transformation assays (Prins and Frisque, 2001). Only with a LT only construct can the addition of T' T antigens be properly evaluated for the effect on transformation. The most direct experiment would be to compare anchorage independent growth of cells expressing LT+sT+T'135 or LTΔVHR+sT+T'135 to determine if the presence of the VHR region has an effect when only these three JCV T antigens are present.

The T' proteins share the same first 132 amino acids with each other and JCV LT, differing only in a short tail at the C-terminus (Bollag et al., 2000). For T'135 and T'136 this short sequence contains three or four out of frame amino acids, respectively. The T'165 C-terminal sequence, on the other hand, is the final 33 amino acids of JCV LT HR domain. Since the majority of the amino acid sequence of the three T' proteins is identical any differences in

behavior would most likely be attributed to the different small C-terminal regions. These could easily be truncated or mutated to test this hypothesis.

The testing of each of these potential mechanisms of transformation is likely to reveal many more questions to pursue to understand how the VHR of JCV T antigen and the combination of T antigen proteins affects transformation. Still, the knowledge gained from these studies in mouse embryo fibroblasts can be transferred to developing experiments in animal models of T antigen tumorigenesis or transformation of human cells, which would be more relevant to human disease.

5.2   DEFINING THE ROLE OF THE LT-p53 COMPLEX IN T ANTIGEN INDUCED TRANSFORMATION

One theme that arises from the work presented here is the relationship between LT and p53. Experiments with MEFs expressing the N-terminal fragments of SV40, BKV, and JCV LT in section 3.0 suggest that p53 interaction with LT is critical for robust dense focus formation and the ability to grow anchorage independent. In the presence of the N-terminal fragment, uninhibited p53 could become activated by stress signals in these environments and induce growth arrest. This would argue that the role of LT-p53 interaction is inhibition of the transactivating functions of p53. Indeed, as discussed in section 1.5.2, many studies have shown that binding of LT to p53 prevents the activation of its normal gene targets.

On the other hand, the direct correlation between levels of p53 and transformation seen with the SV40 and JCV LT VHR truncations suggest that p53 could be acquiring a gain of function activity when in a complex with LT. It is possible that LT could redirect the activities of

bound, activated p53 to directly activate non-canonical genes. Another possibility is that LT uses p53 as a bridge for chromatin modifying enzymes, like CBP and p300, which would enhance transcription of genes required for transformation. Further experiments are needed to test this hypothesis, but at least two publications also support this idea. Deppert et al showed that SV40 LT could not efficiently transform p53 knock out MEFs or cells that contained a p53 that had been mutated to remove the transactivation domain (Hermannstadter et al., 2009). Additionally, when p53 transactivation was impaired by expression of a dominant negative p53, SV40 LT mutants that could not bind p53 were not able to transform cells (Sachsenmeier and Pipas, 2001). This experiment argues that inhibition of p53-target gene expression is not the only element of LT-p53 binding required for transformation.

Experiments to better understand if a gain-of-function exists will be complicated but should be pursued. The first thing that comes to mind is to remove p53 from a cell and see if the T antigens can still transform. Small interfering RNA treatment to remove or alter the levels of p53 in an already transformed cell would determine if the amount of LT-p53 complex influenced the transformation state. Studies with SV40 indicate that the p53 bound to LT is very stable; thus, the effect of siRNA knockdown might not be seen since this technique works at the level of translation. p53 might also be removed from the system by using cells from p53 -/- mice. When I put ER and ERΔVHR constructs of SV40 and JCV into p53 null MEFs (received from A. Levine), LT expression decreased dramatically complicating the fact that anchorage independent growth of the cells was also abolished (Figure 27 A and C).

The binding of LT to p53 may stabilize not only p53 as was discussed before, but also LT. However, a preliminary treatment with MG132 did not show a change in LT protein expression, which argues against the loss of stability of LT when p53 is absent (Figure 27 B).

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This further suggests that the absence of p53 affected LT expression at the level of transcription or translation. How p53 could affect LT at these stages is not clear, and it is still possible that other protein degradation pathways are acting on LT when p53 cannot bind.

