IDENTIFICATION OF HUMAN VAM6P AS A NOVEL CELLULAR INTERACTOR FOR MERKEL CELL POLYOMAVIRUS LARGE T ANTIGEN

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Merkel cell polyomavirus (MCV) has been recently described as the cause for most human Merkel cell carcinomas. MCV is similar to simian virus 40 (SV40) and encodes a nuclear large T (LT) oncoprotein that is usually mutated to eliminate viral replication among tumor-derived MCV. In search of novel cellular interactors for MCV LT, we identified the hVam6p cytoplasmic protein involved in lysosomal processing as a binding partner with MCV LT but not SV40 LT. We have shown that hVam6p binds through its clathrin heavy chain homology domain to a unique region of MCV LT adjacent to the retinoblastoma protein (pRB) binding motif. hVam6p and pRB have discrete binding sites on LT. Intriguingly, MCV LT translocates hVam6p to the nucleus, sequestering it from involvement in lysosomal trafficking. A naturally occurring, tumor-derived mutant LT (MCV350) lacking a nuclear localization signal binds hVam6p but fails to inhibit hVam6p-induced lysosomal clustering, suggesting MCV has evolved a novel mechanism to target hVam6p that may contribute to viral uncoating or egress through lysosomal processing during virus replication. In addition, we have investigated the effect of LT-hVam6p interaction on MCV virion production and viral replication. Mutation of the MCV LT-hVam6p binding site enhances encapsidated virion production, which is confirmed by both elevated

subgenomic DNA synthesis and viral particle production. Remarkably, overexpression of hVam6p reduces MCV virion production by >90%, suggesting a previously unrecognized role for this protein in regulating virus replication. Collectively, identification of novel binding partners for MCV LT has provided new insights into the mechanisms underlying the MCV lifecycle.

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PREFACE

My graduate study at the University of Pittsburgh has been full of fine individuals helping me along the way. Their continuous support and friendship has driven me to become the person I am today. To all of you, I really appreciate what you have done and continue to do for me.

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With sincere respect and appreciation,

Xi Liu

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1.0 INTRODUCTION

1.1 VIRUSES AND CANCER

1.1.1 History of Polyomaviruses

Polyomaviruses are a family of viruses characterized by small non-enveloped virions with icosahedral capsids containing a double-stranded DNA genome of about 5 kb. Polyomaviruses and papillomaviruses were historically considered as subfamilies within the now obsolete family *Papovaviridae.* In 2000, the International Committee on Taxonomy of Viruses recognized them as two distinct families, *Polyomaviridae* and *Papillomaviridae.* Polyomaviruses have been identified from various species of animals including humans, monkeys, rodents, rabbits and birds. Each polyomavirus has a restricted host range and generally does not productively infect other species (Cole and Conzen, 2001).

Murine polyomavirus (MPyV) was the first polyomavirus discovered in 1953. Ludwik Gross was working on the cell free transmission of leukaemia in newborn mice and found a filterable agent causing salivary gland carcinoma formation (Gross, 1953). Subsequently, Stewart and Eddy were able to isolate and characterize this virus by tissue culture using mouse embryo cells (Stewart et al., 1958). It was proposed to be named as polyoma virus due to its ability to

develop multiple tumors when inoculated into newborn mice, hamsters or rats (Stewart et al., 1958).

Simian vacuolating virus 40 (SV40) was isolated by Sweet and Hilleman in 1960 from rhesus monkey kidney cells used in the late 1950s to produce polio vaccines (Sweet and Hilleman, 1960). Although studies on whether SV40 plays a role in causing human malignancies has remained controversial over the past 50 years (Poulin and DeCaprio, 2006), SV40 and MPyV have been used extensively as a model system for the study of DNA replication, transcription, oncogenic transformation as well as signal transduction (Ahuja et al., 2005; Bullock, 1997; Simmons, 2000; Stenlund, 2003; Sullivan and Pipas, 2002).

The first two human polyomaviuses, JC virus (JCV) and BK virus (BKV) were characterized in 1971 and named after patients' initials (Gardner et al., 1971; Padgett et al., 1971). JCV was cultured from the brain tissue of a patient suffering from progressive multifocal leucoencephalopathy (PML) (Padgett et al., 1971). BKV was isolated from the urine of a kidney allograft recipient with ureteral obstruction and renal failure (Gardner et al., 1971). Both viruses establish persistent infection in 35-85% of the population worldwide (Knowles et al., 2003) and possess pathogenic properties in immunosuppressed patients.

Unlike the former discoveries based on direct cultivation of patient material, another two human polyomaviruses, Karolinska Institue virus (KIV) and Washington University virus (WUV), were identified using high throughput DNA sequencing of encapsidated DNA from human respiratory secretions from patients with respiratory tract infections (Allander et al., 2007; Gaynor et al., 2007).

Employing a newly developed high throughput methodology, named digital transcriptome subtraction (DTS) (Feng et al., 2007), Merkel cell polyomavirus (MCV) was identified from Merkel cell carcinoma (MCC) using transcriptome sequencing followed by

subsequent *in silico* subtraction of human transcripts (Feng et al., 2008). MCV was shown to be clonally integrated into ~80% of MCCs (Feng et al., 2008) and has a strong association with MCCs.

Two years later, rolling circle amplification (RCA) lead to the discovery of three more human polyomaviruses. This technique involves random primer extension with bacteriophage phi29 DNA polymerase that preferentially amplifies circular target sequences (Johne et al., 2009). Human polyomavirus 6 (HPyV6) and HPyV7 were detected using RCA in the skin of healthy individuals (Schowalter et al., 2010). In the same year, trichodysplasia spinulosa-associated polyomavirus (TSV) was identified from a heart transplant recipient with trichodysplasia spinulosa, a uncommon skin disease particularly seen in immunosuppressed patients (van der Meijden et al., 2010). Most recently, the ninth human polyomavirus (HPyV9) was identified by consensus PCR from kidney secretions of a kidney transplant patient under immunosuppressive treatment (Scuda et al., 2011).

Polyomaviruses are divided into three different subgroups: MPyV subgroup, SV40 subgroup and avian polyomavirus subgroup (Crandall et al., 2006; Feng et al., 2008). A phylogenetic comparison of sequences shows that BKV, JCV, KIV and WUV belong to the SV40 subgroup, while MCV is closely related to MPyV and the African green monkey lymphotropic polyomavirus (LPV) (Fig. 1).

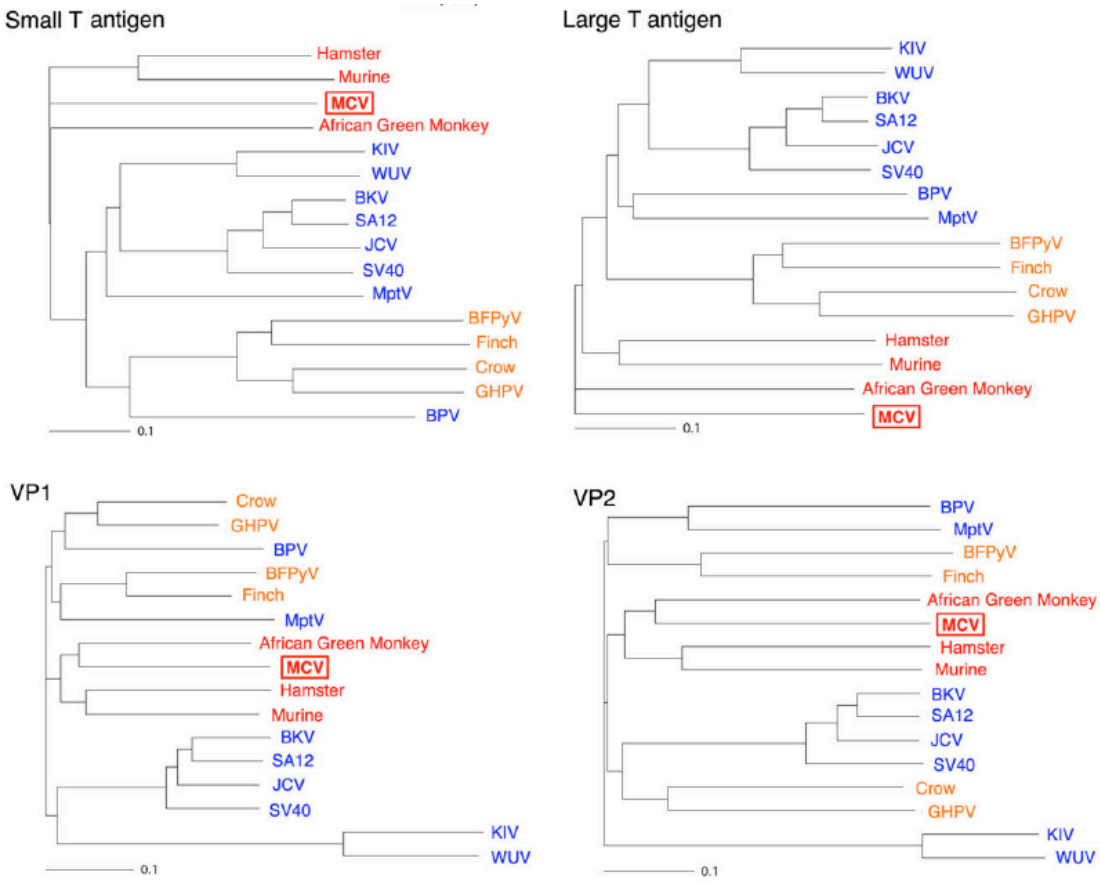

Figure 1. Phylogenetic Trees of Polyomavirus Small T, Large T, VP1 and VP2 Proteins. The MPyV subgroup is indicated in red, the SV40 subgroup is in blue and avian polyomavirus subgroup is in orange. MCV (marked with red rectangle) is most closely related to MPyV subgroup, while BKV, JCV, KIV and WUV group together with the SV40 subgroup. Adapted from (Feng et al., 2008).

1.1.2 Polyomaviruses and Human Cancer

The potential roles of polyomaviruses in the etiology of human malignant diseases have been extensively studied. Among the reported human polyomaviruses, only MCV exhibits strong association with human cancer. The tumorigenic potential of JCV and BKV has been described,

but no consistent correlation with human malignancies has been demonstrated. The pathogenicity of KIV and WUV in respiratory diseases remains speculative because of the co-detection with other respiratory viruses. Due to inadequate evidence, the causal role of SV40 in human tumors is highly controversial.

Merkel cell polyomavirus

There are three lines of evidence implicating MCV's etiological role in MCCs: (1) MCV was found to be clonally-integrated in human genome indicating infection prior to clonal expansion in approximately 80% of MCCs (Feng et al., 2008); (2) Tumor-derived MCV exhibits a signature of LT truncation that eliminates its helicase domain essential for viral replication, whereas retaining the domains required for inducing cell-cycle progression (Shuda et al., 2008); (3) The growth and survival of MCC cell lines are found to be dependent on MCV T antigen expression (Houben et al., 2010). In addition, although MCV is widespread among human populations, it is specific to MCC and is not detected at significant levels in other cancers or in healthy tissues (Kean et al., 2009; Pastrana et al., 2009; Tolstov et al., 2009). The correlation between MCV and MCC has been confirmed by different research groups (Becker et al., 2008; Duncavage et al., 2009; Garneski et al., 2008; Kassem et al., 2008a; Ridd et al., 2009; Sastre-Garau et al., 2009; Sihto et al., 2009; Touze et al., 2009; Varga et al., 2009).

