Update on Human Polyomaviruses and Cancer

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Over 50 years of polyomavirus research has produced a wealth of insights into not only general biologic processes in mammalian cells, but also, how conditions can be altered and signaling systems tweaked to produce transformation phenotypes. In the past few years three new members (KIV, WUV, and MCV) have joined two previously known (JCV and BKV) human polyomaviruses. In this review, we present updated information on general virologic features of these polyomaviruses in their natural host, concentrating on the association of MCV with human Merkel cell carcinoma. We further present a discussion on advances made in SV40 as the prototypic model, which has and will continue to inform our understanding about viruses and cancer.

I. INTRODUCTION

Five human polyomaviruses have been identified to date. In 1971, the first two members, JC virus (JCV) and BK virus (BKV) were concurrently reported in the journal Lancet (Gardner *et al.*, 1971; Padgett *et al.*, 1971).

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JCV was cultured from progressive multifocal leukoencephalopathy (PML) brain tissue in a patient with Hodgkin disease and BKV was isolated from the urine of a renal transplant patient with ureteral stenosis. Both viruses manifested unusual clinical diseases in immunosuppressed patients and were named after their source patients' initials. More than 30 years intervened before two more human polyomaviruses were identified in 2007 (Allander *et al.*, 2007; Gaynor *et al.*, 2007). These two viruses, Karolinska Institute virus (KIV) and Washington University virus (WUV) were named after the institutions where their identification occurred. Both were detected from respiratory samples in symptomatic pediatric patients after DNase enrichment for encapsidated viral particles followed by library construction and mass sequencing of cloned cDNAs. The publication of the most recently identified human polyomavirus, Merkel cell polyomavirus (MCV) occurred in 2008 (Feng *et al.*, 2008). MCV was named for the uncommon, but aggressive Merkel cell carcinoma (MCC) skin cancer, from which viral transcripts were found by digital transcriptome subtraction (DTS) (Feng *et al.*, 2007). DTS involves transcriptome sequencing followed by *in silico* subtraction to exclude human from candidate viral transcripts (Feng *et al.*, 2007). In contrast to other human polyomaviruses, the discovery of MCV did not depend on the presence of replication competent, encapsidated virions.

Research on polyomaviruses began in 1953 with strong ties to cancer biology when Ludwik Gross, during the course of his investigations in transmitting mouse leukemia from cell-free filtrates, isolated an agent that induced tumors in newborn mice (Gross, 1953). This filterable agent, murine polyomavirus (MPyV) became the archetypal member of the *Polyomaviridae* family. In 1960, Sweet and Hilleman found simian vacuolating virus 40 (SV40) infecting lots of rhesus monkey kidney cells used for the production of both Sabin and Salk polio vaccines (Sweet and Hilleman, 1960). The ability of SV40 to cause tumor in experimental animals and the widespread administration of the polio vaccine raised concerns regarding xenotropic exposure in the human population to this agent. The resultant search over the past 50 years to establish a causal relationship between SV40 and human cancers has not been fruitful; however, this does not diminish the contributions that studies of SV40 have made to both viral oncogenesis and cellular biology. In this review, we will examine the biology of the human polyomaviruses concentrating on MCV and its association with human cancer. SV40, and its gene products, serve as well-established models for cancer and provide a context for these discussions due to commonalities in genome, structure, and biochemical properties with the human polyomaviruses.

II. BIOLOGY OF POLYOMAVIRUSES

A. Classification and Phylogeny

Polyomaviruses were historically categorized with the papillomaviruses under the designation of papovaviruses until their separation into two distinct families in 2000. In addition to MPyV, SV40, and the five human members, other polyomaviruses from diverse species of animals including other nonhuman primates, birds, bats, rabbits, and rodents have been found. To date, full genome sequences of over 21 polyomaviruses have been deposited in GenBank. Although variations exist with respect to phylogenetic relatedness when different genes are used for analysis, JCV, BKV, KIV, and WUV appear to group closely with SV40 (Fig. 1). Within this subgroup JCV and BKV are consistently less divergent from each other when compared to either KIV or WUV, which appear to be in a clade of their own. In contrast to the first four identified human polyomaviruses, MCV is more closely related to the archetypal murine polyomavirus (MPyV) and the African green monkey lymphotropic polyomavirus (LPV) (Fig. 1).

B. Genome Organization

The genomes of human polyomaviruses range between ~5.0 and 5.3 kb (JCV-5130 bp; BKV-5153 bp; KIV-5040 bp; WUV-5229 bp; MCV-5387 bp). They exist as circular dsDNA closely associated with histones, and are packaged into chromatin resembling cellular genomes (minichromosomes) within nonenveloped, 40–45 nm icosahedral capsids. The polyomavirus genome is almost evenly divided into an early and a late region encoded on opposite strands.These two regions are separated by a noncoding regulatory region (NCRR) containing the origin of replication and transcriptional control elements. The SV40 genome is depicted in Fig. 2, since we will later use it as a model for polyoma-induced neoplastic transformation. The early region is transcribed from the early promoter immediately upon entry and uncoating of the genome, while the late region is expressed from the late promoter after the onset of viral DNA replication. Early message is differentially spliced to encode at least two proteins, and up to five in some polyomaviruses. The large T (tumor) antigen (LT) and small t antigen (ST) proteins are invariantly expressed in all polyomaviruses including the five human members, although in KIV and WUV these two proteins have been predicted only by open reading frame analysis and not by experimentation (Fig. 3). These early proteins are referred to as tumor antigens, because they were originally detected using antibodies from tumor bearing animals.

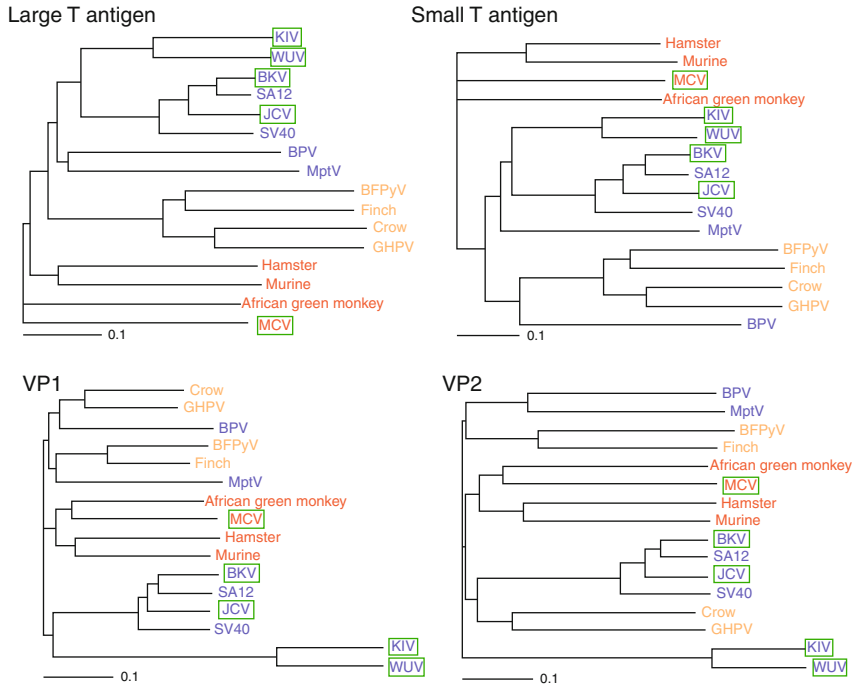


Fig. 1 *Phylogenetic analysis of polyomavirus LT, ST, VP1, and VP2 protein sequences*. The analysis includes: human polyomaviruses (BKV, JCV, KIV, WUV, and MCV, all marked with a green rectangle) as well as simian agent 12 (SA12), SV40, bovine polyomavirus (BPV), murine pneumotropic virus (MptV, also known as Kilham strain of polyomavirus), budgerigar fledgling disease polyomavirus (BFPyV), finch polyomavirus, crow polyomavirus, goose hemorrhagic polyomavirus (GHPV), hamster polyomavirus, murine polyomavirus, African green monkey polyomavirus (also known as lymphotropic polyomavirus), and MCV. The subgroup of SV40 is outlined in blue, of the murine polyomavirus in red, and of the avian polyomavirus in orange. While BKV, JCV, KIV and WUV LT and ST sequences cluster together with SV40, MCV, in contrast, clusters with the murine polyomavirus subgroup (modified from Feng *et al.*, 2008; Fig. 2B).

Analogous to the 17k T antigen of SV40 which is expressed from an alternatively spliced early transcript (Zerrahn *et al.*, 1993), additional T antigen isoforms have also been identified in JCV, BKV, and MCV: JCV encodes three T′ antigens, T′165, T′136, and T′135 (Trowbridge and Frisque, 1995); MCV has a 57 kDa T antigen (57kT) (Shuda *et al.*, 2008) (Fig. 3); and BKV encodes a truncated T antigen close in structure to T′136 of JCV (Abend *et al.*, 2009b). No accessory T antigens have yet been identified for KI or WU. Regardless of the number of T antigen mRNAs, exon 1 is shared in common with all alternatively spliced early mRNAs of each virus (Fig. 3). The functions of the accessory T antigen proteins are still largely unknown. Determination of their function in the viral life cycle awaits

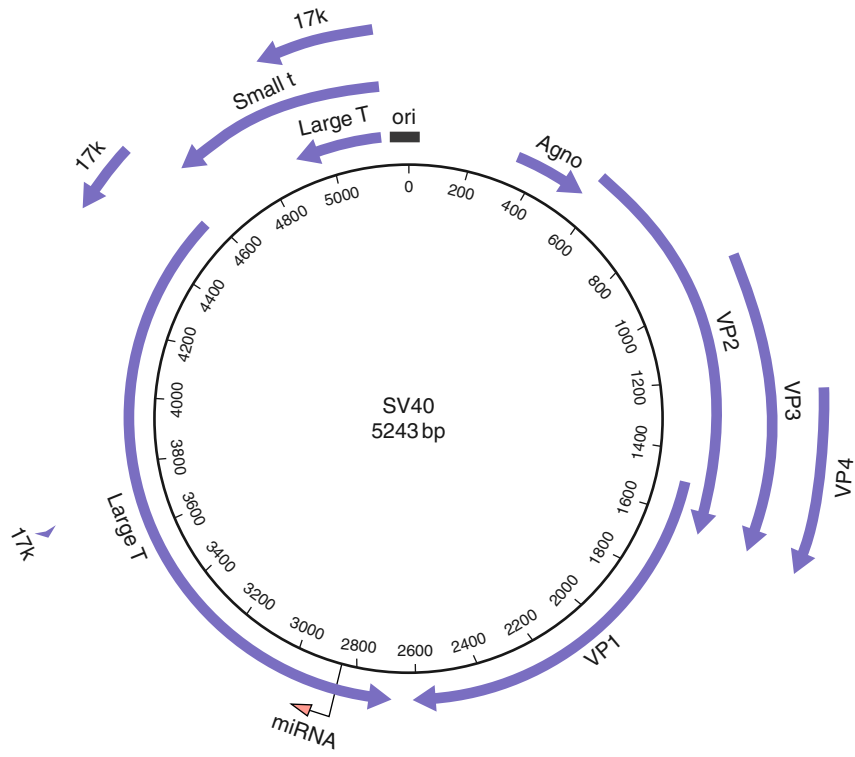


Fig. 2 *SV40 genome organization.* The early region of the viral genome (the left half) encodes LT, ST, and 17k by differential splicing. The respective open reading frames are colored blue. The late region of the viral genome (the right half) encodes agnoprotein and the structural proteins VP1, VP2, VP3, and VP4. These gene products are generated by differential splicing and internal translation. The core origin of replication (ori) is located on top adjacent to transcriptional control elements, together encompassing the NCRR. A red arrow indicates the viral miRNA that targets the early message.

generation of mutant viruses lacking their expression. It is known that the 17k SV40 accessory T is expressed at low levels during SV40 infection, where it has been suggested to fine-tune cell-cycle regulation (Zerrahn *et al.*, 1993). It can bind pRB family members, drive cell-cycle progression, and is capable of causing minimal transformation of F111 rat cells (Boyapati *et al.*, 2003; Zerrahn *et al.*, 1993). A mutant 17k deficient in pRB binding drives normal human fibroblasts into premature senescence (Gjoerup *et al.*, 2007).