Inversely, the LT-p53 complex could be prevented by mutating the p53 binding site with LT protein. This would ensure that only the interaction with p53 is prevented, as compared to the N-terminal fragments used in section 3.0 where any interactions with the C-terminus of LT that would contribute to transformation were removed. I mutated a critical aspartic acid (D) residue to asparagine (N) in SV40 and JCV ERΔVHR constructs to prevent LT-p53 binding (Figure 28). Unfortunately, the MEFs with the SV40 construct repeatedly did not survive drug selection after transduction, suggesting that the combination of the p53 binding mutation and removal of the VHR prevented LT immortalization. In contrast, I was able to create two selected pools of MEFs expressing JCV.ERΔVHR.D403N. Perhaps the many alternative T antigens expressed from the JCV ER as compared to the SV40 ER confer a growth advantage that prevents the lethality seen with the SV40 construct. As seen in the p53 null MEFs, JCV LT protein expression was greatly reduced in the JCV.ERΔVHR.D403N MEFs, again confusing the interpretation of the transformation results (Figure 28 A). Prevention of p53 binding to JCV LTΔVHR greatly reduced transformation (Figure 28 B). In both experiments where the LT-p53 complex was prevented, the LT protein levels and transformation decreased.

At least two questions arise from these observations which will need to be further investigated. First, it still remains unclear as to whether *active* p53 is required to bind to LT to induce transformation since the severe reduction in LT levels in p53 null MEFs may have been below the threshold required to transform cells. In this case, it would be interesting to introduce and compare mutants of p53 that prevent promoter transactivation, mutants that inhibit binding

of p53 to specific chromatin modifying proteins, or wild type p53 into the p53 null MEFs with T antigen. These cells could then be compared in transformation assays and to see if binding of a "dead" p53 would rescue levels of LT. Second, why do both SV40 and JCV LT levels drop when p53 cannot bind to LT? As mentioned before, p53 interaction might be preventing the degradation of the LT protein by non-proteasome dependent pathways. There is evidence that some cellular protein levels are maintained through autophagy (reviewed in Cuervo and Wong, 2014), which supports this theory.
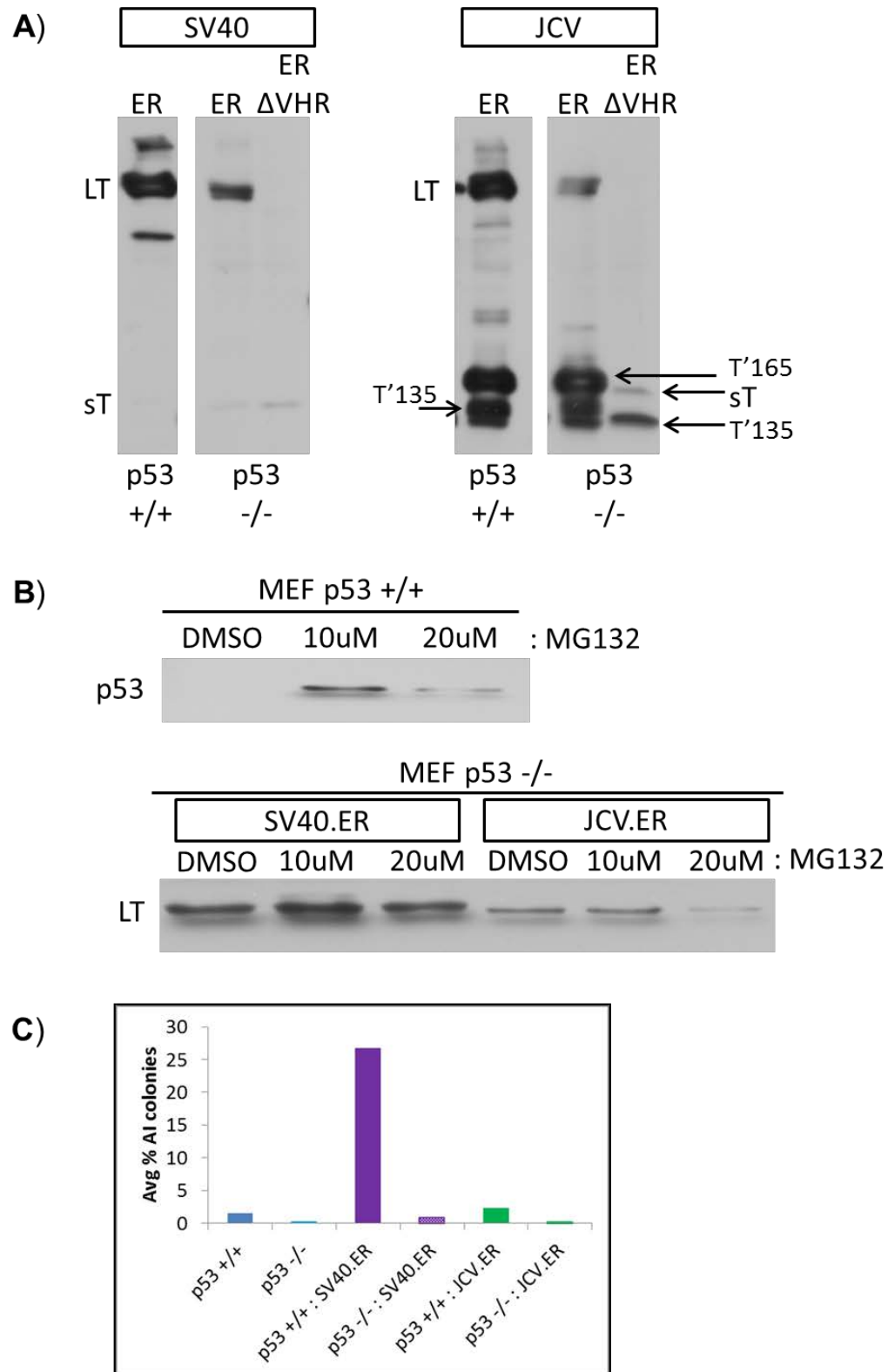
Figure 27. LT expression and transformation are reduced in p53 null MEFs.