JC virus

JCV has a limited tissue tropism infecting the kidney, oligodendrocytes and astrocytes in the central nervous system (CNS) (Maginnis and Atwood, 2009). It establishes a persistent

infection in the kidney and bone marrow (Maginnis and Atwood, 2009). JCV was thought to cause a lytic infection in the CNS and lead to development of the demyelinating disease named progressive multifocal leukoencephalopathy (PML) in immunocompromised individuals (Gibson et al., 1993; Grinnell et al., 1983; Stoner et al., 1986; Taoufik et al., 1998). Experimentally, it was shown that JCV causes tumor formation when inoculated into rodent animals and non-human primates (Houff et al., 1983; London et al., 1978; London et al., 1983; Ohsumi et al., 1986; Walker et al., 1973). Although some studies reported that JCV associates with various human cancers such as brain tumors, colorectal cancers and gastric cancers (Caldarelli-Stefano et al., 2000; Jung et al., 2008; Laghi et al., 1999; Murai et al., 2007; Rencic et al., 1996; Ricciardiello et al., 2001; Shin et al., 2006), other studies showed no correlation (Maginnis and Atwood, 2009). So far, there is no clear relationship between JCV and development of human malignancies.

BK virus

BKV ubiquitously infects the human population and establishes a persistent infection in the kidney and urinary tract (Chesters et al., 1983; Heritage et al., 1981). BKV can reactivate and cause hemorrhagic cystitis (HC) or polyomavirus nephropathy (PVN), particularly in bone marrow and renal transplant patients (Dropulic and Jones, 2008; Nickeleit and Mihatsch, 2006). BKV was suspected to be a tumor virus because the expression of its early region is able to transform rodent cells in culture and immortalize human cells (Costa et al., 1977; Grossi et al., 1982a; Grossi et al., 1982b; Major and Di Mayorca, 1973; Portolani and Borgatti, 1978). Additionally, inoculation of BKV into newborn mice, rats and hamsters, promotes formation of various types of tumors, including ependymoma, neuroblastoma, glioma, nephroblastoma,

fibrosarcoma, liposarcoma, and osteosarcoma (Tognon et al., 2003). Despite numerous studies, the etiological role of BKV in human cancer remains controversial. Many contradictory results were reported on the presence of BKV DNA and/or proteins in various tumor types (Jiang et al., 2008).

KI virus and WU virus

KIV and WUV were originally detected in respiratory samples, however, their causative roles in respiratory disease have not been proved due to the fact that viral sequences were detected at similar frequencies in asymptomatic patients (Abed et al., 2007; Han et al., 2007; Jiang et al., 2008; Norja et al., 2007), and that other pathogens can be co-detected (Abedi Kiasari et al., 2008; Allander et al., 2007; Bialasiewicz et al., 2008; Gaynor et al., 2007; Han et al., 2007; Le et al., 2007; Neske et al., 2008).

Simian vacuolating virus 40

Concerns regarding the health risk of SV40 have arisen from the discovery of this virus as a contaminant in poliovirus vaccine (Sweet and Hilleman, 1960). The oncogenic potential of SV40 lies in its transformation ability in a variety of human cells as well as inducing tumor growth in animal models (Girardi et al., 1965; Jensen et al., 1963; Koprowski et al., 1963; Pipas, 2009; Ponten et al., 1963; Shein and Enders, 1962). However, conflicting reports linking SV40 with human malignancies does not provide sufficient evidence that SV40 plays a role in human cancer (Poulin and DeCaprio, 2006).

1.1.3 Other Viruses and Human Cancer

It is estimated that viral infection accounts for approximately 15% of human cancer cases worldwide (Bouvard et al., 2009; Parkin, 2006; zur Hausen, 1991). Therefore, the discovery of human viruses as etiological agents for human malignancies is a milestone in cancer research (Javier and Butel, 2008). To date, seven human tumor viruses have been identified, including Epstein-Barr virus (EBV/HHV4), Hepatitis B virus (HBV), human T-lymphotropic virus-1 (HTLV-1), high-risk human papillomaviruses (HPV) 16 and HPV18, Hepatitis C virus (HCV), Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) and Merkel cell polyomavirus (MCV) (Moore and Chang, 2010) (Liao, 2006; Javier and Butel, 2008) (Table 1).

Table 1. The Human Cancer Viruses. Adapted from (Moore and Chang, 2010)

Virus	Genome	Notable cancers	Year first described
Epstein–Barr virus (EBV; also known as human herpesvirus 4 (HHV4))	Double-stranded DNA herpesvirus	Most Burkitt's lymphoma and nasopharyngeal carcinoma, most lymphoproliferative disorders, some Hodgkin's disease, some non-Hodgkin's lymphoma and some gastrointestinal lymphoma	1964
Hepatitis B virus (HBV)	Single-stranded and double-stranded DNA hepadenovirus	Some hepatocellular carcinoma	1965
Human T-lymphotropic virus-I (HTLV-I)	Positive-strand, single-stranded RNA retrovirus	Adult T cell leukaemia	1980
High-risk human papillomaviruses (HPV) 16 and HPV 18 (some other α-HPV types are also carcinogens)	Double-stranded DNA papillomavirus	Most cervical cancer and penile cancers and some other anogenital and head and neck cancers	1983–1984
Hepatitis C virus (HCV)	Positive-strand, single-stranded RNA flavivirus	Some hepatocellular carcinoma and some lymphomas	1989
Kaposi's sarcoma herpesvirus (KSHV; also known as human herpesvirus 8 (HHV8))	Double-stranded DNA herpesvirus	Kaposi's sarcoma, primary effusion lymphoma and some multicentric Castleman's disease	1994
Merkel cell polyomavirus (MCV)	Double-stranded DNA polyomavirus	Most Merkel cell carcinoma	2008

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EBV, also known as HHV4, is the first human tumor virus. It was discovered by electron microscopy in 1964 in cultured cell lines derived from Burkitt's lymphoma (BL) patient (Burkitt, 1962; Epstein et al., 1964). EBV has a worldwide distribution among human populations and persists as a lifelong, asymptomatic infection (Young and Rickinson, 2004). EBV has been implicated to have a central role in the etiology of Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma (Thompson and Kurzrock, 2004). However, the signature chromosomal translocation of BL still occurs with the absence of EBV, suggesting that EBV may not be necessary or sufficient to induce lymphoma (Kelly and Rickinson, 2007).

HBV was discovered by Baruch Blumberg in 1965, when he screened blood samples and found that one Australian sample contained an antigen reacting with the antibody from an American hemophilia patient serum (Blumberg et al., 1965). Subsequently, a series of studies showed that hepatitis sera specifically contained this antigen (Blumberg et al., 1967; Prince, 1968), which was later on shown to be the surface antigen of HBV. In 1975, Blumberg's study suggested a link between chronic HBV infection and hepatocellular carcinoma (HCC) (Blumberg et al., 1975). This finding was confirmed by another independent study demonstrating that HBV infection increases 200-fold the risk for HCC development compared to non-infected individuals in a Taiwanese cohort (Beasley et al., 1981). HCC is one of the most common cancers in the world, and HBV-induced HCC causes more than 300,000 deaths each year (Javier and Butel, 2008). The discovery of Australia antigen has led to the development of a HBV vaccine (Buynak et al., 1976) and Blumberg was awarded the Nobel Prize in 1976.

HTLV-1 is the first oncogenic human retrovirus reported. It was originally isolated from the lymphocytes of a cutaneous T-cell lymphoma patient (Poiesz et al., 1980). Shortly after, it was shown that HTLV antigen could be visualized by indirect immunofluorescence in an adult T-cell leukemia (ATL) cell line (Hinuma et al., 1981). More evidence has been found supporting

the correlation of HTLV-1 with ATL (Levine, 1992). It is estimated that HTLV infects 10~20 million people worldwide, but less than 5% of people develop ATL (Matsuoka and Jeang, 2007).

In 1974, zur Hausen proposed that HPV might be the causal agent for cervical cancer, due to its role in sexually transmitted genital warts (zur Hausen, 1976; zur Hausen et al., 1974). Subsequently, in 1983 and 1984, this hypothesis was substantiated by the identification of two novel genotypes of HPV DNA that were detected in cervical cancers (Boshart et al., 1984; Durst et al., 1983). Intriguingly, these two types of HPV, high-risk papillomaviruses HPV-16 and HPV-18, were found to integrate into the host genome and be present in approximately 70% of cervical cancers (Boshart et al., 1984; Durst et al., 1983; Frazer et al., 2007). More recently, the generation of virus-like particle (VLP)-based HPV vaccines has contributed to the prevention of more than 300,000 cervical cancer cases per year (Javier and Butel, 2008).

HCV was the second human virus linked to hepatocelluar carcinoma (HCC). After the discovery of HBV, in search of additional causal agents for transfusion-associated hepatitis, Houghton and colleagues generated a cDNA library made from the sera of chronically infected chimpanzees, which led to the identification of a genome fragment from HCV (Choo et al., 1989). Subsequent radioimmunoassay studies showed that HCV is a critical factor associated with HCC and non-A, non-B hepatitis (Colombo et al., 1989). HCV infects more than 170 million people worldwide and approximately 20% of such individuals develop cirrhosis (Javier and Butel, 2008).

KSHV, also known as HHV8, was the second tumorigenic human herpesvirus discovered. It was found by representational difference analysis using Kaposi's sarcoma and healthy tissue samples from the same patient (Chang et al., 1994; Lisitsyn et al., 1993). The viral DNA was detected in 90% of Kaposi's sarcoma tissues from AIDS patients but not in non-AIDS patients

(Chang et al., 1994). Numerous studies indicate that KSHV plays a causal role in the development of Kaposi's sarcoma (Sarid et al., 1999).

Collectively, compelling evidence has accumulated that these seven human viruses play etiologic roles in human malignancies (Butel, 2000; Javier and Butel, 2008; McLaughlin-Drubin and Munger, 2008; Moore and Chang, 2010). Although cancer development is a multistep process and a virus is not the only contributing factor, these viruses have been proved to serve as powerful tools for understanding cancer biology.

1.1.4 General Polyomavirus Biology

1.1.4.1 Polyomavirus Genetics and Genome Organization

Polyomaviruses are small, noneveloped, double-stranded DNA viruses with a genome of around 5Kb, which is packaged with cellular histone proteins H2A, H2B, H3 and H4 within 40-45 nm icosahedral capsids (Ahsan and Shah, 2002). The circular genome of polyomaviruses can be divided into three functional elements: an early coding region, a late coding region and a non-coding control region (NCCR) encompassing the origin of DNA replication. The early genes and the late genes are transcribed in opposite directions along the genome. The early coding region encodes a large T (tumor) antigen (LT) and a small t antigen (ST), which are translated from alternatively spliced transcripts. LT is a master regulator for the production of early mRNA, initiator of viral DNA replication and activator of late gene expression (Ahuja, 2005). Both LT

and ST play critical roles in cellular transformation and tumorigenesis. The late region encodes four structural capsid components VP1, VP2, VP3 and VP4. VP3 and VP4 are generated by internal translation of VP2. VP4 has only been described for SV40 and it promotes cell lysis as well as viral egress (Daniels et al., 2007). Agnoproteins, encoded by SV40, BKV and JCV, are small regulatory proteins involved in virion maturation (Khalili et al., 2005; Ng et al., 1985).