The late message, by differential splicing and internal translation, produces three to four capsid proteins VP1, VP2, VP3, and VP4. VP3, and VP4 when present, is generated by internal translation of VP2. VP4 has so far only been detected in SV40, where it promotes lysis of the cell and egress of

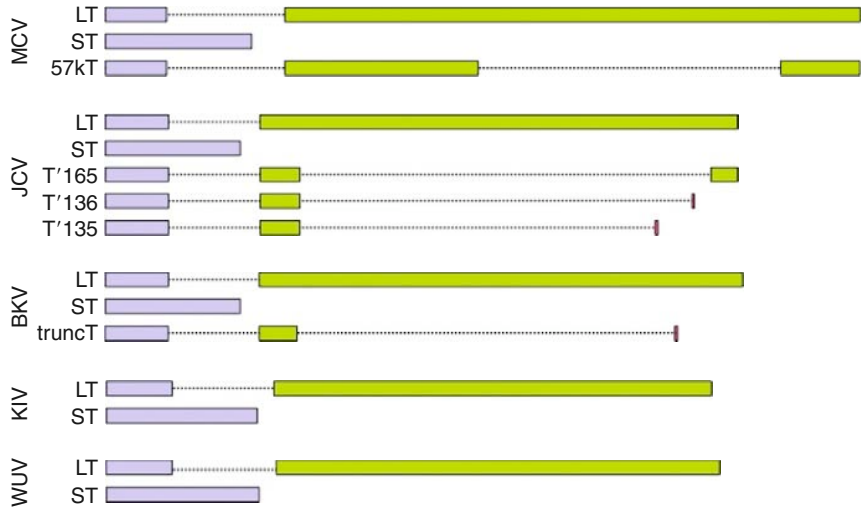


Fig. 3 *Splicing arrangement for the human polyomavirus T antigens*. Colored rectangles indicate coding sequences, whereas the broken line depicts intron sequences. Different color rectangles refer to distinct reading frames after splicing events. All T antigens of each polyomavirus share the sequence encoded within exon 1. The accessory T antigens in addition share fragments of their respective LT sequences.

the virus (Daniels *et al*., 2007). The viral capsid is composed of 72 pentamers of the major capsid protein VP1 that contacts 72 copies of the minor capsid proteins VP2/3. In BKV and JCV, the leader region of the late message additionally encodes the agnoprotein, which may be involved in virion maturation (Khalili *et al*., 2005; Ng *et al*., 1985). KIV, WUV, and MCV are without known agnoproteins. Only murine and hamster polyomaviruses are known to encode a middle T antigen, which is their principal transforming protein (Cheng *et al*., 2009).

Several polyomaviruses have been found to express miRNAs derived from the late transcript. SV40, MPyV, JCV, and BKV each has a single pre-miRNA from which two miRNAs of opposing orientations are processed (Seo *et al*., 2008; Sullivan *et al*., 2005, 2009). The JCV miRNAs have been detected in PML lesions (Seo *et al*., 2008). These miRNAs are predicted to autoregulate early gene expression at late times in infection. For SV40, mutants that cannot produce miRNA show increased expression of viral T antigens and are more susceptible than wild-type virus to lysis by cytotoxic T cells (Sullivan *et al*., 2005). In contrast, no *in vivo* differences in antiviral CD8 T cell responses can be discerned between infections with wild-type MPyV and MPyV lacking miRNA (Sullivan *et al*., 2009).

C. Viral Life Cycle

The polyomaviruses generally have a narrow host range and limited cell type tropism. They are able to infect cells of their natural hosts, giving rise to a productive life cycle that results in cell lysis. In addition, these viruses are also able to establish a persistent infection rarely associated with disease except when immunodeficiency is induced. The full, infectious viral life cycle has only formally been studied in JCV and BKV as no infectious system has yet been devised for KIV, WUV, or MCV. However, it is generally thought that polyomaviruses are internalized by the interaction of VP1 with specific cellular receptors and co-receptors. It is known that BKV uses gangliosides GD1b and GT1b (Low *et al*., 2006); JCV uses GT1b and the serotonin receptor, 5HT2AR (Elphick *et al*., 2004); and GT1b has been proposed as a putative host cell receptor for MCV (Erickson *et al*., 2009). Virus then traffics through caveolae and the endoplasmic reticulum to the nucleus, where it is uncoated and the early message is transcribed. In the case of JCV, clathrin-dependent endocytosis precedes localization in caveosomes (Eash *et al*., 2006). After translation, LT then initiates DNA replication of the viral genome from the origin in the NCRR. The shift to late viral expression is not fully elucidated but likely involves LT transcriptional activation of the late and repression of the early promoter. VP1 is expressed and assembled together with VP2 and VP3 and then imported into the nucleus where encapsidation of viral genomes takes place.

D. Natural Infection, Reactivation, and Clinical Disease

All five human polyomaviruses have high prevalence in the human population and infections start in childhood; however, variability has been reported in the age patterns of polyomavirus acquisition. A study of 2435 sera from English and Welsh individuals ranging from 1 to 69 years showed an overall seroprevalence rate of 81% for BKV with seroconversion occurring very early in childhood, peaking at 91% in the 5–9 age range, and dampening in elderly individuals. For JCV, the overall seroprevalence was 35% with a steady increase of 11% from children below 5 years up to 50% in the 60–69 age group (Knowles *et al*., 2003). These findings were largely replicated in a study examining 400 consecutive healthy blood donors from Basel, Switzerland. Egli and colleagues found an overall IgG seroprevalence of 82% for BKV and 58% for JCV in this cohort (Egli *et al*., 2009). The first large serosurvey of KIV and WUV in 1501 adults using GST-VP1 capture ELISA showed rates of 55% and 69%, respectively (Kean *et al*., 2009). Age-specific prevalence studies have not been performed for KIV and WUV;

however, the detection of viral DNA in respiratory specimens from children suggests that initial exposure occurs at a relatively early age. Similar findings are emerging for MCV. Using wild-type MCV strain sequences in VLP-based ELISA, Tolstov and colleagues reported an age associated increase in MCV prevalence from 50% among children 15 years or younger up to 80% among persons older than 50 years (Tolstov *et al.*, 2009). Pastrana and colleagues found 88% MCV seropositivity in adults without MCC. These studies additionally demonstrate that MCC patient sera showed markedly elevated MCV IgG responses with the geometric mean titers in controls 59-fold lower than in the MCC patient group (Pastrana *et al.*, 2009; Tolstov *et al.*, 2009). Carter and colleagues reported the following seroprevalence results in 451 general population adults: 92% for BKV, 45% for JCV, 90% for KIV, 98% for WUV, and 59% for MCV (Carter *et al.*, 2009). Although rates of infection for JCV and BKV found in various studies are in general agreement, additional studies on KIV, WUV, and MCV will more precisely define overall and age-specific prevalence rates.

Fecal–oral, oral, and respiratory routes of transmission have been proposed for different human polyomaviruses. For JCV and BKV, studies of urban sewage samples and rivers show significant numbers of stable virus particles from divergent geographical areas suggesting the possibility of virus acquisition through fecally contaminated water, food, and fomites (Bofill-Mas and Girones, 2003; Bofill-Mas *et al.*, 2000; McQuaig *et al.*, 2006). Initial studies of KIV, WUV, and MCV have also shown viral DNA in stool samples (Allander *et al.*, 2007; Babakir-Mina *et al.*, 2009a; Loyo *et al.*, 2009). The detection of salivary shedding of BKV and productive infection of salivary cell lines implicates oral transmission as another possibility for this virus (Jeffers *et al.*, 2009). MCV is also detected at high level in saliva (Loyo *et al.*, 2009). In contrast to JCV and BKV, which are only rarely detected from respiratory sources, KIV and WUV can be isolated from respiratory secretions of children worldwide. Curiously, these viruses cannot be detected in the respiratory specimens of adults except in the setting of immunosuppression (Bialasiewicz *et al.*, 2009; Loyo *et al.*, 2009; Norja *et al.*, 2007; Ren *et al.*, 2008). MCV DNA can also be detected in respiratory specimens from symptomatic patients and occurs in nasal swabs and nasopharyngeal aspirates at a frequency similar to or even higher than that of KIV and WUV (Bialasiewicz *et al.*, 2009; Kantola *et al.*, 2009). Significantly more adults than children are positive for respiratory MCV in contrast to KIV and WUV (Goh *et al.*, 2009).

The process by which polyomaviruses gain access to and establish persistent infections in distal body compartments is not well established. However, DNA of all human polyomaviruses has been detected in tonsillar tissue, a possible point of entry (Babakir-Mina *et al.*, 2009b; Kantola *et al.*, 2009; Monaco *et al.*, 1998a). For JCV, virus has been localized to tonsillar stromal

cells and B lymphocytes with the latter cell type implicated in circulatory dissemination to other anatomic sites (Monaco *et al*., 1998a,b). Since all viruses can be detected at increased frequencies in blood and lymphoid tissues during host immunosuppression (Sharp *et al*., 2009), it is likely that hematolymphoid cells can carry or harbor polyomaviruses. Recently, Mertz and colleagues detected MCV in (CD14+/CD16−) inflammatory monocytes but not in lymphocytes or granulocytes (Mertz *et al*., 2009).

JCV and BKV establish persistent infections in renal tissue and virus is shed into the urine. In a recent study examining 400 consecutive healthy blood donors from Basel, Switzerland, Egli and colleagues found urinary shedding of BKV in 7% of these individuals compared to 19% for JCV (Egli *et al*., 2009). Reactivation of JCV and BKV, as reflected by increased viruria, occurs during immunosuppression, but only BKV levels correlate with the degree of immunosuppression (Behzad-Behbahani *et al*., 2004). The bone marrow is another possible site of persistent infection for JCV and may be the source of virus positive cells detected in the circulation (Tan *et al*., 2009). JCV can additionally gain access to glial cells of the central nervous system where reactivation or new infection can result in PML and virus can be detected in the cerebral spinal fluid (Drews *et al*., 2000; Vago *et al*., 1996).