The SV49 and JCV ER and ERΔVHR constructs were expressed in p53 -/- MEFs and compared to the MEF p53 +/+ SV40 and JCV ER pools described in section 3.0. **A**) Protein expression of T antigens by Western blot. All lanes are from the same blot, but some lanes were removed for clarity. A mixture of SV40 and JCV antibodies was used to detect the T antigens. Note the decrease in LT expression for both SV40 and JCV constructs when p53 is absent. The levels of the smaller T antigens in the JCV.ER cells do not change regardless of p53 expression. **B**) Inhibition of the proteasome by MG132 treatment does not significantly change LT levels in p53 -/- MEFs. MG132 was dissolved in DMSO and added to cell culture medium at a concentration of 10 or 20 µM. Negative controls received the same amount of DMSO without MG132. After a short incubation, cells were collected and LT expression was observed by Western blot. The same procedure was performed on p53 +/+ MEFs with no T antigen to determine if the concentrations of MG132 used were effective. p53 was monitored in these cells since it is known to have a very short half-life and be degraded by the proteasome. These experiments were performed by Memphis Hill. **C**) Anchorage independent (AI) colonies formed in a preliminary soft agar assay. MEFs with (+/+) or without (-/-) p53 did not form a significant number of colonies without T antigen. AI growth was essentially abolished in cells expressing SV40 or JCV ER, but lacking p53.
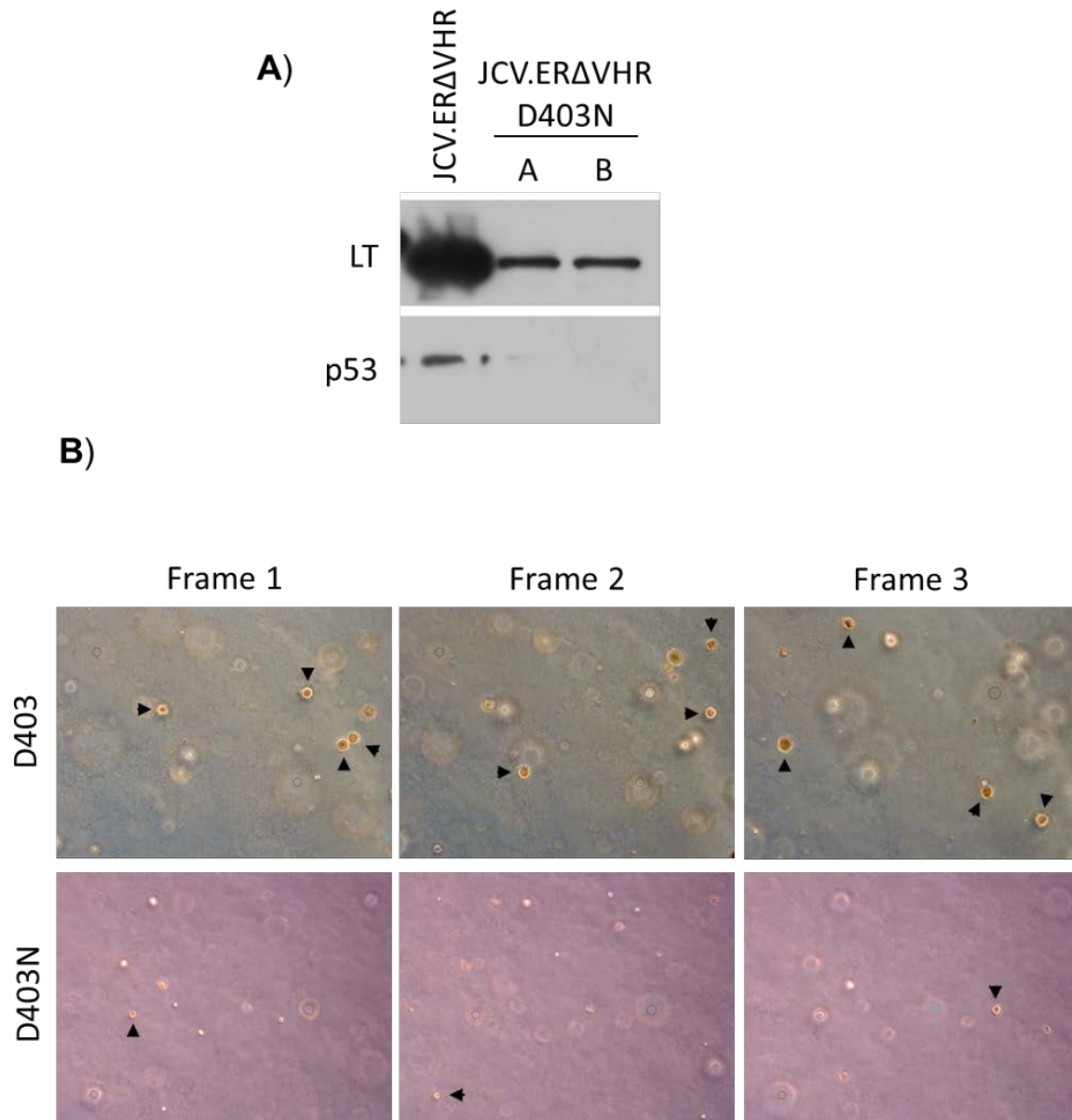
Figure 28. The D403N mutation prevents p53 binding to JCV LTΔVHR and reduces its ability to induce anchorage independent growth.