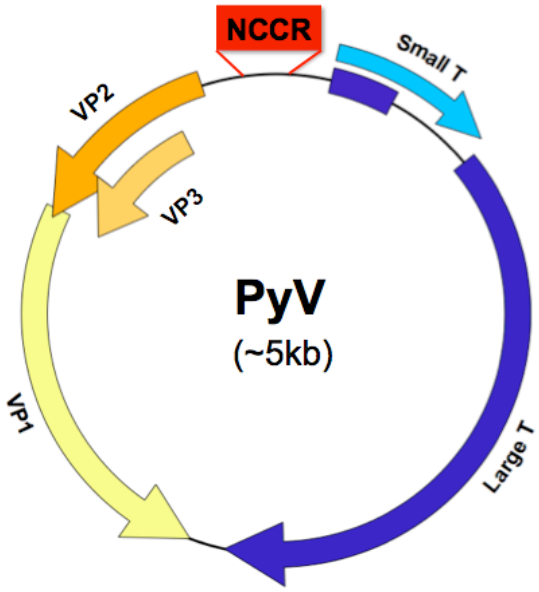


Figure 2. Polyomavirus (PyV) Genome Organization.

In addition to LT and ST, some polyomaviruses also encode various accessory T antigens. Murine and hamster polyomaviruses possess a middle T antigen, which functions in cellular transformation (Cheng et al., 2009). SV40 encodes a 17k T antigen (17kT) from an alternatively spliced early transcript that induces minimal transformation in rat fibroblasts (Boyapati et al., 2003; Zerrahn et al., 1993). Additional T antigen isoforms, analogous to SV40 17kT, have also been described for JCV, BKV and MCV: JCV has three T' antigens: T'165, T'136 and T'135 (Trowbridge and Frisque, 1995); BKV contains a truncated T antigen similar to

JCV's T'136 (Abend et al., 2009); and MCV encodes a 57k T antigen (Shuda et al., 2008). No accessory T antigens have been reported for KIV or WUV.

Several polyomaviruses (SV40, MPyV, BKV, JCV and MCV) express microRNAs (miRNA) during lytic infection, predicted to autoregulate early gene expression at late times during infection (Seo et al., 2008; Sullivan et al., 2005; Sullivan et al., 2009). Recent studies demonstrate that SV40 miRNA downregulates the expression of viral T antigen, thus evading cytotoxic T cells (Sullivan et al., 2005).

1.1.4.2 Polyomavirus Life Cycle

There are two alternative consequences when a polyomavirus infects a cell. If the host is permissive to viral replication, virus will initiate viral DNA amplification, produce progeny virions and release viral particles through cell lysis. If the host is nonpermissive to viral replication, the infection will be abortive and transiently cause cell transformation by expression of the early genes (Imperiale, 2000, 2001; Tognon et al., 2003).

The infectious lytic lifecycles of polyomaviruses have both early and late stages. The early stage involves the absorption of the virion onto the cell surface, internalization by the target cell, transportation to the nucleus, release of the viral genome and initiation of viral DNA replication. The late stage involves the production of capsid proteins, the subsequent assembly of new virions and the release of viral progeny (Fig. 3).

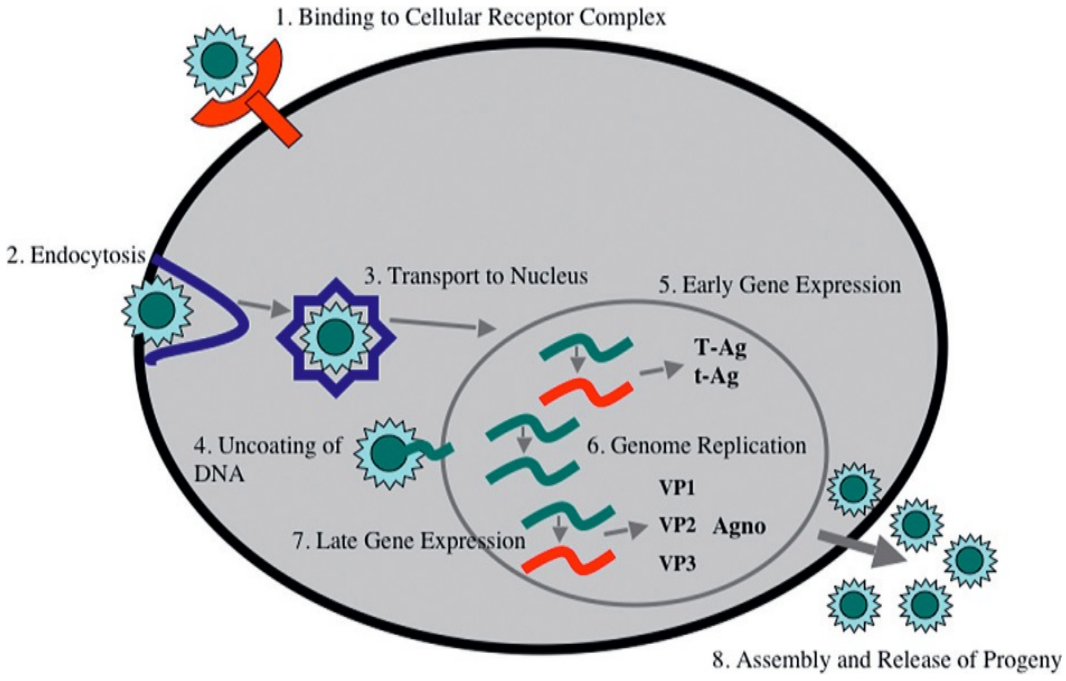


Figure 3. Schematic of Polyomavirus Life Cycle. (1) Attachment: polyomaviruses attach the host cell through interaction with cellular receptor; (2) Internalization: polyomaviruses are internalized into the target cell by endocytosis; (3) Nuclear transport: polyomaviruses are transported from the cytosol to the nucleus; (4) Uncoating: viral genome is exposed for early gene expression; (5) Early gene expression: translation of large T and small t antigens; (6) Viral replication: viral DNA is synthesized in the nucleus; (7) Late gene expression: translation of viral capsid proteins; (8) Assembly and release of new virion progeny: this stage marks a completed infectious viral lifecycle. Adapted from (Eash et al., 2006).

At the beginning of the viral lifecycle, virions attach to the cell surface through the interaction of capsid protein VP1 with cellular ganglioside receptors (Campanero-Rhodes et al., 2007; Low et al., 2006; Smith et al., 2003; Tsai et al., 2003). Gangliosides are molecules composed of glycosphingolipids with one or more sialic acids. They are components of the plasma membrane that regulate signaling transduction (Ledeen and Yu, 1982). The number of sialic acid residues is used to classify the ganglioside species (Svennerholm, 1994).

Polyomavirues use different receptors to gain entry into the cell. Specifically, SV40 binds to ganglioside GM1 (Campanero-Rhodes et al., 2007; Neu et al., 2008; Tsai et al., 2003), MPyV binds to GD1a and GT1b (Smith et al., 2003; Tsai et al., 2003), BKV binds to GD1b and GT1b (Low et al., 2006), and MCV binds to GT1b (Erickson et al., 2009). It was revealed that JCV uses serotonin receptor $5HT_{2A}$ to infect cells (Elphick et al., 2004).

Upon attachment, polyomaviruses take advantage of the cellular endocytic machinery to penetrate the plasma membrane. BKV and JCV utilize different mechanisms to enter the host cell (Eash et al., 2004; Pho et al., 2000; Querbes et al., 2004). BK virus enters cells by caveolae-mediated endocytosis through a caveolin-1 scaffolding domain (Eash et al., 2004), whereas the JCV uptake utilizes clathrin-dependent endocytosis (Pho et al., 2000).

After penetration, polyomaviruses are transported from the cytosol to the nucleus. Viruses depend on the active cytoskeletal transport machinery for the intracellular migration (Dohner and Sodeik, 2005). In the case of BKV and JCV, the microtubule network plays a critical role during early infection (Ashok and Atwood, 2003; Dohner and Sodeik, 2005).

With the help of endocytic and cytoskeletal transport, polyomaviruses arrive at the nucleus, where viral replication and virion assembly take place. Uncoating of polyomaviruses happens inside the nucleus. Viral chromatin is released and transcription of early genes is initiated (Drachenberg et al., 2003; Nakanishi et al., 1996). Initiation of transcription takes place at the NCCR, which contains sequences necessary for transcriptional regulation and the origin of viral DNA replication (Imperiale, 2001). Early coding genes are first transcribed to ST and LT, driving infected cells to S-phase. After translation, LT performs functions to initiate viral genome replication, including binding and unwinding the viral origin and recruiting host factors such as DNA polymerase α/primase complex (Waga et al., 1994). Meanwhile, LT represses the

transcription from early promoter and stimulates the transcription from late promoter (Cole and Conzen, 2001).

The expression and nuclear localization of viral structural proteins VP1, VP2 and VP3 leads to the assembly of the virion capsid. Subsequently, viral DNA is packaged with histone proteins and generates a mini-chromosome structure similar to the host's chromatin. New viral particles are then realeased by host cell lysis, although some reports imply that virions can be secreted from the plasma membranes of intact cells (Clayson et al., 1989)

1.1.4.3 Polyomavirus and DNA Replication

Polyomavirus DNA replication occurs in the nucleus utilizing host replication machinery in conjunction with LT. LT is a multifunctional nuclear protein that is required for initiating viral replication (Myers and Tjian, 1980; Tegtmeyer, 1972). The replication takes place when LT binds to viral origin of replication (*ori*) as a double hexamer and functions as a bi-directional helicase hydrolyzing ATP as well as unwinding the DNA genome (Fanning and Knippers, 1992; Smelkova and Borowiec, 1997; Wright et al., 2009). This process involves the recruitment of cellular proteins that are required for DNA synthesis including DNA toposisomerase I (Top1), replication protein A (RPA) and DNA polymerase α-primase (pol-prim) (Dean et al., 1987; Dornreiter et al., 1990; Melendy and Stillman, 1993; Stahl et al., 1986). Upon initiation of replication, pol-prim is replaced by replication factor C (RF-C), proliferating cell nuclear antigen (PCNA) and DNA polymerase δ for elongation (Maga et al., 2000; Mossi et al., 2000).

1.1.4.4 T Antigen Interactions with Cellular Proteins

Polyomaviruses have been used as model systems to understand fundamental biological processes including DNA replication and oncogenic transformation. Tumor (T) antigens, the early gene products encoded by polyomaviruses, associate with key cellular targets to alter signaling pathways. LT interacts with heat shock chaperone Hsc70, pRB family proteins, p53, as well as other binding partners to cause malignant transformation. ST also possesses transforming ability through association with protein phosphatase PP2A. Figure 4 represents the cellular binding domains on SV40 LT and ST antigens.