PML is an acquired demyelinating disease pathologically characterized by a triad of findings: 1) oligodendrocytes having enlarged nuclei with viral inclusion bodies, 2) bizarre, atypical astrocytes, and 3) loss of myelin with accompanying phagocytic infiltrate. PML underscores how critical the host cell environment is in determining the outcome of a viral infection. Although both oligodendroglial and astroglial cells of the central nervous system are infected, only the oligodendrocytic myelin forming cells support full lytic replication of the virus causing the hallmark loss of myelin seen in PML. By contrast, infection of the astroglial population results predominantly in changes in nuclear morphology, size, and ploidy. The bizarre, atypical astrocytes which are visually indistinguishable from tumor cells in high-grade glial neoplasia may represent an infection by JCV of a cell type that is unable to support a fully lytic viral life cycle. Viral DNA and LT protein expression is detectable in astrocytes but at a much lower frequency and quantity. Whether the JCV infected astroglial population is transiently transformed is an intriguing conjecture. There are scattered, but convincing case reports in the literature demonstrating expression of JCV T antigen in tumors of the central nervous system (Krynska *et al*., 1999; Pina-Oviedo *et al*., 2006); however, as a group, the glial neoplasms have not been associated in a consistent way with JCV infection. Uncommon cases and case series of JCV association with other human neoplasia have also been published (see review Maginnis and Atwood, 2009). PML has recently been diagnosed in patients taking natalizumab (Tysabri) (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould *et al*., 2005; Van Assche *et al*., 2005), causing its temporary

withdrawal from the market (Major, 2010). Several mechanisms have been proposed for the effect of natalizumab on JCV including restriction of leukocyte trafficking across the blood–brain barrier or direct inhibition of T cell reaction against JCV (Chen *et al.*, 2009).

For BKV, important clinical diseases occur in the posttransplant setting and relate to type of tissue transplanted. Hemorrhagic cystitis, hematuria, and renal impairment are seen with hematopoetic stem cell transplantation (HSCT) (O'Donnell *et al.*, 2009); and BK-associated nephropathy occurs in 2–5% of renal transplant patients with graft loss in nearly half of these cases (Bonvoisin *et al.*, 2008). Viral reactivation during these complications is robust and can be monitored by DNA-based techniques in blood and urine (Bonvoisin *et al.*, 2008; Cimbaluk *et al.*, 2009; O'Donnell *et al.*, 2009).

III. MERKEL CELL POLYOMAVIRUS AND HUMAN CANCER

A. Human Polyomaviruses and Cancer

Of the five human polyomaviruses, only MCV demonstrates a robust correlation with human cancer. Studies showing MCV T antigen interactions with cellular proteins such as pRB, Hsc70, and PP2A have been performed, but demonstration of the biochemical effects of these interactions have not been published. As noted above, JCV and BKV are associated with important nonneoplastic clinical diseases that have significant morbidity and mortality in immunocompromised individuals; but despite their potential to act as transforming viruses in rodent and *in vitro* cell culture models, no consistent association with human cancers have been found. The reader is referred to excellent reviews on the potential role of these viruses in human cancers (see Abend *et al.*, 2009a; Maginnis and Atwood, 2009; White *et al.*, 2005). KIV and WUV have not yet been found to be associated with human disease and although both of these viruses were originally detected in the respiratory samples of symptomatic children, attributing a causal role for them in respiratory diseases is difficult because of the significant rates of co-detection with other respiratory pathogens (Bialasiewicz *et al.*, 2008; Norja *et al.*, 2007).

B. Merkel Cell Polyomavirus

MCC is an uncommon cancer with an overall age adjusted incidence of 0.24 per 100,000 person-years (Agelli and Clegg, 2003). Overall incidence of MCC has increased three fold from 1986–2001 (Hodgson *et al.*, 2005).

There is a slight male predominance and a strong association with whites/fair-skinned individuals, advanced age, and sun exposure. The 5-year relative survival is 75%, 59%, and 25% for localized, regional, and distant MCC, respectively (Agelli and Clegg, 2003). Unfortunately, most cases of primary MCC are diagnosed when the disease is no longer localized.

MCC is derived from resident Merkel cells of the skin, which along with associated terminal sensory neuritis, comprise the epidermal mechanoreceptors that allow touch discrimination of fine surface textures (Maricich *et al*., 2009). Historically, Merkel cells have been thought to be of neuroendocrine derivation due to their elaboration of various neurosecretory markers; however they also express the low molecular weight cytokeratin (CK) 20 suggestive of epithelial origin. MCC typically affects the elderly, but studies have shown that it may occur in younger ages and at an increased frequency in immunosuppressed individuals. Engels *et al*. noted that MCC was increased in both AIDS and posttransplantation populations (Engels *et al*., 2002), a striking epidemiologic feature that suggests strong immunologic surveillance in controlling MCC development. In 2008, Feng and colleagues specifically sought for an infectious agent in MCC using the DTS technique (Feng *et al*., 2008). Pooling cDNA libraries made from four MCC lesions, a DTS candidate was detected that showed a significant degree of similarity to the LPV LT. The 5387 bp polyoma genome (MCC350 strain) was sequenced by PCR walking using primers designed from the DTS viral transcript.

Initial studies of MCC lesions from 10 patients showed that although 80% contained Southern blot detectable MCV DNA, there exists a minority subset of MCC cases which do not contain MCV. This estimate has held up in subsequent studies from various laboratories and has been generalized to geographically diverse populations (Becker *et al*., 2009; Duncavage *et al*., 2009b; Foulongne *et al*., 2009; Kassem *et al*., 2008; Sihto *et al*., 2009; Touze *et al*., 2009; Varga *et al*., 2009). An exception is the finding of only 24% correlation between virus genome and MCC in Australian populations (Garneski *et al*., 2009). These findings suggest that MCC is a heterogenous disease having at least two pathoetiologies. Pathological and immunophenotypic studies of virus positive MCC cases compared to virus negative cases demonstrate no other markers that can distinguish between these two types of MCC (Shuda *et al*., 2009). However, data are beginning to accumulate on clinical correlates for MCV positive MCC compared to virus negative cases. Patients with MCV positive MCC tumors had better overall survival than those with MCV negative tumors (5-year survival: 45% vs. 13%, respectively) (Andres *et al*., 2009b; Sihto *et al*., 2009). These findings are preliminary since the uncommon nature of these tumors precludes easy assessment of clinical outcomes and treatment responses.

Determination of viral load in MCC lesions by quantitative PCR shows the following average copy number per tumor cell: 5.2 (range 0.8–14.3)

(Shuda *et al.*, 2009) and 10 (range 0.05–173) (Loyo *et al.*, 2009). In contrast, although MCV DNA can be detected in many non-MCC tissues including appendix, colon, lung, peripheral blood mononuclear cells, and skin, the calculated copy number per cell equivalent of DNA is orders of magnitude lower (Loyo *et al.*, 2009; Shuda *et al.*, 2009). Associations have been sought for MCV and other human neoplasia: neuroendocrine tumors from a variety of anatomic sites (Duncavage *et al.*, 2009a; Touze *et al.*, 2009; Wetzels *et al.*, 2009), colon cancer (Kassem *et al.*, 2009), prostate cancer (Bluemn *et al.*, 2009), mesotheliomas (Bhatia *et al.*, 2010), hematopoietic neoplasms (Shuda *et al.*, 2009), breast cancer, ovarian cancer, and skin cancers including basal cell carcinomas, melanomas, and Kaposi's sarcoma (Andres *et al.*, 2009a; Katano *et al.*, 2009; Sastre-Garau *et al.*, 2009; Varga *et al.*, 2009). Results suggest that MCV is specifically associated only with MCC.

Using 3′ RACE, Feng and colleagues detected T antigen–human fusion transcripts in one of the cases of MCC they studied which mapped to a viral integration site in a receptor tyrosine phosphatase, type G (PTPRG) gene intron on Chromosome 3 of the host genome (Feng *et al.*, 2008). The banding pattern on Southern blots indicated monoclonal MCV integration in six of eight MCV-positive MCCs, suggesting that infection and subsequent integration preceded clonal expansion of tumor cells, consistent with MCV being causally linked to MCC. For one MCC with a monoclonal MCV integration pattern, both primary and metastatic tumor samples were available. These two samples exhibited an identical integration pattern, indicating that MCV integration occurred before tumor metastasis (Feng *et al.*, 2008). Sastre-Garau also found clonal integration in all 10 MCV positive MCC tumors they examined. The restriction patterns and integration sites when identified varied between different MCC tumors suggesting that this event is likely to occur at random sites in the host genome (Feng *et al.*, 2007; Sastre-Garau *et al.*, 2009). Sastre-Garau found viral integration in the 3′ end of the T antigen, in the regulatory control region and in the VP1 open reading frame of informative cases (Sastre-Garau *et al.*, 2009).

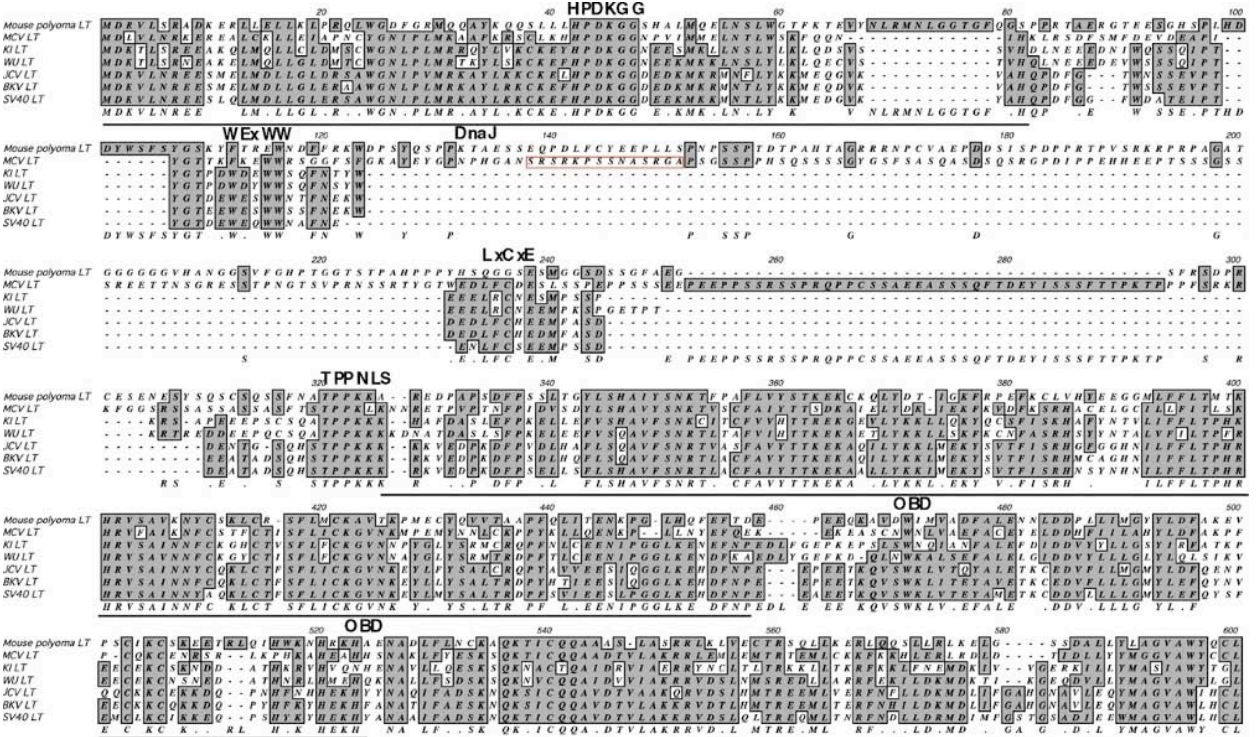
In nearly all tumor-derived MCV genomes sequenced, missense mutations or deletions in the early region result in the expression of truncated LT antigens (Sastre-Garau *et al.*, 2009; Shuda *et al.*, 2009). These LTs all retain their pocket protein binding motifs, but lose their helicase domains (see below for explanation of functions). By contrast, sequences from the early regions of MCV strains derived from non-MCC tissues show intact T antigen ORF (Shuda *et al.*, 2009). Shuda and colleagues have shown that both tumor- and nontumor-derived MCV LT have LxCxE and DnaJ domains capable of binding pRB and Hsc70 proteins (Shuda *et al.*, 2008). The functional consequence of the tumor-derived T antigen mutations is the retention of pocket protein interactions and the loss of viral DNA replication

capability. This is important, because the loss of replication activity upon integration in MCC demonstrates that MCV is not simply a "passenger virus" that happens to grow well in MCC cells. None of these mutations affect the expression of an intact ST (Sastre-Garau *et al.*, 2009; Shuda *et al.*, 2009) which contributes to malignant transformation in the case of SV40 (see Section IV.B). Clearly, adventitious firing from the origin of an integrated viral genome would activate signaling pathways leading to cell death and inhibit tumor outgrowth. The presence of these mutations support the tenet in polyomaviruses biology that lytic replication is incompatible with tumor formation.