A point mutant was introduced into the ATPase domain of the JCV.ERΔVHR construct to prevent binding of p53 to LT ΔVHR (D403N). **A**) Protein expression analysis by Western blot showed a decrease in LT expression of the D403N mutant compared to the unaltered JCV.ERΔVHR expressing MEFs. p53 was undetected in the D403N samples, suggesting that p53 was not binding to the LT as expected. **B**) Both unaltered (D403) and mutated (D403N) JCV.ERΔVHR were tested for the ability to induce anchorage independent growth in MEFS. Each image in a row is from the same field of view of a soft agar plate, but each is focused at a different depth of the agar layer. Black arrows indicate unique AI colonies. It is clear that the lack of p53 binding to LT has reduced both the number and size of AI colonies.

5.3 HOW DO ALTERNATIVE T ANTIGENS OF OTHER POLYOMAVIRUSES AFFECT TRANSFORMATION?

Several of the newly discovered human polyomaviruses produce T antigens in addition to LT and sT. While SV40 also produces an alternative T antigen, 17kT, it was shown not to be important for transformation, and so interest in the alternative T antigens fizzled out. The work presented in this thesis points to potential roles at least for JCV, but also possibly for BKV, in transformation.

JCV early region produces three proteins, T'135, T'136, and T'165, in addition to LT and sT. All three proteins contain a J domain, N-terminal linker, and a small tail that is either in frame with the JCV LT VHR region (T'165) or out of frame (T'135, T'136). This makes these proteins similar to the JCV N137 fragment used in Chapter 3. Congruent with the JCV N137 effect on p130, all three T' proteins have been shown to bind pRB; however, they may bind to pRB, p107, and p130 with different affinities (Bollag et al., 2000). Previous work has shown that the T' proteins have some transformation potential when expressed alone – including growth in low serum and cooperation with activated Ras to form dense foci (Bollag et al., 2006), which is similar to what was seen with JCV N137 fragment. Moreover, when only the LT was expressed (because all splice sites for T' proteins and small T were mutated) only 1 clone out of 30 survived the selection process and LT expression was low (Bollag et al., 2006), suggesting these proteins grant an advantage when expressed with LT. Other work presented in Chapter 4 suggests that T'165, whose C-terminal tail is part of the JCV VHR region, may confer a negative effect on transformation. In fact, one possibility for the increase in transformation seen when the JCV VHR was removed could be that expression of T'165 and T'136 was prevented, leaving

only LTΔVHR, sT, and T'135. The combination of T' proteins, LT, and sT may be an important factor in JCV early region induced transformation.

Human Merkel Cell PyV, BKV, and STLPyV also produce T antigen proteins besides LT and sT. BKV produces one additional T antigen protein, called mini T (Abend et al., 2009), and it's sequence closely resembles JCV T'135 or T'136. STLPyV and MCPyV "other" T antigens appear to be unique in sequence and could therefore also have unique functions in transformation as well as viral replication. STLPyV also creates one additional T antigen protein, 229T, that contains the entire sT coding region with an additional 38 amino acids that are not in frame with the LT (Lim et al., 2013). PyV small T antigens are important transforming proteins and an added 38 a.a. may allow for additional cellular proteins to bind that have