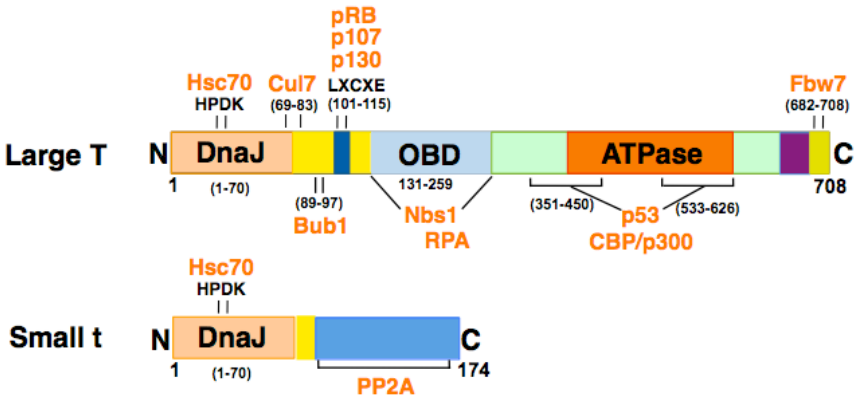


Figure 4. Schematic of Binding Domains on SV40 LT and ST. Reproduced from (Gjoerup and Chang, 2010).

LT Interactions with Cellular Proteins:

LT is a nuclear phosphoprotein targeting a number of cellular proteins to exert its activity in viral replication and neoplastic transformation. LT is composed of distinct functional domains mediating association with binding partners. These motifs include the DnaJ domain (Campbell et

al., 1997a), pRB-binding LXCXE motif (Kim et al., 2001b), the origin-binding domain (Luo et al., 1996), the ATPase/helicase domain and the C-terminal host range domain (Li et al., 2003)(Figure 4). LT interactors include Hsc70, pRB family proteins and p53, as well as other host proteins such as CBP/p300, Cul7, IRS-1, Bub1 and Nbs1.

DnaJ Domain

The LT DnaJ domain is a ~70 amino acid fragment located at the *N*-terminus (Brodsky and Pipas, 1998; Kim et al., 2001b) and homologous with other known DnaJ domains containing HPD motif (Campbell et al., 1997a; Kim et al., 2001b). It is responsible for associating with heat shock protein Hsc70 and enhancing its ATPase activity (Campbell et al., 1997a; Sawai and Butel, 1989; Sawai et al., 1994; Srinivasan et al., 1997; Sullivan and Pipas, 2002). The conformation of Hsc70 is changed upon ATP hydrolysis, thus affecting the bound substrates (Sullivan and Pipas, 2002) and performing functions including protein transport, refolding of denatured proteins and disruption of protein complexes such as the replication machinery of phage λ (Brodsky, 1996; Hartl, 1996; Polissi et al., 1995; Sullivan and Pipas, 2002). Mutations within the DnaJ HPD motif (such as H42Q or D44N) have been shown to abolish the DnaJ/Hsc70 association (Campbell et al., 1997a).

It was shown that the DnaJ domain is critical for viral replication (Pipas et al., 1983). Point mutants in this domain significantly reduce viral DNA synthesis as well as virus production (Collins and Pipas, 1995; Gluzman and Ahrens, 1982; Peden and Pipas, 1992). Its essential role for viral replication is further supported by the fact that a chimeric T antigen, in which the DnaJ

domain is replaced with the human Hsj1 DnaJ domain, retains the ability to promote DNA replication (Campbell et al., 1997a; Sullivan and Pipas, 2002).

Aside from promoting viral DNA replication, the DnaJ domain is capable of contributing to oncogenic transformation through inactivation of the pRB family members (Campbell et al., 1997b; Srinivasan et al., 1997; Stubdal et al., 1997; Stubdal et al., 1996; Zalvide et al., 1998). It has been shown that DnaJ domain deletion mutants fail to cause foci formation although retaining the ability to bind pRB family proteins and p53 (Pipas et al., 1983; Srinivasan et al., 1997). Focus formation assays demonstrated that DnaJ mutant dl1135 (deletion of residue 17-27) is completely defective in inducing transformation (Srinivasan et al., 1997). Interestingly, the D44N mutant is less defective in the context of full-length LT compared to T1-136 (Beachy et al., 2002; Gjoerup et al., 2000; Srinivasan et al., 1997), possibly due to conformational effects, or other transforming function existing in the LT N-terminus (Gjoerup and Chang, 2010). Notably, DnaJ domain seems to be a unique feature for polyomavirus T antigens, which is not acquired by other viral oncoproteins such as adenovirus E1A/E1B or HPV E6/E7 (Gjoerup and Chang, 2010).

pRB Family

The retinoblastoma (RB) gene was demonstrated to be a tumor suppressor gene since homozygous null mutations in humans cause tumor formation in the retina (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987), based on Knudson's two-hit hypothesis (Knudson, 1971). Mutations in RB have been found in a broad range of tumors (Burkhart and Sage, 2008). The Rb protein (pRB) was reported as a nuclear 110 kDa phosphoprotein (Lee et al., 1987), guarding the

cell cycle G1/S checkpoint and inhibiting S-phase entry by associating with E2F through its transactivation domain. The E2F family of transcription factors regulates genes involved in cell cycle progression including cyclins A and E, DNA polymerase α, and proliferating cell nuclear antigen (PCNA) (DeGregori and Johnson, 2006; Nevins et al., 2001). The retinoblastoma family of proteins contains three members, pRB, p107, and p130, which are referred to "pocket proteins" based on a conserved pocket structure essential for function (Felsani et al., 2006).

The first report of virus targeting pRB for inactivation was adenovirus E1A oncoprotein (Whyte et al., 1988). Subsequently, SV40 LT was shown to specifically bind to pRB, p107 and p130, which is essential for oncogenic transformation (Chen and Paucha, 1990; DeCaprio et al., 1988; Ewen et al., 1989; Kalderon and Smith, 1984; Ludlow et al., 1989; Manfredi and Prives, 1994). Mutagenesis studies showed that LT residues 106-114 are crucial for transformation (Chen and Paucha, 1990), and a LT mutant containing a single point substitution at residue 107 (E107K) is completely defective for transformation (Kalderon and Smith, 1984). Similar to adenovirus E1A and SV40 LT, HPV E7 also interacts with pRB through a conserved LXCXE motif (DeCaprio et al., 1988; Dyson et al., 1989; Munger et al., 1989; Whyte et al., 1988).

Extensive mutational analysis demonstrated that the DnaJ domain is also required to inactivate pRB family tumor suppressor function, by acting together with LT LXCXE motif to disrupt the pRB/E2F complex (Harris et al., 1996; Pipas et al., 1983; Sheng et al., 1997; Srinivasan et al., 1997; Zalvide et al., 1998). Interestingly, a LT truncation mutant containing only the LXCXE motif and the DnaJ domain retains the ability to induce limited foci formation and hyperplasia in transgenic mice (Fromm et al., 1994; Kim et al., 1994; Srinivasan et al., 1989; Tevethia et al., 1997).

SV40 LT preferentially binds to the hypophosphorylated form of pRB in G1 phase and inhibits its growth suppressing function (Ludlow et al., 1989). The interactions between the LT

and the pRB family proteins lead to the activation of the E2F family of transcription factors, which contribute to the expression of target genes required for DNA synthesis and cell entry into the S phase (Das and Imperiale, 2009).

p53

p53 is a DNA damage responsive transcription factor acting as a "guardian of the genome" that affects DNA synthesis and repair, cell cycle arrest, senescence and apoptosis (Lane, 1992; Levine, 1997). p53 is inactivated in more than 50% of all human cancers (Soussi and Lozano, 2005). Under normal conditions, p53 remains at low levels, but its protein stability is elevated in stressed cells. Activation of p53 occurs upon the exposure to DNA damage, or virus-induced unscheduled DNA synthesis, which leads to a cascade of events including the transcriptional activation of the CDK/cyclin kinase inhibitor p21 and the E3 ubiquitin ligase MDM2 (Coutts and La Thangue, 2007; Momand et al., 2000).

The p53 tumor suppressor was initially identified as a binding partner for LT in SV40-transformed and infected cells (Lane and Crawford, 1979; Linzer and Levine, 1979). Other viruses were also reported to target p53. For instance, adenovirus E1B binds and inhibits p53 function, and E1B/E4 targets p53 for degradation (Nevels et al., 1997; Sarnow et al., 1982; Yew and Berk, 1992). Interestingly, unlike adenovirus E1B/E4 and HPV E6, SV40 LT stabilizes p53 instead of degrading it (Deppert et al., 1989; Oren et al., 1981; Tiemann and Deppert, 1994). LT mutants defective in p53 binding lose the ability to transform (Kierstead and Tevethia, 1993; Peden et al., 1989; Peden et al., 1998; Zhu et al., 1991). It was demonstrated that LT binds the p53 DNA binding domain, thus preventing its transactivation functions (Bargonetti et al., 1992; Jiang et al., 1993). Although LT-p53 interaction is essential for transformation in most systems,

it is not sufficient (Conzen and Cole, 1995). It was shown that a LT mutant lacking the p53-binding domain but containing the DnaJ and the pRB-binding motif, still retains the ability to prevent p53-mediated growth arrest (Michael-Michalovitz et al., 1991; Quartin et al., 1994). Similar to SV40 LT, JCV and BKV LTs also bind to p53 and inhibit the upregulation of p21 upon DNA damage (Bollag et al., 1989; Harris et al., 1998; Krynska et al., 1997).

Other host proteins

Although it has been clearly demonstrated that the DnaJ domain, pRB and p53 binding domains are crucial for LT-directed cellular transformation and tumorigenesis, many studies have also shown that additional interactions could possibly lead to oncogenesis as well (Cavender et al., 1995; Gjoerup and Chang, 2010; Sachsenmeier and Pipas, 2001).

CBP/p300

CREB-binding protein (CBP)/p300 are transcriptional co-activating proteins acting potentially as tumor suppressors that mediate a number of biological functions, including cell growth and transformation (Gayther et al., 2000; Goodman and Smolik, 2000). CBP/p300 were first discovered as binding partners for E1A, and this was linked to adenovirus transformation and regulation of cellular DNA synthesis (Egan et al., 1988; Howe et al., 1990; Wang et al., 1993). Subsequent studies showed that SV40 wild-type LT, but not DnaJ domain mutants, can complement CBP/p300 binding-defective mutants of E1A to restore transformation (Yaciuk et

al., 1991), suggesting that LT and E1A may have analogous function against CBP/p300. It was later shown that the LT C-terminal (amino acids 251-708) region is responsible for binding, although with decreased efficiency compared to wild-type LT (Lill et al., 1997). It was unclear whether or not LT-p300/CBP association was direct (Avantaggiati et al., 1996; Eckner et al., 1996; Lill et al., 1997), until more recently, it was shown that this interaction was indirect and bridged by p53 (Borger and DeCaprio, 2006; Poulin et al., 2004). Although CBP is able to acetylate LT on K697 in a p53-dependent manner, the biological significance remains elusive (Borger and DeCaprio, 2006; Poulin et al., 2004). Recent studies suggested that LT also directly targets CBP/p300, which is essential for oncogenic transformation (Ahuja et al., 2009).

Cul7

SV40 LT was initially found to associate with an unknown protein named p185 (Kohrman and Imperiale, 1992). Subsequently, an independent group reported the interaction of LT and p193, an apoptosis-inducing protein (Tsai et al., 2000). Using mass spectrometry, p185 and p193 were identified to be Cul7, a member of the cullin family of E3 ubiquitin ligases that mediate ubiquitination-dependent proteosomal degradation (Ali et al., 2004). The Cul7 binding site on LT was mapped to residues 69-83 (Kasper et al., 2005). More importantly, it was demonstrated that LT-induced transformation also depends on the inactivation of Cul7 (Kasper et al., 2005). Recent report indicated the insulin receptor substrate 1 (IRS1) as a candidate substrate for Cul7-mediated degradation (Xu et al., 2008). Additionally, LT targets the Mre11-Rad50-Nbs1 (MRN) complex for degradation through Cul7 during SV40 infection (Zhao et al., 2008).