In early studies of SV40 transforming potential, it was determined that efficiency of transformation of human fibroblasts was much higher using artificially produced origin defective SV40 mutants and that only defective SV40 could be isolated from transformed monkey, human, and hamster cells (Huebner *et al.*, 1975; Small *et al.*, 1982). For MCV, viral replication has been mapped to a 71 bp core origin (Kwun *et al.*, 2009). All but one of the core origins examined showed invariant wild-type sequences (Kwun *et al.*, 2009). It is possible that overlapping early transcriptional control functions in this region need to be maintained for robust T antigen expression in MCC tumors and alternative mechanisms, such as truncation of C-terminus of T antigen or viral capsid mutations would be required to ablate replication.

PCR detection used in studies to establish an association between various polyomaviruses and human cancers suffers from the exquisite sensitivity as well as susceptibility for template contamination of the technique. A monoclonal antibody, CM2B4, has been developed based on a peptide sequence in exon 2 of MCV LT that also detects 57kT but not ST. The epitope site of CM2B4 is in a unique span of exon 2 not present in other human polyomaviruses (Fig. 4), and therefore not cross-reactive with their T antigens (Shuda *et al.*, 2009). Using this antibody, a high percentage of MCC lesions with PCR detectable MCV DNA show expression of MCV LT antigen localized diffusely to the nuclei of tumor cells, but not in adjacent normal cells (Busam *et al.*, 2009; Shuda *et al.*, 2009). The discrepancy between the few cases which are MCV DNA positive by PCR and T antigen negative by CM2B4 immunoreactivity may be due to very low amounts of T antigen expression, mutational loss of the CM2B4 epitope, or false positive PCR results.

Although many new insights have been gained about MCV, many questions remain outstanding about its biology and the molecular mechanisms whereby it facilitates oncogenesis. Recent evidence indicates that MCV may be widespread in the human body, which raises the question: Are Merkel cells particularly susceptible to transformation by MCV? Alternatively, this tropism might be restricted by transcriptional control from enhancer sequences. Thus far we have substantial evidence that MCV can be found integrated in MCCs, but there is also some indication that it can be found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WExWW
DnaJ
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Mouse polyoma LT
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SV40 LT

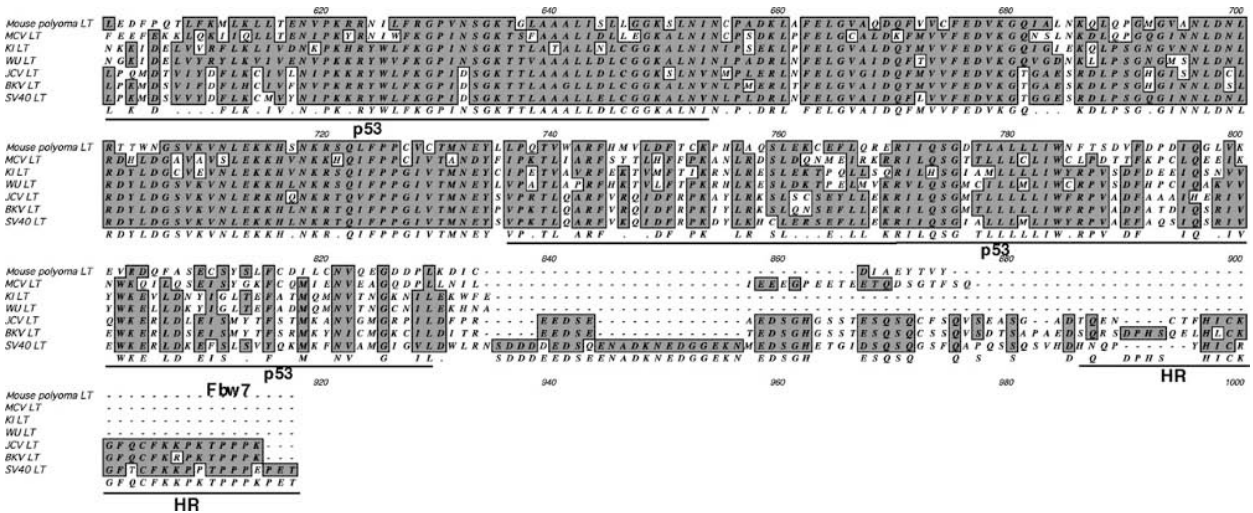

Fig. 4 *Alignment of polyomavirus large T antigens*. The protein sequences of the human polyomavirus, SV40, and MPyV LTs are aligned for comparison. Known domains and motifs are depicted. The first ~70 amino acids constitute the DnaJ domain with the embedded canonical "HPDKGG" motif. The WExWW sequence is a conserved motif required for binding Bub1 in SV40 LT. A red rectangle illustrates the epitope for the CM2B4 MCV LT monoclonal antibody. An intact LxCxE motif is necessary for binding pRB family members. Note that the program (MacVector 10.6.0) did not correctly align the mouse polyoma LxCxE sequence with the others. Both mouse polyoma and MCV LT contain large inserts in their sequence shortly after the first exon. The sequence "TPP" is a conserved phosphorylation site motif, which is proceeded by a canonical nuclear localization signal (NLS). The OBD line indicates the boundaries of the conserved origin-binding domain. The line labeled with "Zn" illustrates the location of a Zn^{2+}-coordination element required for hexamer formation. The line labeled "p53" shows the boundaries of the bipartite p53-binding site in SV40. At the extreme C-terminus the boundaries of the host range (HR) region, which allows SV40 to replicate in certain cell types, is outlined. The extreme C-terminus also contains a binding site for the F-box protein Fbw7 via a phosphodegron sequence.

episomally as well. Other tumor viruses such as HPV are known to be maintained episomally at the precancerous stage; however, in most tumors the genome is integrated resulting in loss of replication activity. Is there a form of MCV "latency" and reactivation, or is the viral genome persistently maintained at low levels? Much future work will depend on development of cell systems that allow us to grow the virus.

Likewise, *in vitro* cell culture systems, and eventually transgenic model systems that recapitulate MCV oncogenic transformation will be paramount. Many outstanding questions relate to the activities of T antigens expressed in MCCs. Given the near universality of viral interference with p53 tumor suppressor signaling, it will be of great interest to find out if, or how, it is perturbed by MCV. Sequence comparisons between different polyoma LTs reveal that, similarly to MPyV LT, MCV has a $\sim$200 amino acids insert immediately following the first exon and before the start of the origin-binding domain. This insert region exhibits no significant homology to other polyomavirus T antigens, suggesting it might confer unique functions (Fig. 4). We are now entering a phase of MCV research where causality for MCC is widely accepted, but the mechanistic details of transformation and the search for therapeutic interventions are underway. A comparison to SV40-induced transformation provides an obvious roadmap; however, MCV promises to reveal additional insights into human oncogenesis.

IV. SV40 AS A CLASSIC MODEL SYSTEM

The human genome is extraordinarily complex. In direct studies that compare the differences between normal cells and tumor cells, for example, by microarray analysis, it becomes extremely challenging to identify the relevant changes that drive malignant progression. Viruses, on the other hand have targeted key molecules and pathways, so-called "hubs" in cellular signaling. Polyomaviruses and their gene products have served as model systems for understanding cellular immortalization and oncogenic transformation. These studies have led to important paradigms and fundamental insight into key biological processes over the last 50 years since they were discovered. The size of polyomaviruses makes them highly amenable to genetic manipulations, and these viruses have historically been easy to grow in culture. Mutants can be tested for proficiency in driving viral replication or transformation, or expression of the viral gene products can be carried out in transgenic models to study tissue-specific tumorigenesis. However, tractable culture systems for the most recently discovered new human polyomaviruses have yet to be developed.

Because of their small genome size, polyomaviruses rely heavily on the cellular replication machinery to replicate their genome. Therefore, these viruses reprogram the host cell cycle to induce progression into S-phase from quiescence and thus create a suitable environment for viral replication. Of relevance to cancer biology, the molecules targeted by the virus to promote unscheduled DNA replication or to inhibit innate immune signaling in the setting of viral replication are often the same as those involved in oncogenesis. In this section we will review and discuss the current knowledge of the biochemical properties of LT and ST using SV40 as our model.

A. Large T Antigen

LT is a multifunctional nuclear phosphoprotein with genetically separable functions in promoting viral DNA replication and the induction of oncogenic transformation. LT initiates replication by specifically binding to the viral origin and recruiting cellular replication factors. LT must also prepare the cell for replication by stimulating cell-cycle progression from G1 (or G0) into S-phase. It is this latter function of LT: the breakdown of cellular control mechanisms associated with LT induced cell-cycle progression that are likely to be main contributors to oncogenic transformation.

Transformation elicited by SV40, or its early gene products, can be assayed in multiple ways. Viral infection leads to transformation of a range of cultured rodent and human cells and induces tumors in newborn hamsters (Eddy *et al*., 1962; Manfredi and Prives, 1994; Todaro *et al*., 1966). Transfection of origin-deficient SV40 genomic DNA significantly enhances *in vitro* transformation of human cells, suggesting that viral replication presents an obstacle to stable transformation (Small *et al*., 1982). The expression of LT alone, or in combination with ST, will oncogenically transform most rodent cell types. Studies have shown that expression of LT often allows cells to become immortalized as well as grow in reduced serum and until higher saturation density. Furthermore, these transformed cells escape contact inhibition as shown in focus formation assays. LT alone, or more frequently in conjunction with ST, induces anchorage-independent growth in soft agar and tumors in nude mice. Previously, several excellent reviews on LT have been published, some general in scope (Ahuja *et al*., 2005; Cheng *et al*., 2009; Fanning and Knippers, 1992; Pipas, 1992, 2009), while others emphasized transformation in different cell types (Manfredi and Prives, 1994), function of the DnaJ domain (Sullivan and Pipas, 2002), or transgenic models for tumorigenesis (Saenz Robles and Pipas, 2009).

Structure determination has yielded substantial insight to the different modules that together make up LT. The structure is known for the DnaJ

domain and adjacent LxCxE motif (Kim *et al.*, 2001), the origin-binding domain (Luo *et al.*, 1996; Meinke *et al.*, 2007), the Zn binding domain and the ATPase/helicase domain (Li *et al.*, 2003). While it is believed that the independent domains are interconnected via flexible linkers, their relative positioning during replication is not known in detail. The structure of the host range domain at the extreme C-terminus is also not known. Insights to the structure has unraveled much greater detail about the functioning of the LT multicomponent machine, in part by revealing important contacts with known interactors like p53, and predicting binding surfaces for novel interactors (Ahuja *et al.*, 2009). Many activities of LT can be ascribed to discrete, linear regions of the protein. This is important for the mutational analysis of LT, which has yielded remarkable insight to its function in basic biological processes. We will traverse the LT molecule from the N- to the C-terminus to discuss each functional entity or binding protein (see Fig. 5 for a graphic depiction).