not been seen before for other sT proteins. Most fascinating is MCPyV, which makes 57kT and ALTO T antigens in addition to LT and sT. 57kT is similar to JCV T' in that it contains the J domain and N-terminal linker of LT; however, 57kT also codes for a portion of the OBD and the last 100 a.a. of the MCPyV LT (Spurgeon and Lambert, 2013). This last 100 a.a. includes part of the helicase domain and a 26 a.a. C-terminal tail which may be akin to the VR region of JCV LT. Another interesting observation is that in Merkel cell tumors MCPyV LT is frequently truncated to remove the helicase domain and C-terminal tail, and therefore the 57kT product (Borchert et al., 2014). 57kT could potentially have a negative effect on transformation alone or when in combination with other MCPyV T antigens, similar to that hypothesized for JCV T'165. In fact, 57kT imparted a growth inhibitory effect to cells when expressed independently compared to full length LT or a truncated LT (Cheng et al., 2013). The mechanisms of action for these observations remain to be uncovered. Finally, MCPyV ALTO protein is the first T antigen of its kind (Carter et al., 2013). ALTO is transcribed from an alternative start site within the early

region. The protein spans the entire N-terminal linker region but is frame-shifted so that the protein sequence is entire different from the other T antigens. So far no biological function has been assigned to the ALTO protein, but it was shown to be expressed during, but not required for, MCPyV DNA replication, pointing to a possible role in reprogramming of the cellular environment.

Taken together, the hypothesis put forth for JCV T' proteins and the published work discussed here suggest that "other" T antigens from human PyVs have the potential to have both positive and negative contributions to transformation. Therefore, more careful consideration of these alternative T antigens is needed in transformation and tumorigenesis studies.

5.4   C-TERMINAL TAILS OF LARGE T ANTIGENS ARE LIKELY TO HAVE A MORE SIGNIFICANT ROLE IN POLYOMAVIRUS BIOLOGY

The identification of the variable linker and host range region as a 4th region of large T antigen to contribute to transformation prompted a closer look at sequences following the ATPase domains of other T antigens (termed C-terminal tails). The goal was to identify any conserved regions or motifs. Conserved regions would likely be important for some aspect of polyomavirus biology, and, therefore, mutation of these regions would provide the best chance to observe a phenotype. It should be noted that not all PyV LT proteins have a C-terminal tail.

I analyzed LT sequences with C-terminal tails from 48 different polyomaviruses over a range of host-specificities. For clarity of alignments, I removed the HR-specific portions from SV40, BKV, JCV, and SA12, leaving just the VR region. (As a side note, these are the only LTs that contain an HR-like sequence). Sometimes there were subgroups within polyomaviruses that

infect the same type of host (primate and humans), whereas others showed no identifiable similarities in the C-terminal tails (bats and birds); however, grouping the sequences by similar host species did not reveal anything unique. As a whole, the C-terminal tails fell into two major groups, those that began with proline-leucine (PL) or isoleucine-leucine (IL) amino acids (Figure 29 A and B). A third group was made up of all non-PL/IL sequences (Figure 29 C). A few common themes were found associated with each group.