IRS-1

Insulin receptor substrate 1 (IRS-1) plays a critical role in transmitting signals from the insulin and insulin-like growth factor I (IGF-I) receptors (IGF-1R) (Keller and Lienhard, 1994). It was initially shown that LT fails to transform IGF-1R-deficient cells (Sell et al., 1993). Subsequently, it was demonstrated that LT binds to IRS-1 and this interaction causes transformation in IGF-IR null cells (D'Ambrosio et al., 1995; Fei et al., 1995). Interestingly, both SV40 and JCV LTs were shown to translocate IRS-1 to the nucleus (Lassak et al., 2002; Prisco et al., 2002). More recently, it was reported that the pRB binding mutation disrupts LT interaction with IRS-1, leading to the loss of activation of PI3K/Akt signaling (Yu and Alwine, 2008). However, a specific IRS-1 binding mutant has not yet been identified.

Bub1

The mitotic checkpoint kinase Bub1 was revealed as an interactor for LT using yeast two-hybrid screening (Cotsiki et al., 2004). A LT mutant deleting residue 89-97 was shown to be defective in Bub1 binding and a WEXWW motif, which is conserved among SV40, JCV, BKV, and bovine polyomavirus T antigens, was critical for efficient binding (Cotsiki et al., 2004). It was demonstrated that LT-Bub1 interaction strongly correlates with cellular transformation, but not immortalization (Cotsiki et al., 2004). Bub1 was shown to be essential for the spindle checkpoint control by promoting the formation of stable kinetochore–microtubule attachments (Meraldi and Sorger, 2005; Perera et al., 2007). Interestingly, Bub1 mutation has been detected in human cancers, and reduced Bub1 expression in mice causes impaired chromosome segregation and increased aneuploidy leading to tumorigenesis (Cahill et al., 1998; Jeganathan et al., 2007; Schliekelman et al., 2009).

Nbs1

LT associates with Nbs1, the Nijmegen breakage syndrome protein, which is a component of MRN (Mre11, Rad50, Nbs1) complex that mediates double-strand break repair (Lee and Paull, 2005; Wu et al., 2004; Zhao et al., 2008). LT binding to Nbs1 allows chromosomal hyper-replication and this binding requires the LT origin binding domain (Wu et al., 2004). Additionally, mutation analysis reveals that an LT deletion mutant of residues 147-259 is defective in binding to Nbs1. Although the MRN complex is degraded by LT via Cul7 during infection (Zhao et al., 2008), the significance of LT-Nbs1 interaction in cellular transformation or genomic instability is not fully understood.

Small T Interactions with Cellular Proteins:

Small t (ST) antigen shares N-terminal sequences with LT, including the DnaJ domain. It cooperates with LT to transform both mouse and human cells (Bikel et al., 1987; Rundell et al., 1998). The unique C-terminal region of ST associates with protein phosphatase 2A (PP2A) family of serine/threonine phosphatases that regulate a variety of signal transduction pathways (Janssens and Goris, 2001a; Pallas et al., 1990; Sontag, 2001). PP2A is made of a scaffold A subunit, a regulatory B subunit and a catalytic C subunit (Janssens and Goris, 2001b; Sontag, 2001). More than 100 different PP2A heterotrimeric complexes can be found through combination of various subunits (Sablina and Hahn, 2008). ST binds to the A and C subunits but displaces the B subunit, which leads to the inhibition of PP2A activity (Chen et al., 2004; Pallas

et al., 1990; Walter et al., 1990; Yang et al., 1991). Mutations in ST that disrupt the interaction with PP2A are defective in ST-mediated transformation (Mungre et al., 1994; Porras et al., 1996). The ST residues 97-103 are crucial for the interaction with PP2A (Mungre et al., 1994; Porras et al., 1996; Yu et al., 2001). Additionally, ST mutant containing only the PP2A binding domain (amino acids 88–174) retains the ability to promote transformation, suggesting that the inhibition of PP2A activity is required for ST transforming activity (Hahn et al., 2002).

1.2 MERKEL CELL POLYOMAVIRUS

1.2.1 Merkel Cell Carcinoma (MCC)

Merkel cells are originally described as “touch cells” by the German anatomist Friedrich Sigmund Merkel in 1875 (Merkel, 1875). They are specialized cells located at the basal layer of the epidermis, characterized by numerous membrane-bound granules with dense cores, suggesting a neuroendocrine function (Pearse, 1980).

Merkel cell carcinoma (MCC) was initially described by Toker in 1972 as “trabecular carcinoma of the skin” (Toker, 1972). It is an uncommon but aggressive skin cancer of neuroendocrine origin that typically affects elderly or immunosuppressed patients (Engels et al.,

2002). Although MCC is rare, its incidence rate has tripled during 1986-2001 to 1500 cases per year in the United States (Hodgson, 2005; Lemos and Nghiem, 2007). MCC frequently appears on sun-exposed skin (e.g., face, neck and extremities) as a fast-growing, painless and firm lump (Swann and Yoon, 2007). Risk factors associated with the development of MCC include UV-radiation, advancing age, and immune suppression (Heath et al., 2008).

Microscopically, MCC consists of small blue cells with sparse cytoplasm and round, medium-size nuclei. MCC tumor cells can be distinguished by the characteristic perinuclear expression of low molecular weight cytokeratin 20 (CK20), which is an intermediate filament protein responsible for the structural integrity of epithelial cells. In addition, MCC cells show negative stains for TTF-1 and CK7 (positive in small cell lung carcinoma), S-100 (positive in melanoma), and LCA (positive in lymphoma) (Nghiem and Jaimes, 2008; Sarma et al., 2010). Current treatment approaches for MCC patients include surgery, radiation therapy and chemotherapy. Prior to discovery of MCV, studies on the molecular origins of MCC were unsuccessful, limiting the development of MCC therapies (Lemos and Nghiem, 2007).

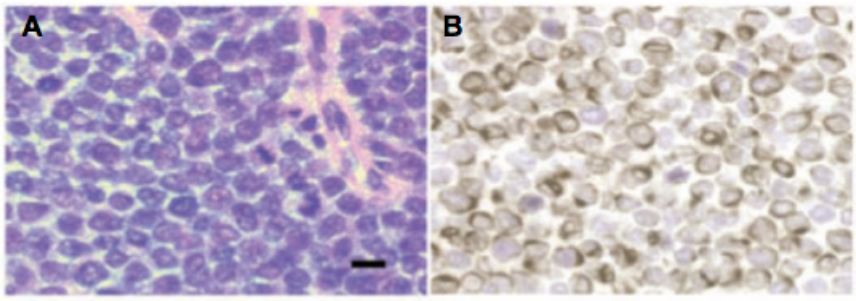


Figure 5. Merkel Cell Carcinoma Tissue Staining with H&E and CK20. (A) Hematoxylin-eosin staining. (B) Cytokeratin-20 (CK20) staining. Reproduced from (Feng et al., 2008).

1.2.2 Discovery of Merkel Cell Polyomavirus (MCV)

The susceptibility of MCC to immune surveillance is suggestive for an infectious origin. A newly developed technique called digital transcriptome subtraction (DTS) has led to the discovery of Merkel cell polyomavirus (MCV) (Feng et al., 2008). DTS is a high-throughput cDNA sequencing methodology used to identify foreign transcripts from human cancers (Feng et al., 2008).

To search for the viral agent in MCC, two cDNA libraries were generated from four MCC tumors, followed by pyrosequencing. Out of 2395 high-fidelity candidate sequences, one transcript showed high homology to the T antigen sequences of African green monkey lymphotropic polyomavirus (LPyV) and BKV (Feng et al., 2008). This transcript was subsequently extended by rapid amplification of cDNA ends (RACE). By genome walking, MCV was revealed to have a closed circular genome of 5387 bp, which is similar to, but distinct from all known polyomaviruses.

To investigate the association between MCV infection and MCC, 10 MCC tumor samples from different patients were screened by PCR and 8 (80%) were positive for MCV sequences (Feng et al., 2008). Southern blotting revealed that the MCV genome is integrated into the human genome before the clonal expansion of tumor cells (Feng et al., 2008).

1.2.3 MCV Genome Organization

Similar to other polyomaviruses (see section 1.1.4.1), MCV genome consists of an early and a late coding region that are separated by non-coding regulatory region (NCCR) (Fig. 6). The early gene expression region encodes LT, sT and a splicing variant named 57kT, all of which share a common N-terminal region (residue 1-78). The late region contains VP1, VP2 and VP3 open reading frames. Unlike SV40, BKV and JCV, MCV does not encode an agnoprotein.

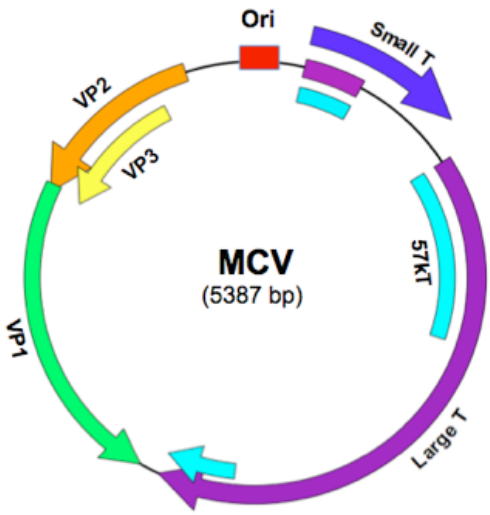


Figure 6. Schematic of MCV Genome. Similar to other polyomaviruses, MCV genome encodes T antigens and capsid proteins: large T antigen (purple), small T antigen (dark blue), 57kT (light blue), VP1 (green), VP2 (orange), and VP3 (yellow).

1.2.4 MCV Tumor Antigens

As generally described for polyomaviruses (see section 1.1.4.1), MCV T antigens are expressed from variably spliced viral transcripts, LT, sT and 57kT, which is analogous to SV40 17kT (Shuda et al., 2008) (Fig. 7). MCV LT sequence retains all major conserved functional motifs of other polyomavirus LTs, including DnaJ, pRB-binding, origin-binding and helicase/ATPase domains (Shuda et al., 2008). Notably, tumor-derived LTs were found to have premature stop codon mutations that ablate full-length LT expression and eliminate the helicase/ATPase domain required for viral replication (Shuda et al., 2008). This finding is consistent with previous studies showing that polyomavirus-induced transformation does not require episomal viral replication (Gish and Botchan, 1987; Lania et al., 1981). In addition, studies also reported mutations in the MCV viral origin and VP1 region that inhibit virus production, implying a strong selective pressure in the tumor (Kassem et al., 2008b; Kwun et al., 2009). To investigate the MCV T antigen expression in MCC, a monoclonal antibody named CM2B4 was developed against a peptide epitope in exon 2 of the T antigen locus (Shuda et al., 2009a) (Fig. 7). CM2B4 recognizes MCV wild-type LT and 57kT as well as tumor-derived LT.T339 and LT.T350, but not the putative ST (Fig. 7). It was shown that CM2B4 antibody is highly specific for MCV and does not have reactivity to T antigens from BKV, JCV or SV40 by immunofluorescence or immuoblotting (Shuda et al., 2009a). Studies demonstrated that MCV T antigen expression is required for the maintenance of MCC cells. Knock down of MCV T antigen using short hairpin RNA (shRNA)-expressing vectors targeting exon 1 region leads to the growth arrest and/or cell death in MCV-positive MCC cells, strongly indicating that MCV is the causative agent of MCV-positive MCC (Houben et al., 2010).