1. DnaJ DOMAIN

The demonstration that LT has recruited chaperone power to its repertoire stands as a landmark achievement (Campbell *et al.*, 1997; Cheetham *et al.*, 1992; Kelley and Landry, 1994; Srinivasan *et al.*, 1997). Previous data had strongly implicated the common region between LT and ST (referred to as T/t common domain) in transformation, but the mechanism was unknown (Marsilio *et al.*, 1991; Montano *et al.*, 1990; Peden and Pipas, 1992; Peden *et al.*, 1990; Pipas *et al.*, 1983; Zhu *et al.*, 1992). The LT N-terminus (first $\sim$70 amino acids) exhibits significant sequence homology with known DnaJ domains, including the canonical HPDK motif (Campbell *et al.*, 1997). Structural analysis of the LT N-terminus also indicates that it folds into a traditional DnaJ structure (Kim *et al.*, 2001). DnaJ proteins are molecular co-chaperones that recruit DnaK family chaperones (heat shock family) to perform functions such as protein folding, protein transport, or remodeling of protein complexes. The energy released from ATP hydrolysis by the DnaK family protein is used to act on a target protein. For LT it was demonstrated that the DnaJ domain binds the constitutively expressed Hsc70 and, as expected for a chaperone, significantly stimulates its ATPase activity (Campbell *et al.*, 1997; Srinivasan *et al.*, 1997; Sullivan and Pipas, 2002). Classical mutants in the DnaJ domain such as H42Q or D44N disrupt this binding. Functional assays demonstrated clearly that the DnaJ domain is critical for two distinct LT functions: to stimulate viral replication and to enhance oncogenic transformation via functional inactivation of the p107/p130 pRB family members (Campbell *et al.*, 1997; Srinivasan *et al.*, 1997; Stubdal *et al.*, 1996, 1997; Zalvide *et al.*, 1998). Strikingly, the LT DnaJ domain can be replaced by the cellular DnaJ protein HSJ1, and the chimeras

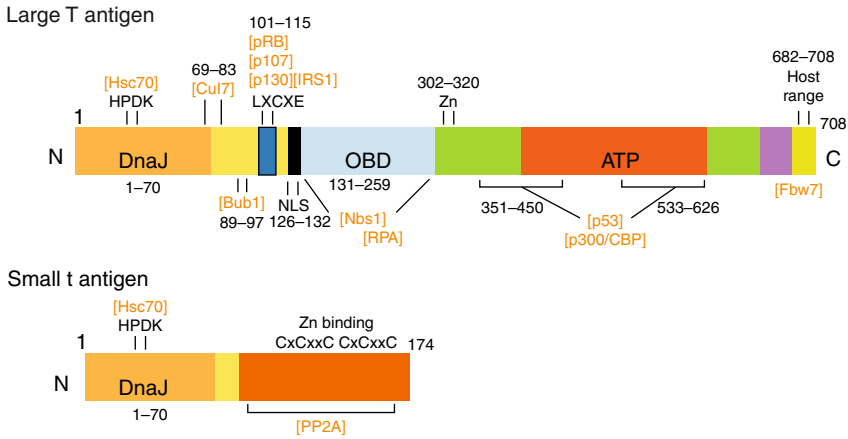

Fig. 5 *Schematic drawings of SV40 LT and ST. Landmark features of LT and ST.* At the N-terminus, the first ~70 amino acids contain the DnaJ domain with its canonical "HPDK" motif required for binding of Hsc70 chaperones. In LT, this is followed by binding sites for the Cul7 and Bub1 proteins, requiring amino acids 69–83 and 89–97, respectively. The LxCxE motif is critical for binding of pocket proteins pRB, p107, and p130, but also contributes to binding of IRS1. The NLS resides between amino acids 126 and 132. The OBD (origin-binding domain), besides conferring specific DNA binding activity to LT, also mediates binding of Nbs1 and replication protein A (RPA). The Zn^{2+}-binding element requires coordinating cysteine/histidine residues from amino acids 302 to 320 and allows LT to assemble into hexamers in an ATP-dependent manner. A bipartite binding site for p53 is present that requires amino acids 351–450 and 533–626; binding of p300/CBP transcriptional co-activators is bridged by p53. The ATPase domain labeled "ATP" is critical for LT helicase activity. The extreme C-terminus contains the host range domain as well as a binding site for the F-box protein Fbw7. The ST protein diverges from LT after the DnaJ domain, having instead at its C-terminus a binding site for the phosphatase PP2A. Cysteine clusters are responsible for coordinating Zn^{2+} and conformational stability.

retain LT DnaJ-dependent functions (Campbell *et al.*, 1997). However, there is a species-specific component, since the yeast Ydj1p or *E. coli* DnaJ proteins fail to functionally replace the LT DnaJ domain (Sullivan *et al.*, 2000b). This failure appeared to be because stimulation of Hsc70 ATPase was compromised, despite normal binding.

A contribution of the DnaJ domain to LT transformation is not observed in all assays for the transformed phenotype, but rather a subset. In assays for anchorage-independent growth, the D44N mutant shows no defect, and it also appears to immortalize mouse embryo fibroblasts normally (Stubdal *et al.*, 1997). However, to promote growth in low serum and to high saturation density, LT absolutely requires the DnaJ domain in cis with the pRB family binding site to disrupt complexes of p107/p130 with E2F4 (Stubdal *et al.*, 1997; Sullivan *et al.*, 2000a; Zalvide *et al.*, 1998).

The hyperphosphorylated forms of p107/p130 are also decreased via the DnaJ domain, and p130 is targeted for degradation (Stubdal *et al*., 1996, 1997). In human diploid fibroblasts, focus formation induced by LT + ST requires an intact DnaJ in LT, but not in ST, as shown using point mutants (Boyapati *et al*., 2003; Porras *et al*., 1996). A different study found no requirement for the LT DnaJ domain in human cell immortalization, transformation, or tumorigenesis; however, this was based on a combination of LT, ST, hTert, and oncogenic H-ras (Hahn *et al*., 2002).

In REF52 cell focus formation assays different DnaJ mutants exhibit variable defects. While the dl1135 mutant (deletion of residues 17–27) is completely defective, the D44N mutant only shows a modest defect in the context of full-length LT (Srinivasan *et al*., 1997). Strikingly, DnaJ point mutants like D44N exhibit much more pronounced defects when expressed in the context of LT1–136 (Beachy *et al*., 2002; Gjoerup *et al*., 2000; Srinivasan *et al*., 1997). The reason for the modest defects of DnaJ point mutants in a full-length background could be because D44N still retains partial DnaJ function (unlikely given data in the literature), because the deletion mutant is conformationally unfolded leading to loss of other LT functions, or because another transforming function exists within the LT N-terminus. Based on sequence homology it has been suggested that LT has a CR1 (conserved region 1 in adenovirus E1A) like sequence, which would be lost in dl1135 (Yaciuk *et al*., 1991). In E1A, the CR1 is important for transformation, because it is required for pRB and p300 binding (Berk, 2005). However, there is no evidence that the CR1-like sequence in LT functions in a similar manner. In fact, based on LT structural analysis, it is likely that the CR1-like sequence is buried within a hydrophobic core (Sullivan and Pipas, 2002).

While all the polyomavirus T antigens appear to have a functional DnaJ domain based on significant sequence homology, this is a unique feature that other viral oncoproteins like adenovirus E1A/E1B or human papillomavirus E6/E7 have not acquired. This is rather striking given the convergence of viral oncoproteins on targets like p53, p300/CBP, and the pRB family.

2. Cul7

Initial reports indicated that LT interacts with a 185 kDa cellular protein (Kohrman and Imperiale, 1992). The protein was subsequently identified as Cul7 from large-scale immunoprecipitations of LT1–135 from mouse NIH3T3 cells coupled with mass spectrometry (Ali *et al*., 2004). Cul7 is a member of an SCF (skp1, cullin, F-box) type E3 ubiquitin ligase complex that targets cellular proteins for proteasomal degradation. Cul7 assembles into complexes with Skp1, Fbxw8, and the Rbx1 ring finger protein (Sarikas *et al*., 2008). Mutations in Cul7 have been identified in the 3-M syndrome

characterized by severe growth retardation (Huber *et al*., 2005). Subsequent genetic analysis mapped the binding site for Cul7 to LT residues 69–83 (Kasper *et al*., 2005). A deletion mutant from 69 to 83 is defective for Cul7 binding but exhibits normal binding of Bub1, pRB, and p53. Importantly, this mutant has a defect in growth to high density and in soft agar (Kasper *et al*., 2005). Strikingly, the defect of the dl69-83 mutant in transformation can be rescued in Cul7-deficient mouse embryo fibroblasts, suggesting that LT inactivates at least some of the Cul7 functions. The critical substrates targeted for Cul7-mediated degradation and that are relevant for LT transformation have not been identified, but IRS1, a critical player in the insulin signaling pathway, was reported as a candidate (Xu *et al*., 2008). Moreover, recent evidence revealed that LT via Cul7 targets the Mre11–Rad50–Nbs1 complex for degradation in an ATM-dependent manner during an SV40 infection (Zhao *et al*., 2008). Currently it is not known if the interaction between LT and Cul7 occurs in a wide variety of cell types across species, and it is unclear if LT only acts to inhibit Cul7 or also to redirect it toward other substrates.

3. Bub1

A yeast two-hybrid screen, using the first 136 amino acids of LT as bait, yielded an interacting clone corresponding to the C-terminal kinase domain (amino acids 600–1085) of the mitotic checkpoint kinase Bub1 (Cotsiki *et al*., 2004). Co-immunoprecipitation analysis has verified that the interaction occurs *in vivo* in mouse and human cells. A deletion mutant of LT, dl89-97, was identified that fails to bind Bub1 (Cotsiki *et al*., 2004) yet retains binding of pRB, p53, and Cul7 (Hein *et al*., 2009). Point mutagenesis identified tryptophans W91, W94, and W95 within a conserved sequence motif "WExWW" to be important for efficient binding. Focus formation assays in rat-1 cells demonstrated a strong correlation between LT binding to Bub1 and transformation (Cotsiki *et al*., 2004). However, Bub1 binding was not required for cellular immortalization. Bub1 has primarily been shown to participate in the spindle checkpoint, a cellular surveillance mechanism that monitors whether kinetochores are properly attached to spindle microtubules (Meraldi and Sorger, 2005; Perera *et al*., 2007) and delays the metaphase to anaphase transition. Thus, Bub1 is critical for maintaining genomic integrity. Interestingly, sporadic Bub1 mutations have been found in colorectal cancer, and reduced expression of Bub1 in mouse models leads to increased spontaneous tumorigenesis concomitant with aneuploidy (Cahill *et al*., 1998; Jeganathan *et al*., 2007; Schliekelman *et al*., 2009).