The majority of "PL" group sequences contained a motif at the N-terminus, P L x x I/LV I/L/V. This motif is often followed by a high density of negatively charged amino acids. A search for other viral or cellular proteins that contain a similar motif was so varied that no significance could be seen. However, the conservation of this motif in so many C-terminal tails makes it a candidate for mutation and further testing. About half of the PyV "PL" sequences also contained a partially conserved motif at the C-terminal end that begins with DSG followed by some combination of F, Q, and T.

"IL" group C-terminal tails were correlated with higher serine (S) and glutamine (Q) content. Three sequences of the "IL" group also contained high proline content. I could not find any information about SQ repeats or content within known proteins; however, there is a gene found in humans and animals called glutamine serine rich 1 (QSER1). Microarray analysis found expression of QSER1 to be significantly increased in livers from patients with recurrent HCV hepatic tumors (Das et al., 2013). Additionally, QSER1 protein was found associated with mitotic RNA polymerase II in a proteomic analysis of this complex (Moller et al., 2012). Although it is hard to draw links between QSER1 and the SQ content of the PyV C-terminal tails, the information about QSER1 suggests that this grouping of amino acids has an important biological function that PyVs could be mimicking. Finally, DSG-beginning motifs were also

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found in this group; however, the motif was followed by different amino acids from those of the "PL" group. The DSGHGSS motif, shown to bind to cellular protein β-TRCP 1/2 in JCV LT, was conserved in SA12 and BKV. An alternate motif, D-S-G-hydrophobic-x-S, was found in 3 sequences in this group.

There were a few outlier C-terminal tails that did not begin with "PL" or "IL". These sequences did not share significant homology with each other, but I did identify degenerate DSG motifs in three out of five. HuPyV 6 and 7 had an identical motif, E-S-G-I-G-S, while California sea lion PyV contained the D-S-G-hydrophobic-x-S motif seen in the "IL" group.

Research into DSG motifs revealed that they are commonly found in human proteins as "phosphodegrons" (Jiang et al., 2011; Lau et al., 2014). Phosphodegrons are motifs containing either a phosphorylated serine or threonine residue, which is bound by the substrate-specificity component of the ubiquitin ligase complexes. The phosphorylated phosphodegron therefore marks a protein for ubiquitination, and ultimately degradation. There are 69 putative Fbox proteins in humans, and at least three have been shown to be mutated or overexpressed in some cancers (Lau et al., 2014). Interestingly, two Fbox proteins are already known to bind PyV LT proteins in the C-terminal tails: JCV binds β-TRCP 1/2 (aka Fbxw-1 and Fbxw-11) (Reviriego-Mendoza and Frisque, 2011), and SV40 binds Fbxw7 (Welcker and Clurman, 2005). Neither of these interactions targets the LT for ubiquitination, favoring a sequestration model, although the effect of sequestration is not understood.

Clearly, C-terminal tails of PyV LTs play a significant role in the biology of some of these viruses. The conservation of DSG phophodegron-like motifs across PyV species suggests an important function for Fbox protein binding in the life cycle of these PyVs. Moreover, the identification of subgroups of conserved motifs and patterns of amino acids indicates that some

PyVs have gained additional, important functions in this region. The biological role of these conserved regions remains to be seen, but promises to unlock a whole new arm of cellular and polyomavirus-related biology.

A)