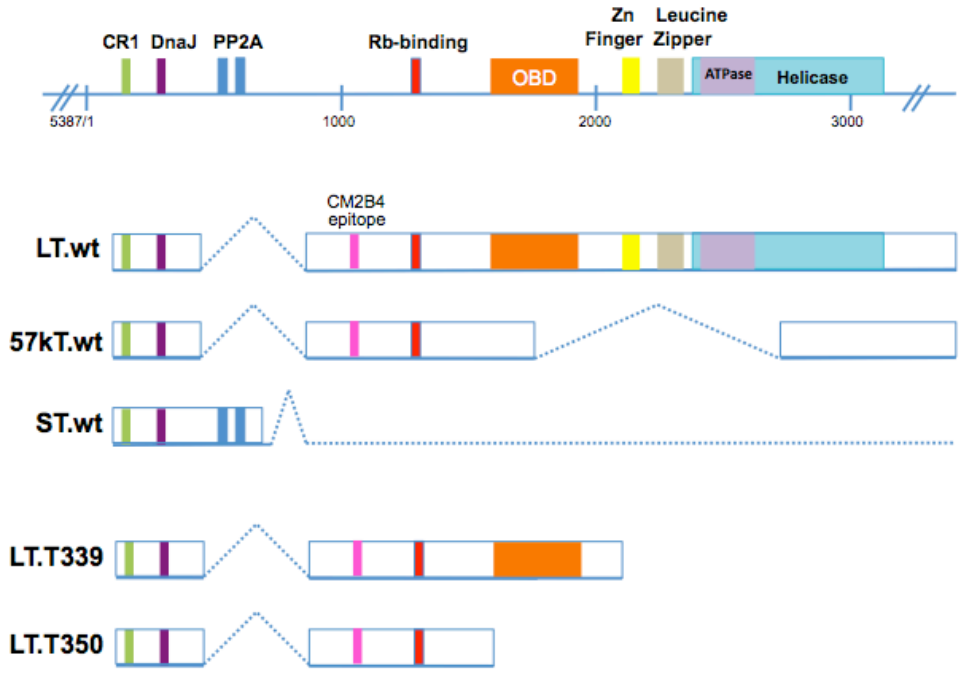


Figure 7. Transcript Diagram of MCV T Antigens. Wild-type (LT.wt, 57kT.wt, and ST.wt) and tumor-derived (LT.T339 and LT.T350) T antigen loci are shown. CM2B4 epitope is indicated in pink.

1.2.5 MCV Origin Replication

Polyomavirus origins are located within the NCCR, containing promoters for both early and late transcription and enhancers that regulate *cis* activation of early gene transcription (Cole and Cozen, 2001). It was shown that MCV NCCR contains a minimum core origin of 71 bp, which is sufficient for viral DNA replication in the presence of wild-type LT (Kwun et al., 2009). This region includes three subdomains: an AT-rich tract that leads to DNA melting, an LT-binding site composed of eight GAGGC-like pantanucleotide sequences (PS), and an early enhancer region (Kwun et al., 2009). Mutation analysis within the core region revealed that three

of the eight PS (PS1, PS2, and PS4) are essential for origin replication (Kwun et al., 2009). Additionally, a single point mutation was found in one PS from a naturally-occurring tumor-derived strain (MCV350) which results in significant reduction of LT binding to the origin and consequent elimination of viral replication (Kwun et al., 2009). Engineering this mutation into the full-length replicating MCV consensus genome abolishes the clone's ability to replicate and to produce virions (H.Feng et al., accepted by *PLoS ONE*).

1.2.6 MCV Tumor Cell Evolution Model

An MCC evolution model was proposed that MCV first integrates into the human genome to be sustained in the tumor cell, but secondary mutations or deletions must occur to abolish the replication capacity (Shuda et al., 2008) (Figure 8). The explanation is that wild-type T antigen expression can initiate unlicensed DNA replication, which causes replication fork collisions and DNA damage (Shuda et al., 2008). Under natural selection pressure, viral replication needs to be eliminated upon viral integration prior to MCC development. Similarly, when the HPV E1 helicase is expressed in cervical cancer cells, DNA damage response occurs (Kadaja et al., 2007). Therefore, loss of replication capacity is likely to be a general feature acquired by integrated DNA tumor viruses.

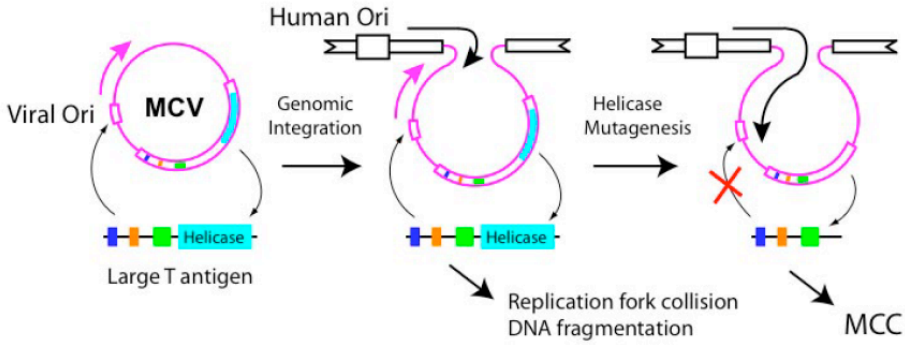


Figure 8. Schematic of MCC Tumor Cell Evolution Model. MCV integration into host chromosomes can be expected to lead to autonomous viral origin DNA replication when wild-type T antigen is expressed. Newly replicated virus DNA strands may collide with cellular replication forks unless secondary mutations eliminate viral LT antigen helicase activity. Adapted from (Shuda et al., 2008)

1.3 ENDOLYSOSOMAL SYSTEM

As a major portion of my thesis, we identified hVam6p, a protein component involved in lysosomal machinery as a binding partner for MCV large T antigen. Thus, in this section we will discuss the cellular endolysosomal system, which will provide us insights into the interplay between host and virus during virus lifecycle.

1.3.1 Endocytic Pathway

Endocytosis is a process in which the cell internalizes extracellular molecules by invagination of the plasma membrane and formation of closed vesicles. It functions to uptake essential nutrients, defend against invading microorganisms and maintain cellular homeostasis. Endocytosis takes place by different mechanisms that fall into two categories, 'phagocytosis' (cell-eating) and 'pinocytosis' (cell-drinking) (Conner and Schmid, 2003). Phagocytosis in mammals is primarily restricted to specialized cells, whereas pinocytosis occurs in all types of cells. The internalization mechanisms include macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (Conner and Schmid, 2003)

Endotytosed cargos pass through a variety of functionally distinct membrane organelles including early endosomes, late endosomes and lysosomes (Fig. 9). These intermediates are distinguished by their molecular content, morphology, pH and kinetics (Sachse et al., 2002). Early endosomes (EEs) are the main recipients of internalized vesicles from the plasma membrane. They are membrane-bound organelles responsible for sorting cargos toward recycling or degradation. Outgoing trafficking pathways from EEs include recycling of membrane components to the plasma membrane, delivery of vesicles to the Golgi apparatus and maturation of EEs into endosomal carrier vesicles (ECV) or multivesicular bodies (MVB) that subsequently fuse with late endosomes and lysosomes as the final destination (Barysch et al., 2009).

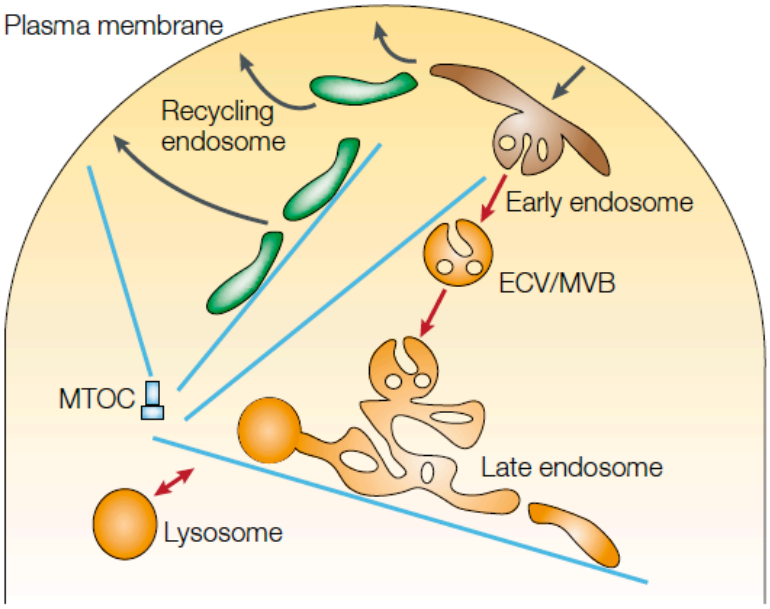


Figure 9. Endocytic Pathway. Internalized molecules are delivered to early endosomes for protein sorting, and downregulated receptors are transported to late endosomes and lysosomes for degradation. Some receptors are recycled back to the plasma membrane to be reused. Degradation pathway is indicated in red and the recycling pathway is in green. Microtubules and the microtubule-organizing centre (MTOC) are in blue. ECV, endosomal carrier vesicle; MVB, multivesicular body. Adapted from (Gruenberg, 2001).

1.3.2 Lysosomes and Lysosome Fusion

As terminal compartments of the endocytic pathway, lysosomes and their fusion with late endocytic organelles are critical for maintaining the integrity of the macromolecule degradation process.

Lysosomes are large (~0.5μm) vacuoles containing electron-dense cores and more than 40 hydrolytic enzymes to break down delivered cargos into simple compounds (De Duve et al., 1955; Holtzman, 1989). Lysosomes are distinguished from endosomes by the absence of the two mannose-6-phosphate receptors (MPRs) and recycling cell surface receptors (Luzio et al., 2000).

Two types of proteins are critical for lysosome function: soluble lysosomal hydrolases and integral lysosomal membrane proteins (LMPs) (Saftig and Klumperman, 2009). Each of the 50 known lysosomal hydrolases is responsible for degrading specific substrates. They are involved in antigen processing, breaking down extracellular matrix and initiating apoptosis (Conus and Simon, 2008). Lysosomes also contain more than 25 types of LMPs present at lysosomal limiting membranes (Callahan et al., 2009; Lubke et al., 2009; Schroder et al., 2007). Their diverse functions include protein import from the cytosol, export of degradation products to the cytoplasm and membrane fusion (Eskelinen et al., 2003). The most abundant LMPs are lysosome-associated membrane protein 1 (LAMP1), LAMP2, lysosome integral membrane protein 2 and tetraspanin CD63 (Saftig and Klumperman, 2009).

Lysosomes represent sites of convergence for multiple pathways including endocytosis, phagocytosis and autophagy. Lysosomes can fuse with themselves, late endosomes, phagosomes and autophagosomes (Fig. 10).