It was demonstrated that LT via Bub1 binding leads to a compromise of the spindle checkpoint (Cotsiki *et al*., 2004), not a total loss, which is known

to be lethal (Perera *et al.*, 2007). Furthermore, when the spindle checkpoint is activated with nocodazole, cells expressing LT, not the dl89-97 mutant, are able to bypass the checkpoint and accumulate with >4 N DNA content. Recent results implicate Bub1 binding in the ability of LT to induce tetraploidy in BJ/tert human diploid fibroblasts (Hein *et al.*, 2009). Subsequent experiments revealed that shRNA-mediated knockdown of Bub1 expression leads to p53-dependent premature senescence in BJ/tert (Gjoerup *et al.*, 2007). Interestingly, expression of 17k with a pRB binding site mutation (designated "K1") also drove cells into premature senescence in a Bub1 binding-dependent manner, mimicking the outcome of Bub1 knockdown. Later experiments demonstrated that 17k K1 induces a DNA damage response via Bub1 binding (Hein *et al.*, 2009), consistent with recent reports that senescence in general is tied to an activated DNA damage response (Bartkova *et al.*, 2006; Di Micco *et al.*, 2006). These experiments also integrated LT function with p53, since the dl89-97 mutant is partially defective in stabilizing p53, likely because of a defect in p53 Ser15 phosphorylation (Hein *et al.*, 2009). The mechanism whereby LT is able to mount a DNA damage response via Bub1 binding is not known. Since the LT-induced DNA damage response occurs in the absence of the viral origin, one may speculate that the damage is associated with a deregulation of cellular DNA replication. Further experiments are likely to reveal exactly how LT acts on Bub1. It is unlikely to be a similar scenario to p53 or pRB where complete inactivation occurs, since total loss of Bub1 is lethal, even in somatic cells, due to catastrophic mitosis (Perera *et al.*, 2007). LT might redirect the Bub1 kinase toward a different set of substrates, of which other LT bound proteins are prime candidates.

It has long been known that LT induces both structural and numerical chromosome instability, but the mechanism has been elusive (Chang *et al.*, 1997; Friedrich *et al.*, 1992; Levine *et al.*, 1991; Ray and Kraemer, 1993; Ray *et al.*, 1990, 1992; Stewart and Bacchetti, 1991; Woods *et al.*, 1994). The interaction of LT with Bub1 may explain some of these observations, but more studies are clearly needed as additional LT binding proteins are likely to contribute. Whether LT-induced genomic instability, associated with gains and loss of specific chromosomes, contributes to long-term malignant transformation is unclear but of significant interest. Two prior observations suggest that it might. Most temperature-sensitive mutants of LT revert to the nontransformed phenotype at the nonpermissive temperature, but so-called A-type transformants remain transformed (Rassoulzadegan *et al.*, 1978; Seif and Martin, 1979). Likewise, when LT was expressed transgenically using an inducible system, hyperplasia and polyploidy could be reversed after 4 months, but no longer after 7 months, even though LT was turned off in both cases (Ewald *et al.*, 1996).

4. pRB FAMILY

The pRB protein is inactivated either by somatic or hereditary mutations leading to the pediatric tumor of the eye called retinoblastoma. The tumor suppressor concept underlying retinoblastoma was founded on Knudsen's original two-hit hypothesis (Knudson, 1971). By now, we know that every tumor virus has targeted pRB, or components of the pRB pathway, for inactivation. Indeed, almost every tumor, including those of nonviral origin, contains mutations in pRB or somewhere else within the pathway (Burkhart and Sage, 2008). Shortly after the report describing the cloning and characterization of the retinoblastoma protein as a 110-kDa nuclear phosphoprotein (Lee *et al.*, 1987), it was demonstrated to be a critical transformation target of adenovirus E1A (Whyte *et al.*, 1988). This was the first demonstration of a viral oncoprotein targeting a known tumor suppressor for inactivation. Subsequently, it was demonstrated that LT also binds pRB, and this is also required for malignant transformation by LT in a wide variety of cell types and assay systems (DeCaprio *et al.*, 1988; Kalderon and Smith, 1984; Manfredi and Prives, 1994). Mutational analysis of LT clearly showed that a transforming function resides within amino acids 106–114 (Chen and Paucha, 1990). Taken together, it was found that LT from SV40 and MPyV, E1A and E7 all bind pRB (DeCaprio *et al.*, 1988; Dyson *et al.*, 1989; Munger *et al.*, 1989; Whyte *et al.*, 1988), and this requires a conserved sequence motif LxCxE.

The pRB protein consists of a number of domains, of which the A and B domains that together form the "short pocket" are critical for tumor suppression (Burkhart and Sage, 2008). Mutations and deletions in human malignancies map to this region, and it constitutes the binding site for the LxCxE motif. The pRB protein functions mainly as a transcriptional repressor. Although as many as 110 cellular proteins have been reported to bind pRB (Morris and Dyson, 2001), the E2F family of transcription factors appears to be the most important target in tumor suppression and cell cycle control. E2F members normally heterodimerize with members of the DP family to facilitate DNA binding. pRB effects transcriptional repression in large part by recruiting chromatin remodeling factors. These include histone deacetylases, hBRM, BRG1, and SUV39H1. The binding sites for LxCxE-containing proteins and E2F are distinct, although they both map within the pRB pocket domain. Thus, pRB can be simultaneously bound to E2F and chromatin remodeling factors to exert transcriptional repression.

After pRB was discovered, it was soon realized that two other members of the family exist, namely p107 and p130, and that these share structural and functional similarities with pRB. The pRB family is also referred to as "pocket proteins" due to their conserved interaction module. Interestingly, although partially redundant with pRB, p107 and p130 also possess unique,

more specialized properties. Thus, a central spacer domain between A and B in p107 and p130, but not in pRB, binds to cyclins A and E in complexes with CDK2. While pRB is mutated in many types of cancer, mutations in p107 and p130 have rarely been observed in human malignancies. Although all of the pRB family members can bind E2F, they each bind different family members. Thus, pRB preferentially binds the "activating" E2Fs (E2F1-3), whereas p107/p130 preferentially bind "repressing" E2Fs (E2F4/5). Activating versus repressing species of E2F refers to whether they preferentially activate or repress their target genes. The downstream targets of E2F are extremely diverse, including genes involved in DNA replication, mitosis, DNA repair, differentiation, development, and apoptosis. The initial emphasis was on E2F targets involved in DNA replication, since these are *bona fide* targets of viruses creating an S-phase-like environment for viral replication.

Pocket proteins are clearly key players in negative growth regulation. Notably, different complexes form in different stages of the cell cycle. For example, p130-E2F complexes are found in G0, E2F4-p107 and E2F4-pRB complexes in G1, and free E2F1, -2 and -3 in S-phase. The levels of p107/p130 as well as several E2F family members are also cell cycle regulated. The pRB family is clearly involved in control of the restriction point, which is the point of no return in G1, when cells are committed to progression into S-phase. Additionally, pocket proteins are required for the G1–S checkpoint triggered following DNA damage. Recent experiments with mouse knockouts have demonstrated that loss of all three pocket proteins causes a failure to arrest in G1 following serum starvation, contact inhibition or DNA damage and is associated with cellular immortalization (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). pRB is normally inactivated by sequential cyclin-dependent kinase phosphorylation, which leads to derepression of E2F. LT only targets the hypo- or underphosphorylated form of pRB, causing a loss of G1–S checkpoint control.

Accumulated evidence indicates that LT by binding all three pRB family members via its LxCxE motif causes the disruption of most, but perhaps not all, of their repressive complexes with E2F family members (Sullivan *et al.*, 2000a, 2004; Zalvide and DeCaprio, 1995; Zalvide *et al.*, 1998). As a consequence, many E2F target genes are activated or derepressed. Significantly, data from p107/p130 knockout cells has established very clearly that they are equally important targets for LT transformation as pRB. The DnaJ domain is absolutely required in addition to the LxCxE for inactivation of p107/p130 (Stubdal *et al.*, 1997; Sullivan *et al.*, 2000a; Zalvide *et al.*, 1998). Taken together, these observations have served to reinforce the notion that p107 and p130 are important in preventing tumorigenesis just like pRB.

Finally, it is important to take into consideration that there are certain effects of LT that are LxCxE dependent, but not DnaJ dependent, for example, the ability of LT to override p53 growth suppression and induce

some properties of transformed cells (Gjoerup *et al*., 2000; Tevethia *et al*., 1997b). It has been demonstrated that LT through the LxCxE motif can partially overcome pRB-mediated repression of a heterologous promoter, possibly by disrupting interaction of pRB with HDAC corepressors that also bind via an LxCxE (Gjoerup *et al*., 2000).

Recent studies on E1A suggest that perhaps our model of LT acting merely to disrupt pRB/E2F complexes is too simplistic (DeCaprio, 2009). Reports indicate that E1A can be found at a number of different cellular promoters using chromatin immunoprecipitation techniques (Ferrari *et al*., 2008; Horwitz *et al*., 2008). E1A, like LT, binds a large number of cellular proteins including the pRB family and p300/CBP, which are histone acetyltransferases and coactivators (Berk, 2005). The implications are that E1A can recruit p300/CBP to pRB bound and repressed promoters with E2F sites and thereby turn on transcription. The reverse is likely also occurring. E1A could be recruiting repressive pRB complexes to promoters via an interaction of p300 with transcription factors bound upstream, effectively silencing the promoter. In other words, E1A by acting as an assembly platform that binds both transcriptionally repressive and activating factors can epigenetically reprogram the expression pattern of cellular genes, effectively tailoring the cellular environment to support viral replication. We can speculate that LT also may be bound to promoters repressed by pRB family members, and it might by recruitment of p300/CBP via p53 also be able to switch repressed promoters to an active state (DeCaprio, 2009). Currently there is little data to support this scenario for LT, but it is very plausible. Finally, it remains unclear if there are significant E2F-independent contributions to tumor suppression by pRB (Sellers *et al*., 1998), and whether these are specifically targeted by LT.

5. IRS1

Initial experiments demonstrated that LT cannot transform insulin-like growth factor I receptor (IGF-IR)-deficient cells (DeAngelis *et al*., 2006; Sell *et al*., 1993). Subsequently, it was shown that insulin receptor substrate 1 (IRS1), a key downstream target of IGF-I, is bound by LT, and together LT and IRS1 can transform IGF-IR null cells (D'Ambrosio *et al*., 1995; Fei *et al*., 1995). This implicated LT in IGF-I/insulin signaling pathways. A mutant lacking the first 250 amino acids failed to bind IRS1. LT causes the translocation of IRS1 from the cytoplasm to the nucleus (Prisco *et al*., 2002). Recently it was further shown that the pRB binding mutant K1 (E107K) is also defective for binding IRS1 and consequently defective in Akt activation (Yu and Alwine, 2008). It is possible that LT activation of Akt, leading to protection against apoptosis, is the main mechanism whereby IRS1 binding

contributes to LT transformation. A mutant has not been identified yet that uniquely affects IRS1 binding.

6. Nbs1

LT binds the Nbs1 protein, a component of the MRN (Mre11, Rad50, Nbs1) complex that functions in DNA repair and possibly as a sensor of DNA double strand breaks (Lee and Paull, 2005; Wu *et al*., 2004; Zhao *et al*., 2008). Nbs1 is mutated in the Nijmegen breakage syndrome associated with chromosomal instability and increased cancer susceptibility. LT binding to the Nbs1 protein is believed to allow unrestrained firing of the viral origin, unlike cellular origins that are licensed to fire only once during S phase (Wu *et al*., 2004). The binding of LT to Nbs1 requires the LT origin-binding domain. An internal deletion mutant from 147 to 259 failed to bind Nbs1, whereas a deletion from 147 to 201 retained binding. During an SV40 infection, the MRN complex was shown to be degraded by LT in a Cul7 binding-dependent manner (Zhao *et al*., 2008). The MRN complex is also targeted for degradation by adenovirus (Stracker *et al*., 2002). Once a LT point mutant is generated that selectively affects only Nbs1 binding, a role of the LT/Nbs1 interaction in oncogenic transformation or genomic instability can be investigated.