PyV Name	Accession	Sequence
NJ PyV	NJPyMv_4	PLTDL-----IDEGDN
Chimp PyV	YP_004046683	PLTDI-----IDEGDIA
Red Colobus	YP_0071195258	PLTNI-----LQEDEPDD
Vervet	YP_0071188590	PLTNI-----LQEDEPDD
N. Freetail Bat	YP_0073465954	PLHGI-----IEEEDTGDD
Bat (Otomops)	YP_0073465959	PLTDI-----LEEYEDTDS
Bat (Cardio.)	YP_0073465964	PLKGI-----TEDDQSEDDSNQ
Bat (Molos.)	YP_0073465984	PLHGI-----LEYEEDEED
Bat (Eidolon)	YP_0073465969	PLRGVDPVEEEEENDDTTQ
Merkel Cell	ACI25294	PLLNI---LIEEEGPEE-TEETQDSGTFSQ
P. Troglodytes 4	YP_0071195285	PLHDI---VVCEDDNDE-TED----SGNFTQ
P. Troglodytes 3	YP_0071195281	PLYGL---VVEDENEQD--------SGNYTQ
TSV	YP_003800007	PLDSV---VIEVEDEEE-------EEFSETNDSGFQTQ
Orangutan	YP_003264534	PLDGI---VIEVEDEEE-------EFSETND-SGFQTQ
Sider Monkey	YP_0071195273	PLEGL---VIEVEEVG-GNS-ENGNDSM-NDSGFQTQ
Murine PyV	NP_041264	PLKDI---CDIAEYTVY
Hamster PyV	NP_056730	PLEAL
HuPyV12	YP_007684356	PLHDI---VTEADE
HuPyV9	YP_004243706	PLHGI---VIEEQA
P. Troglodytes 5	YP_0071195289	PLHGI---VIEEQI
Long tail Macaque	YP_0071195262	PLDGI---VIEEQM
AGM (LPV)	NP_848008	PLHGI---VIEEQM
P. Troglodytes 2	YP_0071195293	PLDGL---VIEEE
MW PyV	YP_006495789	PLKDI--LICVDADED-TQQ----DSGINSQ
STL	YP_007354885	PLKNI--LICEDTENN-ETQ----DSAFCTQ----DSDNE

B)

```
            WU YP_001285488  ILEKHNA-------------------------------------------
            KI YP_001111259  ILEKWFE-------------------------------------------
  Bat (Mini) YP_007346974  ILTKRAGGTPPASPLSSPPSSPQPPTQSTKHSCQENDSGFDSMSMTQ---
  Bat (Pter) YP_007346979  ILDEEEDEMQTDCQRMPPPGPPPYTPVDPRTQSQPNSQTSQETPDSGVFSQASQ---TS
           BK YP_717940    ILDITRE-------EDSETEDSGHGSSTESQSQCSSQVSD----TS
         SA12 YP_406555    ILDWARE-------EESETEDSGHGSSTESQSQCFSQASD----TSGSA
           JC NP_043512    ILDFPRE-------EDSEAEDSGHGSSTESQSQCFSQVSE----ASG
         SV40 YP_003708382 VLDWLRN-----SDDDDEDSQENADKNEDGGEKNMEDSGHETGIDSQ----SQGS
       Equine YP_006383692 ILKRETD----------PPSPPESMEDSGVGTESQEPNLSQQM------
    Sq Monkey YP_001531349 ILEKQQD--------SGIFTQTQDTECQQ----------------
  Capu Monkey YP_007195277 ILTKHED-----SGIF------TQSQSQSQDTQCTV---------
       Bovine NP_040788    ILEK---------------------------------------------
     elephant YP_008603286 ILEDEMN---------------NETQDSGLESGLETETDSRDFTD----
```