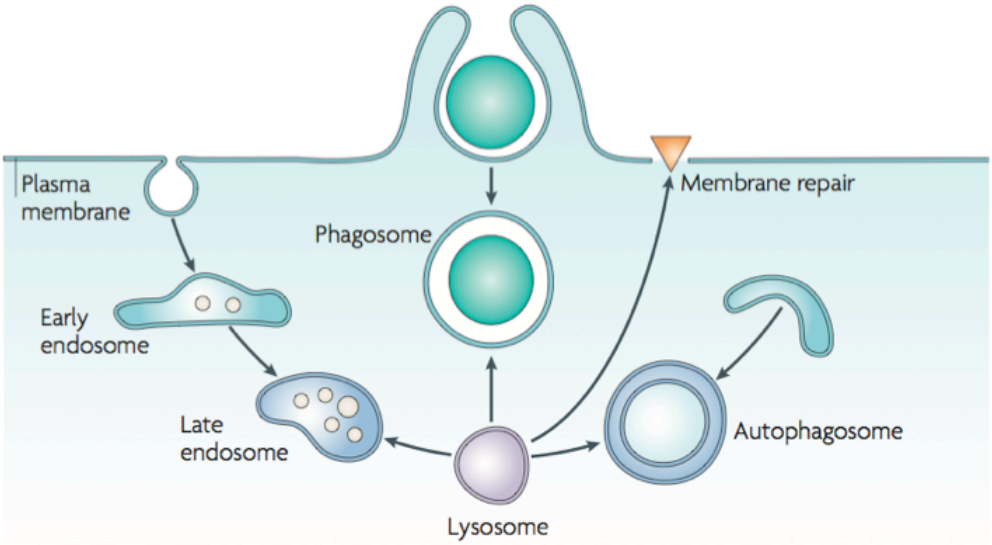


Figure 10. Delivery to Lysosomes and Lysosomal Fusion. Adapted from (Luzio et al., 2007).

Lysosomal fusion events are composed of three sequential steps: tethering and docking, SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) assembly, and membrane fusion (Fig. 11).

Tethering refers to the formation of links between two late endocytic organelles that extend over distances >25 nm. The tethering of late endosomes and lysosomes was observed both in cultured cells and in cell-free systems (Bright et al., 1997; Futter et al., 1996; Mullock et al., 1989; van Deurs et al., 1995). It has been suggested that the mammalian homotypic fusion and vacuole protein sorting (HOPS) complex act as tethers (Luzio et al., 2007). Overexpression of the HOPS complex components Vps18 and Vps39 leads to the clustering of late endosomes and lysosomes, suggesting their role in tethering process (Caplan et al., 2001; Poupon et al., 2003).

Docking refers to the holding of membranes from two vesicles within a bilayer's width (<5-10 nm) (Pfeffer, 1999). This step requires N-ethylmaleimide sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs) (Whiteheart and Kubalek, 1995; Whiteheart et al., 1994). Tethering and docking of vesicles at the target membrane precedes the formation of a tight core SNARE complex (Luzio et al., 2007).

SNARE complex is a four-helix bundle, which is the key driving force for membrane fusion (Weber et al., 1998). The formation of SNARE complex requires syntaxin-7, syntaxin-8 and VTI1B (Vps10 tail interactor-1B) (Luzio et al., 2007). SNARE proteins localizing in opposing membranes are able to drive membrane fusion by using the free energy that is released during the complex formation (Jahn and Scheller, 2006).

Subsequently, the membranes destined to fuse are brought into tight connection and the membrane fusion occurs in a Ca^{2+}- and calmodulin-dependent fashion (Chen and Scheller, 2001; Hay, 2001; Jahn and Scheller, 2006). This process leads to the formation of hybrid organelles responsible for degradation of endocytosed materials (Griffiths, 1996).

Homotypic and heterotypic membrane fusions are characterized based on whether or not two fused vesicles are of the same type. Figure 11 shows the homotypic late endosome fusion and heterotypic late endosome-lysosome fusion.

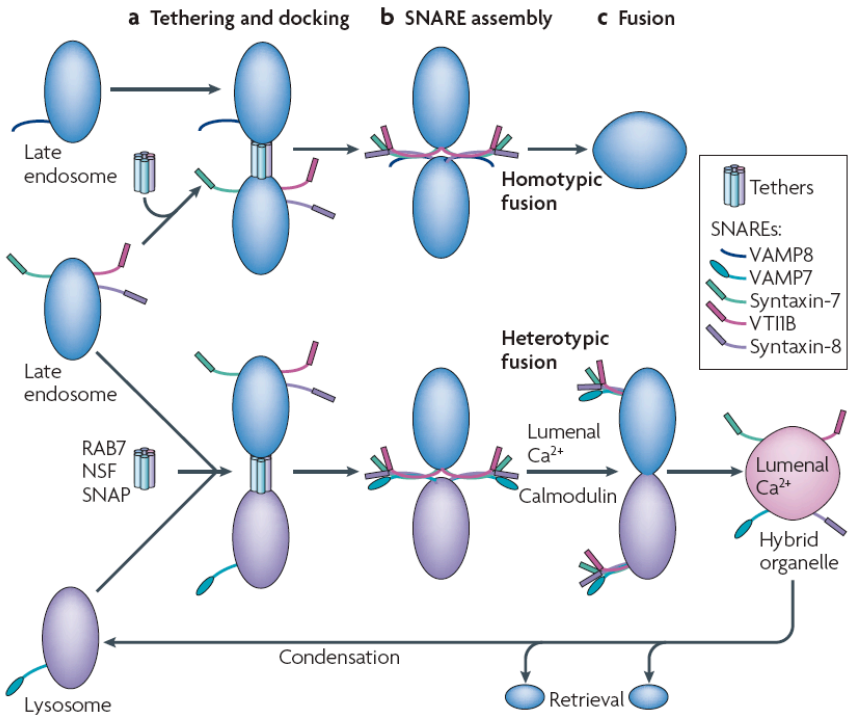


Figure 11. Schematic Models of Homotypic Late Endosome and Heterotypic Late Endosome–Lysosome Fusion. Adapted from (Luzio et al., 2007).

1.3.3 Vacuole Protein Sorting (VPS) Complex

Intracellular protein trafficking is critical for the maintenance of cell homeostasis (Ferro-Novick and Jahn, 1994; Rothman, 1994). Endolysosomal fusion is dependent on correct protein sorting and association of each specific membrane organelle. Proteins involved in this process have been genetically defined in yeast and are called, vacuole protein sorting (VPS) components.

In yeast, vacuoles (equivalent to mammalian lysosomes) play indispensable roles in the turnover of the cytoplasmic organelles as well as cellular components. Genetic screens in yeast

have revealed more than 50 Vps genes that are required for the vacuole biogenesis or vesicular transport (Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989; Rothman and Stevens, 1986). These Vps mutants are categorized into six (A-F) subgroups, based on their vacuolar morphology and functional defects (Banta et al., 1988; Wada et al., 1992).

The class C Vps proteins, including Vps11, Vps16, Vps18 and Vps33, exist in two complexes: the HOPS (homotypic fusion and vacuole protein sorting) complex, which also contains Vps39/Vam6 and Vps41/Vam2, functions at the vacuole (Kim et al., 2001a; Price et al., 2000), and the class C core vacuole/endosome tethering (CORVET) complex which contains Vps3 (Vam6 homolog) and Vps 8 (Vps41 homolog), acting at the endosome (Peplowska et al., 2007). Both of the two homologous complexes interact with Ypt7p, a Rab GTPase, coupling Rab activation and SNARE assembly during fusion (Eitzen et al., 2002; Price et al., 2000; Sato et al., 2000; Seals et al., 2000; Wurmser et al., 2000).

The HOPS complex plays an important role in vesicular trafficking and is functionally conserved in multiple organisms including yeast, *Drosphila*, zebrafish, mouse and human (Huizing et al., 2001; Kim et al., 2001a; Maldonado et al., 2006; Poupon et al., 2003; Pulipparacharuvil et al., 2005; Sadler et al., 2005; Suzuki et al., 2003). In *Drosophila*, mutations in yeast homologues of HOPS components lead to defects in lysosomal trafficking (Lindmo et al., 2006; Pulipparacharuvil et al., 2005; Sevrioukov et al., 1999). Mammalian orthologues of the HOPS proteins also function in late endosomal fusion. A Beclin-1-binding autophagic tumor suppressor, UVRAG (UV-irradiation-resistance-associated gene), was also shown to associate with class C Vps complex (Liang et al., 2008). The resulting UVRAG-Vps complex facilitates autophagosome fusion with late endosomes/lysosomes, thereby stimulating delivery and degradation of both endocytic and autophagic cargos (Liang et al., 2006; Liang et al., 2008).

1.3.4 Vps39/Vam6 Protein

In this thesis, I describe our discovery that MCV LT interacts with human Vps39/Vam6. Vam6 is a ubiquitously expressed cytoplasmic protein containing a citron homology domain (CNH) and a clathrin homology domain (CLH). Three functions of Vam6 have been proposed: Vesicular trafficking, TGF-β (transforming growth factor β) signaling and mTOR (mammalian target of rapamycin) pathway.

1.3.4.1 Vam6 Protein Domain Structure

The vacuolar protein sorting gene product Vps39/Vam6 was first described in *S. cerevisiae* as a component of the HOPS complex acting on the vacuolar membrane to promote protein sorting (Nakamura et al., 1997). Subsequently, a human homologue of Vam6 (hVam6p) was found and shown to contain a CNH and a CLH domain, both of which are required to induce clustering and fusion of late endosomes and lysosomes (Caplan et al., 2001). Vam6 is a typical cytosolic protein ubiquitously expressed among different species, including human, yeast, *Drosophila*, and *C .elegans*. CNH and CLH domains are well conserved among these homologues, except that *S. cerevisiae* Vam6 lacks the CNH domain (Fig. 12).

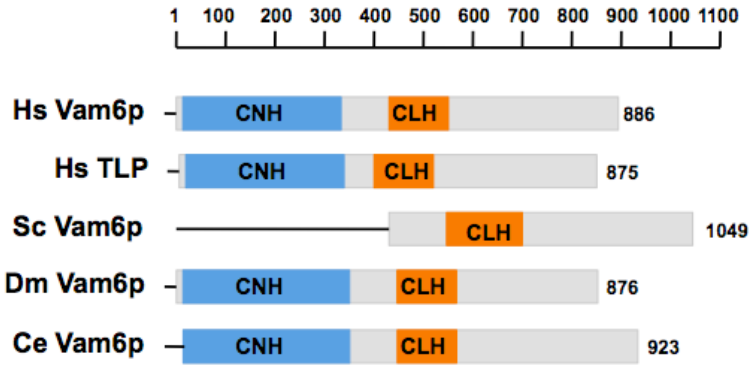


Figure 12. Vam6 Protein Domain Structure. Hs: *Homo sapiens*; Sc: *S. cerevisiae*; Dm: *D. melanogaster*; Ce: *C. elegans*. CNH: citron homology domain; CLH: clathrin homology domain. CLH domain is conserved in all the orthologues. Sc Vam6p doesn't have CNH domain. Reproduced from (Felici et al., 2003)

1.3.4.2 Vam6 and Vesicular Trafficking

Vam6 has been shown to be essential for vacuolar protein sorting in yeast. Vam6-null mutants exhibit inefficient protein processing of many vacuolar proteins and lead to accumulation of numerous vesicular structures in the cytoplasm (Nakamura et al., 1997). As a component of the HOPS complex, Vam6 has also been reported to confer GTPase exchange factor (GEF) activity to Ypt7p, the yeast Rab7 orthologue (Wurmser et al., 2000). Overexpression of hVam6p causes both homotypic and heterotypic clustering and fusion of lysosomes, strongly suggesting that hVam6p is a mammalian tethering/docking factor (Caplan et al., 2001). Although the mechanism is unclear, it is speculated that hVam6p possibly bridges the membranes of two vesicles, or that hVam6p overexpression sequesters other factors, which

prevent the unregulated fusion (Caplan et al., 2001). Since CLH domain is essential for hVam6p self-assembly, the overexpression-induced phenotypic effects on lysosomes are likely to require the homooligomerization of hVam6p (Caplan et al., 2001).