7. p53

The p53 protein was first discovered as a LT binding protein in 1979 in studies that would revolutionize the fields of tumor virology and cancer research (Lane and Crawford, 1979; Linzer and Levine, 1979). Subsequent investigations have revealed that most viruses inactivate p53. Adenovirus E1B 55k binds and transcriptionally represses p53 and together with E4orf6 targets it for degradation (Nevels *et al*., 1997; Querido *et al*., 1997; Sarnow *et al*., 1982; Yew and Berk, 1992). Human papillomavirus E6 targets p53 for proteasomal degradation (Scheffner *et al*., 1990; Werness *et al*., 1990). While initially perceived as an oncogene, p53 was later realized to be in fact a tumor suppressor protein (Baker *et al*., 1990; Donehower *et al*., 1992; Eliyahu *et al*., 1989; Finlay *et al*., 1989; Malkin *et al*., 1990). As a confounding factor, the p53 clones that were used in initial studies contained mutations that made them oncogenic (Levine and Oren, 2009). In fact, p53 was shown to be mutated or deleted in at least 50% of human cancers (Hollstein *et al*., 1994). Thus, it is likely the most frequently mutated gene in human cancer, especially since many other tumors have mutations affecting other components of the p53 pathway. The p53 protein has earned the nickname "Guardian of the Genome" (Lane, 1992), because it responds to many types of cellular stress

(e.g., genotoxic or oncogenic stress) by imposing either cell-cycle arrest, apoptosis, or senescence mediated by transcriptional control of a wealth of target genes. The $p21^{CIP1}$ protein is a critical downstream target that mediates cell-cycle arrest by inhibiting cyclin-dependent kinases, pRB phosphorylation, and cell-cycle progression (el-Deiry *et al*., 1993).

LT binds p53 within its core DNA binding domain leading to loss of target gene activation (Bargonetti *et al*., 1992; Jiang *et al*., 1993; Mietz *et al*., 1992; Segawa *et al*., 1993). The p53-related proteins p63 and p73 do not appear to be targeted, at least not by direct binding (Marin *et al*., 1998). The region of LT required for p53 binding is bipartite residing in residues 351–450 and 533–626 intertwined with the helicase domain (Kierstead and Tevethia, 1993). Detailed insight on specific residues of LT and p53 required for their interaction, as well as the mechanism of p53 inactivation, became apparent when the crystal structure of the LT helicase domain in complex with p53 was reported (Lilyestrom *et al*., 2006). It is generally believed that LT induces unscheduled DNA synthesis via interaction with pRB family members. This aberrant proliferation response triggers p53-dependent apoptosis or growth arrest, which must be thwarted by LT. The p53 binding site is critical for mouse embryo fibroblast immortalization and for extension of lifespan in human diploid fibroblasts (Kierstead and Tevethia, 1993; Lin and Simmons, 1991; Zhu *et al*., 1991). In transformation assays using established cell lines, p53 binding is often not strictly required (Manfredi and Prives, 1994). In fact, LT1–121 can transform in some cell systems such as C3H10T1/2, albeit at reduced frequency (Srinivasan *et al*., 1989, 1997; Zhu *et al*., 1992). Interestingly, p53-mediated cell-cycle arrest is overcome by LT in a mainly pRB binding-dependent manner (Gjoerup *et al*., 2000; Quartin *et al*., 1994). This can be rationalized, because p53 in large part signals via $p21^{CIP1}$ to pRB in order to induce cell-cycle arrest, reflecting important crosstalk between these two major tumor suppressor pathways.

Complexities of the LT/p53 interaction have gradually emerged. The traditional view that LT simply blocks p53 sequence-specific DNA binding via its interaction has been challenged by observations that mouse p53 bound to LT could contact DNA but not activate transcription, and human p53 bound to LT could both contact DNA and activate transcription (Sheppard *et al*., 1999). These contrasting views have not been resolved. There is also evidence that LT1–136, independent of p53 binding, can still inhibit p53-dependent transcription, although less efficiently than wild-type LT (Rushton *et al*., 1997). Perhaps even more confounding is the observation that LT potently stabilizes p53 (Deppert and Haug, 1986; Oren *et al*., 1981; Reich *et al*., 1983). Why would LT stabilize a major tumor suppressor protein, while at the same time functionally inactivating it? It was proposed that LT hijacks p53, essentially converting it from a tumor suppressor into an oncogene. In this scenario, presence of LT is not equivalent to a p53 null

phenotype but actually causes a gain of function in p53 (Deppert *et al.*, 1989; Hermannstadter *et al.*, 2009; Tiemann and Deppert, 1994a,b). Work with LT transgenic mice also concluded that the presence of wild-type p53 enhanced tumor formation, consistent with a p53 gain of function (Herzig *et al.*, 1999). Recently, it was demonstrated that the LT-p53 complex can have growth stimulatory properties, proposed to occur via activation of transcription from the insulin-like growth factor (IGF-1) promoter (Bocchetta *et al.*, 2008). One might hypothesize that LT in part stabilizes p53 to gain access to p300/CBP that in turn act on promoters or acetylate other LT bound proteins (Borger and DeCaprio, 2006). Interestingly, several of the naturally occurring p53 mutants have been shown to exhibit a gain of function (Brosh and Rotter, 2009; Dittmer *et al.*, 1993). Thus, a study of the LT stabilized form of p53 might also reveal insight that is germane to the mechanisms of tumorigenesis in nonviral lesions. The mechanistic basis for p53 stabilization by LT has not been fully elucidated, but likely involves induction of a DNA damage response via Bub1 binding (Hein *et al.*, 2009). Thus, it might be a consequence of LT's strategy to co-opt the DNA damage response to enhance viral replication (Dahl *et al.*, 2005; Hein *et al.*, 2009; Shi *et al.*, 2005; Zhao *et al.*, 2008).

8. p300/CBP AND p400

E1A was first shown to bind p300/CBP, and this was linked to transformation and induction of cellular DNA synthesis in quiescent cells (Egan *et al.*, 1988; Howe *et al.*, 1990; Wang *et al.*, 1993). Later, a pRB binding-deficient LT was shown to complement a p300 binding-deficient mutant of E1A for transformation, whereas the LT dl1135 mutant failed to complement (Yaciuk *et al.*, 1991). This suggested that LT and E1A might target p300/CBP in analogous ways. While the initial studies suggested the binding site is N-terminal requiring the CR1-like sequence of LT, it was convincingly demonstrated later that a carboxy-terminal segment of LT (251–708) sufficed to bind, although at reduced efficiency compared to wild-type LT (Eckner *et al.*, 1996; Lill *et al.*, 1997). Furthermore, somewhat surprisingly, binding is indirect through p53 (Lill *et al.*, 1997). Thus, binding to p300/CBP is absent in p53-deficient cells, but can be restored when p53 is reconstituted (Borger and DeCaprio, 2006). Given what we now know, it remains difficult to explain why dl1135 failed to complement, unless the DnaJ domain has to act on p300/CBP in some way.

CBP and p300 are large scaffolds involved in transcriptional regulation that contribute to many biological processes and are considered potential tumor suppressors (Gayther *et al.*, 2000; Goodman and Smolik, 2000; Iyer *et al.*, 2004). They act as adaptors or co-activators, in part via their intrinsic histone acetyltransferase (HAT) activity. Other proteins than histones can be

acetylated by p300/CBP. Binding of LT to CBP results in specific acetylation of p53 and LT on K697, of which the significance is unclear (Borger and DeCaprio, 2006; Poulin *et al*., 2004). Transcription factors that are co-activated by p300/CBP include p53 and E2F. In one model LT recruits the p300/CBP co-activators to promoters that are normally repressed by pRB, thus eliciting promoter activation. Nevertheless, it remains unclear exactly how LT acts on p300/CBP. The initial report suggested that p300/CBP transcriptional activity is inhibited by LT (Eckner *et al*., 1996), but a complex scenario now seems more likely. With the recent implications that LT binding to p53 causes a gain of function, the most likely candidates to mediate this are p300/CBP, although the details have not yet been worked out. Although LT binds p300/CBP using p53 as an adaptor, recent work based on "patch" surface mutants of LT indicates that LT also directly makes contact with p300/CBP, and this is required for oncogenic transformation (Ahuja *et al*., 2009). The critical targets modulated by LT binding to p300/CBP largely remain to be identified, but one of them appears to be induction of c-myc (Singhal *et al*., 2008).

As with p300, p400 was first identified as a component of E1A immunocomplexes (Barbeau *et al*., 1994). Subsequent experiments confirmed that LT also binds p400, and the C-terminal fragment of LT251–708 retains binding (Lill *et al*., 1997). The binding site for p400 has not been further mapped to attempt to separate it from p300 binding. p400 is a SWI2/SNF2-related chromatin remodeling factor and interacts with a c-myc binding protein called TRRAP (Fuchs *et al*., 2001). Binding of the complex p400/TRRAP is critical for E1A transformation, perhaps because it is required to induce c-myc expression (Tworkowski *et al*., 2008). The p400 protein is also implicated in the p53–p21 cellular senescence pathway and p53-dependent apoptosis (Chan *et al*., 2005; Samuelson *et al*., 2005). Its role in LT-mediated transformation, if any, has not been elucidated.

B. Small t Antigen

Mutant SV40 viruses that fail to produce ST are viable but grow somewhat more slowly and produce less virus (Cicala *et al*., 1994; Sleigh *et al*., 1978). *In vivo*, expression of ST stimulates viral DNA replication (Cicala *et al*., 1994). Significant effects of ST are seen in some transformation assays. While LT expression is sufficient to induce foci in many rodent transformation systems, anchorage-independent growth often requires ST as well (Bikel *et al*., 1986; Bouck *et al*., 1978; Jog *et al*., 1990; Mungre *et al*., 1994; Sleigh *et al*., 1978). In the human cell system, ST is required both for focus formation and growth in soft agar (Chang *et al*., 1985; de Ronde *et al*., 1989; Hahn *et al*., 1999; Porras *et al*., 1996; Yu *et al*., 2001).

The demonstration that human cell transformation can be accomplished by defined genetic elements brought ST back in the spotlight, because the first example comprised a combination of LT, ST, oncogenic H-ras and hTERT (Hahn *et al*., 1999). LT is not sufficient to immortalize human cells (Neufeld *et al*., 1987), but the combination of LT and hTERT bypasses both senescence and crisis arising from telomere shortening, effectively creating immortal cells (Counter *et al*., 1998; Zhu *et al*., 1999).

Due to the splicing arrangement, ST has the first 82 amino acids in common with LT followed by 92 unique ones. As expected, ST has a functional DnaJ domain capable of stimulating the ATPase activity of DnaK proteins (Srinivasan *et al*., 1997). However, unlike the LT DnaJ domain, its function or target has not been elucidated, neither has it been implicated in ST-mediated transformation (Boyapati *et al*., 2003). While LT is mainly a nuclear protein, ST is distributed both in the cytoplasm and nucleus. ST binds Zn via two C-terminal CxCxxC clusters, which confer conformational stability (Turk *et al*., 1993). The majority of ST activities can be attributed to binding of the serine–threonine protein phosphatase PP2A (Rundell and Parakati, 2001; Sablina and Hahn, 2008; Skoczylas *et al*., 2004). PP2A is a heterotrimeric enzyme composed of an A scaffold subunit, a B regulatory subunit, and a C catalytic subunit. It is really a family of phosphatases, since there are two different A subunits, two C subunits and at least 17 B subunits, divided into four classes, that can assemble together into >100 different holoenzyme complexes (Sablina and Hahn, 2008). ST binds the A subunit and primarily acts to displace the B subunit or prevent it from binding the AC core complex (Pallas *et al*., 1990; Yang *et al*., 1991). In most cases, this leads to an inhibition of enzyme activity. However, ST most likely targets a specific subset of PP2A complexes that are still poorly defined (Sablina and Hahn, 2008). Although the DnaJ domain is not strictly required for A subunit binding, recent structural analyses indicate it most likely enhances binding to the AC complex and contributes to inhibition of PP2A activity (Chen *et al*., 2007; Cho *et al*., 2007). There are also examples like histone H1 (Yang *et al*., 1991), androgen receptor (Yang *et al*., 2007) and 4E-BP1 (Yu *et al*., 2005), where ST delivers PP2A to the substrate, thus mediating dephosphorylation rather than inhibiting it. In the case of 4E-BP1 dephosphorylation, this effect on the mTOR pathway causes inhibition of cap-dependent translation, which is consistent with a reduction in overall cellular translation at late stages of SV40 infection (Yu *et al*., 2005).