C)

```
         HuPyV 7 YP_003848924  PFEGVLINDP---TEENTRETQESTESGIGSMNN---------
         HuPyV 6 YP_003848919  PFTNVLVDESFVQPQENDETNDSTQESGIGSMHSM--------
 Mouse eared bat YP_002261489  LFHKR-------------------------------------
   Murine Pneumo NP_041232     ------------------SVCTKYSNANTAQ-----------
     CA sea lion YP_003429323  IRHGDVEEDD---DRSTECSEAMPSDSGVGSEAPSTQETTQQSHE
```
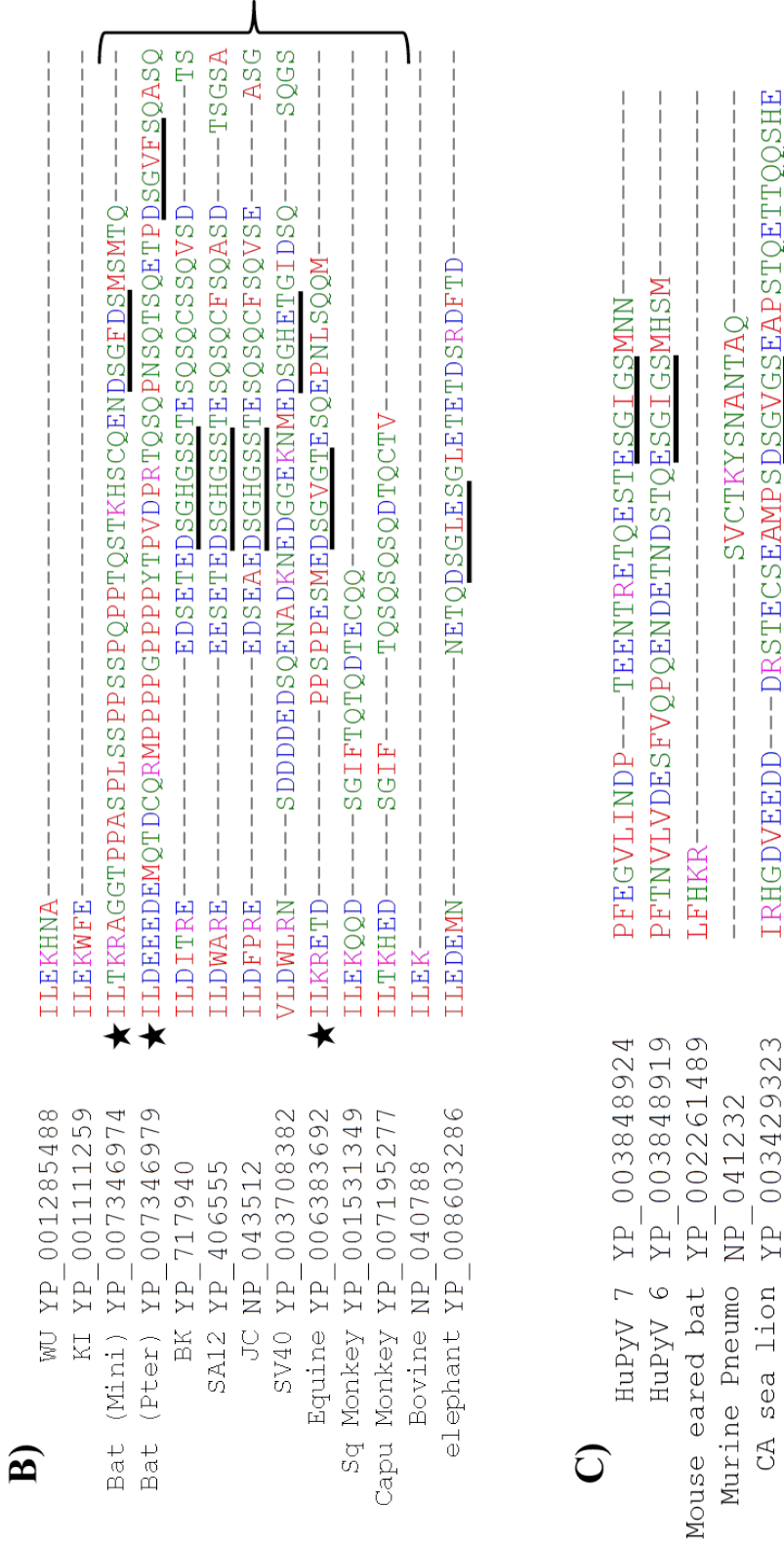
Figure 29. Alignment of C-terminal tails of PyV large T antigens reveals subgroups and conserved motifs.

C-terminal tails comprise any sequence that remains after the last amino acid of the ATPase domain. For SV40, BKV, JCV, and SA12 only the variable region of the C-terminal tail was used. The accession numbers for the LT sequences used are listed along with the common name or host of each PyV. Alignments were performed using Kalign on the EMBL-EBI website. Amino acids are colored according to the chemical properties as described for Figure 9. Putative phosphodegron motifs are underlined. **A)** Alignment of C-terminal tails beginning with "PL". Bracketed sequences contain a high density of negatively charged amino acids. Columns marked with * are conserved in almost all PL containing sequences and may make up a new motif P-L-x-x-I/L/V-I/L/V. **B)** Alignment of sequences beginning with "IL". Bracketed sequences show a high serine and glutamine content. Sequences marked with a black star show a high proline content. **C)** Alignment of outlier sequences that did not begin with "PL" or "IL". No significant patterns were observed within this group.

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