Human cell transformation assays, as well as other transformation assays that require ST, strictly depend on its binding to PP2A as shown using point and truncation mutants of ST (Hahn *et al*., 2002; Mungre *et al*., 1994). The morphological aspects of transformation might be mediated by the ability of ST via PP2A to induce profound disruption of the actin cytoskeleton (Nunbhakdi-Craig *et al*., 2003; Sontag and Sontag, 2006). PP2A acts in a

myriad of proliferative or apoptotic pathways within the cell that are regulated by different isoforms of PP2A. Interestingly, knockdown of the PP2A B56Y subunit mimicked the effects of ST in anchorage-independent growth and tumorigenesis, suggesting that it is a key target (Chen *et al.*, 2004). Importantly, mutations in PP2A subunits have been found in human cancers, suggesting that some of these have tumor suppressor function (Arroyo and Hahn, 2005; Sablina and Hahn, 2008).

Some of the many and diverse targets of PP2A potentially involved in ST-induced tumorigenesis have been identified. One of these is c-myc, which is stabilized by ST via inhibition of PP2A-mediated dephosphorylation at S62 (Yeh *et al.*, 2004). Another important player is the phosphatidylinositol 3-kinase (PI3K) pathway that normally leads to activation of Akt, a prosurvival kinase, and Rac, a small GTP binding protein known to regulate proliferation and cytoskeletal organization (Skoczylas *et al.*, 2004; Zhao *et al.*, 2003). In human mammary epithelial cell transformation assays, ST could be replaced by constitutively active forms of PI3K or by Akt + Rac (Zhao *et al.*, 2003). Interestingly, a dominant negative subunit of PI3K blocked ST-dependent transformation in this system, although it does not appear that ST directly activates PI3K itself. ST also, again via PP2A binding, activates a number of kinases such as MAPK (Sontag *et al.*, 1993), Akt (Rodriguez-Viciana *et al.*, 2006; Yuan *et al.*, 2002), and PKCζ (Sontag *et al.*, 1997), the latter in turn leads to NFκB activation. MPyV ST has been demonstrated to be either pro- or anti-apoptotic dependent on the presence or absence of growth factors and the relative phosphorylation of Akt at sites T308 and S473 (Andrabi *et al.*, 2007). Studies have revealed that more than 30 kinases are regulated by PP2A (Millward *et al.*, 1999). ST can also activate the CAK kinase, resulting in cdk2 T160 phosphorylation, and together with cyclin E, promote active cyclin E/cdk2 complexes and concomitant DNA synthesis in quiescent cells (Sotillo *et al.*, 2008).

These observations may in part explain the long known ability of ST to induce proliferation, either alone or in some cell systems like human diploid fibroblasts, together with LT (Cicala *et al.*, 1994; Howe *et al.*, 1998; Porras *et al.*, 1999; Sontag *et al.*, 1993). Another important contribution comes from ST's ability to activate cellular promoters associated with proliferation control (Loeken *et al.*, 1988; Skoczylas *et al.*, 2004). The cyclin D1 promoter is activated via an AP1 site probably as a consequence of MAPK stimulation (Watanabe *et al.*, 1996). Also, ST activates the cyclin A promoter via a variant E2F site in its promoter (Skoczylas *et al.*, 2005). The cyclin-dependent kinase inhibitor $p27^{kip1}$ is much reduced upon ST expression, probably via proteasomal degradation, although the exact mechanism is unknown (Porras *et al.*, 1999). Thus, ST in a concerted manner targets the cyclin-dependent kinases involved in G1–S phase transition. However, high-level ST expression via an adenovirus vector also induces arrest in

G2 and prophase accompanied by a failure to assemble the spindle and centrosomes (Gaillard *et al.*, 2001). Finally, microarray analysis has identified many genes involved in proliferation, apoptosis, integrin signaling, and immune responses whose expression is altered by ST (Moreno *et al.*, 2004). Many genes are altered in expression via ST interaction with PP2A, but several other alterations are in fact independent of ST binding to PP2A, thus demonstrating that other functions of ST exist, although at the moment other cellular targets have not been identified.

C. Transgenic Model Systems

The transgenic expression of the SV40 early region in a wide variety of different tissues and organs has provided a wealth of insight to malignant progression (Ahuja *et al.*, 2005; Saenz Robles and Pipas, 2009). Dependent on the cell system, LT alone, or in conjunction with ST, can induce either no stimulation of proliferation, hyperplasia, dysplasia, carcinoma, or a full-blown metastatic phenotype. It underscores the importance of cell context for tumor development. One of the most thoroughly analyzed systems involves targeted expression of LT to the choroid plexus epithelium (CPE) of the brain using the LPV transcriptional control elements (Brinster *et al.*, 1984; Symonds *et al.*, 1993, 1994). The mice succumb to aggressive tumors within 1–2 months. Interestingly, when an N-terminal fragment of LT is expressed, encoding amino acids 1–121, identical tumors are observed but they grow more slowly (Chen *et al.*, 1992; Saenz Robles *et al.*, 1994). This is due to apoptosis, and it is p53-dependent, because in a p53-deficient background LT1–121 induces rapid tumor growth with minimal apoptosis (Symonds *et al.*, 1994). Tumor induction by LT1–121 was absolutely dependent on the LxCxE motif, implying a critical contribution from pocket protein inactivation. The underlying notion here is that LT1–121 binds pRB family members and induces unscheduled DNA synthesis. This leads via the E2F1 transcriptional pathway to induction of a p53-dependent checkpoint poised to eliminate the tumor cells. It elegantly demonstrates the necessity in this system of inactivating both pRB and p53 pathways to induce proliferation while maintaining cell viability. Targeted expression of LT1–121 in the mammary gland demonstrated a very similar scenario to the CPE of p53-dependent apoptosis limiting tumor development (Simin *et al.*, 2004). Interestingly, p53 inactivation by LT was also required for induction of B and T cell lymphomas, probably because p53-dependent apoptosis is prominent in these cell types (Saenz Robles *et al.*, 1994; Symonds *et al.*, 1993).

However, in a wide range of other cell types and tissues, p53 inactivation is not a requirement. Expression of LT1–121 in astrocytes leads to aberrant proliferation and extensive apoptosis that is not dependent on p53 but on

functional PTEN (Xiao *et al.*, 2002). Thus, inactivation of the pRB pathway by LT1–121 combined with PTEN loss accelerates astrocytomas. When expressed in the liver, LT1–121 is fully capable of inducing hepatocellular carcinomas (Bennoun *et al.*, 1998). This is dependent on both pRB binding and an intact DnaJ domain. Similarly, another N-terminal construct LT1–127 induces pancreatic acinar carcinomas, with no requirement for p53 inactivation (Tevethia *et al.*, 1997a). Expression of LT alone, or together with ST, via the probasin promoter has also been used in a prostate cancer model. When expressed in the prostate epithelium, hyperplasia, adenocarcinomas, and metastases are observed (Greenberg *et al.*, 1995; Masumori *et al.*, 2001). However, expression of LT1–121 induces prostate intraepithelial neoplasia and eventually adenocarcinoma (Hill *et al.*, 2005). In this system, PTEN, rather than p53, inhibits tumor progression via apoptosis.

Expression of LT in intestinal enterocytes leads to hyperplasia, and with age this progresses to dysplasia (Hauft *et al.*, 1992; Kim *et al.*, 1993, 1994; Markovics *et al.*, 2005). This is dependent on pRB family binding and a functional DnaJ domain, but p53 binding is dispensable since LT1–121 or LT1–136 behaves similarly to full-length LT, although they induce dysplasia with lower penetrance (Markovics *et al.*, 2005; Rathi *et al.*, 2007, 2009). In fact, there is no binding to or stabilization of p53 by LT in enterocytes, and a targeted deletion of p53 does not enhance hyperplasia (Markovics *et al.*, 2005). Recent microarray experiments indicate that in enterocytes LT and LT1–136 regulate an almost identical set of genes via their DnaJ and pRB binding motifs (Rathi *et al.*, 2009). The majority of these are likely targets of E2F, mainly E2F2 and E2F3a. This demonstrates that enterocyte proliferation and tumorigenesis is in large part regulated via the pRB/E2F pathway (Rathi *et al.*, 2009).

Taken together, whereas pRB inactivation is always required for aberrant proliferation and various aspects of tumorigenesis in each LT transgenic model system, p53 inactivation plays a highly variable role in tumor development and progression. Transgenic model systems are powerful because they allow us to directly assess the individual contribution of different genes to tumorigenesis in different cell types. Furthermore, they often provide a model of human cancer in which therapeutic modalities might be tested. It is not entirely clear why LT expression across a range of different cell and tissue types elicits such different responses, but it may reflect the general heterogeneity of cancers. LT could, in addition to the well-characterized targets pRB and p53, interact with other unique binding proteins within each cell type that contribute to specific aspects of tumorigenesis (Saenz Robles and Pipas, 2009). Alternatively, LT interacts with the same set of proteins in each cell type, but the response is different, because these cellular proteins are wired differently dependent on the cell system. Although pRB inactivation is clearly important for tumorigenesis, it remains unclear if additional binding proteins within LT1–136 (Bub1, Cul7, IRS1?) might contribute to the malignant phenotype observed in the various systems.

V. CONCLUSION

In the past 50 years, polyomaviruses such as SV40 and MPyV have contributed in critical ways to our understanding of basic mechanisms and key cellular proteins involved in carcinogenesis (Atkin *et al*., 2009). However, before the discovery of MCV, evidence to support the etiologic role of polyomaviruses in human cancers was tenuous. Since its discovery, accumulating data provides compelling evidence that MCV is causally associated with MCC. It is likely that future investigations into the biology of this new tumor virus will be generalizable to other settings, including gaining insights into the development of nonvirally induced cancers.

The recent surge in discovery of new polyomaviruses suggests that there are more to come. There is long-standing serologic evidence for another LPV-like polyomavirus residing in the human population, although it has not been identified (Brade *et al*., 1981). Studies have suggested that 15–25% of the population is seropositive for an LPV-related virus (Kean *et al*., 2009). For KIV and WUV, studies are very preliminary and have thus far focused only on detection of viral DNA and serologic seroprevalence. Future studies of KIV and WUV are likely to reveal if these viruses are indeed linked to human pathogenesis. The relationship between JCV and BKV and human cancers has been difficult to determine despite extensive investigations, yet *in vitro* assays as well as animal experiments have clearly established their potential for oncogenic transformation. The range of diseases associated with these human polyomaviruses show that they are highly significant human infections. The accumulated knowledge gained from the study of each individual virus synergizes to help understand each in their particular niche.

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