1.3.4.3 Vam6 and TGF-β Signaling

Vam6 has been suggested to act as a modulator of TGF-β signaling. TGF-β is a potent regulatory cytokine that has an active role in cellular functions including proliferation, differentiation, homeostasis and angiogenesis (Massague, 1998; Piek et al., 1999). Altered TGF-β signaling contributes to tumor growth and invasion, evasion of immune surveillance, and cancer cell metastasis (Massague, 2008).

TGF-β superfamily members signal through cell membrane receptor serine/threonine kinases to activate downstream targets. Initially, TGF-β binds to type II receptors (TβRII), which recruit and phosphorylate type I receptors (TβRI). The activated TβRI phosphorylates receptor-regulated Smads (R-Smads), Smad2 and Smad3 that act as transducers of TGF-β signaling. R-Smads dissociate from the receptor after phosphorylation and bind to Smad4. The nucleus translocation of the resulting complex then regulates the transcription of target genes (Massague and Chen, 2000; Miyazono et al., 2000).

An isoform of hVam6p, TGF-β receptor I-associated protein-1 (TRAP-1)-like protein (TLP), was described to be identical to hVam6p except for an 11 amino acid deletion (Felici et al., 2003). TLP was shown to constitutively interact with TGF-β and activin receptors (Felici et

al., 2003). In addition, TLP represses the ability of TGF-β to induce transcription from Smad3, while it potentiates transcription from Smad2 reporter (Felici et al., 2003).

1.3.4.4 Vam6 and mTOR pathway

A third function described for Vam6 is involved in the mTOR pathway. The target of rapamycin (TOR) is a serine/threonine kinase that regulates cell growth and metabolism in response to diverse signals, including amino acids, energy levels and mitogenic growth factors (De Virgilio and Loewith, 2006; Wullschleger et al., 2006). Dysregulation of mammalian TOR (mTOR) signaling is frequently found in human cancers (Sarbassov et al., 2005). TOR exists in two distinct multiprotein complexes, TORC1 and TORC2, each having different subunit compositions and physiological functions (Loewith et al., 2002). mTORC1 controls cell growth and proliferation by phosphorylating the eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4EBP1) and the ribosomal protein S6 kinase (S6K1) (Guertin and Sabatini, 2007; Hay and Sonenberg, 2004). The phosphorylation of these two downstream effectors is inhibited by rapamycin, thus measuring 4EBP1 or S6K phosphorylation is commonly used as a readout of mTORC1 activity.

Studies have shown that the Rag family GTPases are key upstream regulators of TORC1 activation and play critical roles in coupling signals in both *Drosophila* and mammalian cells (Kim et al., 2008; Sancak et al., 2008). The Rag GTPases are conserved from yeast to mammals. Four Rag genes (Rag A-D) exist in humans, and Gtr1 and Gtr2 are yeast homologues to RagA/B and RagC/D, respectively. The EGO complex in yeast, consisting of Ego1/Meh1, Ego3/Slm4, Gtr2 and Gtr1, functions directly upstream of TORC1 (Loewith et al., 2009).

Vam6 colocalizes with the EGO complex at the vacuole membrane and acts as a guanine nucleotide exchange factor (GEF) for Gtr1 in yeast, suggesting that Vam6 may integrate amino acid signals to coordinate the control of TORC1 activity (Loewith et al., 2009). In mammalian cells, knockdown of hVam6p inhibits insulin and amino acid stimulated mTORC1/S6K1 activation, indicating that the integrity of late endosomes is essential for mTORC1 signaling (Flinn et al., 2010).

1.3.5 Endocytosis Utilized by Viruses

Viruses take advantage of cellular processes to gain entry into the host cell. Although some viruses (e.g. herpes simplex virus, poliovirus) are able to penetrate the plasma membrane by pore formation and enter into the cytosol directly, most viruses depend on endocytic pathway for uptake, transport and delivery to intracellular organelles (Agirre et al., 2002; Mercer et al., 2010; Ojala et al., 2000). Endocytosis occurs by different mechanisms including phagocytosis, macropinocytosis, clathrin-mediated endocytosis (CME), caveolin/raft-dependent endocytosis, and clathrin-/caveolae-independent endocytosis (Conner and Schmid, 2003)

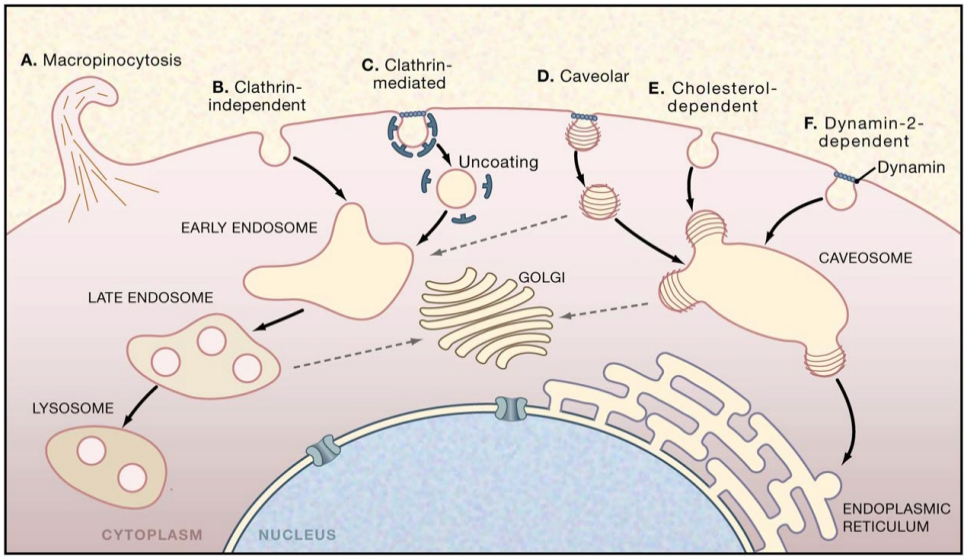


Figure 13. Endocytic Pathways Utilized by Viruses. Adapted from (Marsh and Helenius, 2006).

Phagocytosis happens in specialized mammalian cells, including macrophages, monocytes and neutrophils. The process is triggered by the attachment of large pathogen particles to the cell surface receptors, followed by a dynamin 2- and actin-dependent formation of tight-fitting endocytic vacuoles around the particles (Aderem and Underhill, 1999; Kinchen and Ravichandran, 2008; Melendez and Tay, 2008; Mercer et al., 2010; Swanson, 2008). Herpes simplex virus and acanthamoeba polyphaga mimivirus can be taken up by this mechanism (Clement et al., 2006; Ghigo et al., 2008).

Macropinocytosis refers to the formation of large endocytic vesicles (macropinosomes) generated by actin-dependent membrane ruffling of the plasma membrane (Conner and Schmid, 2003; Swanson, 2008). Unlike phagocytosis, macropinocytosis is independent of receptors or dynamin 2 (Kerr and Teasdale, 2009; Mercer and Helenius, 2009; Swanson and Watts, 1995). Viruses shown to utilize macropinocytosis as an entry route include vaccinia virus (Mercer and

Helenius, 2008), Kaposi's sarcoma-associated herpesvirus (Raghu et al., 2009), human immunodeficiency virus (HIV) (Liu et al., 2002), Ebola virus (Nanbo et al., 2010), and human adenovirus serotype 3 (Amstutz et al., 2008).

Clathrin-mediated endocytosis (CME), the most well-characterized mechanism, involves the concentration of transmembrane receptors and their bound ligands into clathrin-coated pits on the plasma membrane, followed by invagination to form endocytic vesicles (Conner and Schmid, 2003). Semliki Forest virus (SFV) was the first virus shown to use CME as part of the productively infectious pathway (Helenius et al., 1980). More recently, other viruses, such as influenza A, hepatitis C, adenovirus 2 and 5 have also been demonstrated to enter the cell through CME (Chen and Zhuang, 2008; Gastaldelli et al., 2008; Helle and Dubuisson, 2008; Medina-Kauwe, 2003; Meertens et al., 2006; Rust et al., 2004; Stewart et al., 2003).

Caveolin/raft-dependent endocytosis is recognized by the formation of endocytic vesicles composed of cholesterol and lipid rafts. Caveolin/raft-dependent endocytosis is ligand triggered and followed by activation of a phosphorylation-dependent signaling cascade involving tyrosine kinases (Pelkmans and Helenius, 2002). Polyomaviruses (SV40, mPy and BKV) are reported to make use of this pathway for internalization (Eash et al., 2006). Other viruses, such as filovirus, coronavirus, respiratory syncytial virus, are also involved in this pathway (Empig and Goldsmith, 2002; Nomura et al., 2004; Werling et al., 1999).

Some viruses exploit multiple strategies to enter host cells. For instance, HIV and many other retroviruses undergo fusion with the plasma membrane, but they are also endocytosed (Gruenberg, 2009). Another example of enveloped virus, influenza virus, utilizes both the clathrin-mediated endocytosis and a clathrin-independent pathway (Rust et al., 2004). Additionally, SV40 can be internalized by either caveolin-dependent or caveolin-independent pathway (Damm et al., 2005).

In this thesis, we sought to identify novel cellular partners for MCV LT unique region. I identified human Vam6 protein (hVam6p) as a specific interactor and found that this lysosomal protein binds to LT through a domain adjacent to pRB-binding motif. hVam6p, a cytoplasmic protein, is relocalized to the nucleus in the presence of LT harboring nuclear localization signal. Mislocalization of hVam6p by LT was found to prevent hVam6p-induced lysosome clustering and fusion. Our study showed that MCV antagonizes lysosomal machinery through a novel nuclear sequestration mechanism. The experimental data will be present in Chapter 2. To further investigate the functional significance of MCV LT-hVam6p interaction, we assessed its effect on MCV lifecycle using a full-length replicating MCV molecular clone. We found that the mutation of hVam6p-binding site leads to an increase in viral replication and virion production. Additionally, hVam6p overexpression significantly reduces nuclease-resistant MCV virion production suggesting a previously unrecognized role for hVam6p in regulating virus replication. The experimental data will be present in Chapter 3.

2.0 IDENTIFICATION OF HUMAN VAM6P AS A NOVEL CELLULAR INTERACTOR FOR MERKEL CELL POLYOMAVIRUS LARGE T ANTIGEN

Work described in this section was published in the *Journal of Biological Chemistry* (JBC 2011, 286 (19): 17079-90)

with authors Xi Liu, Jennifer Hein, Simon C. W. Richardson, Per H. Basse, Tuna Toptan, Patrick S. Moore, Ole V. Gjoerup and Yuan Chang.

J. Hein performed initial GST pull-down experiments. S. C. W. Richardson provided hVam6p antibody. P. H. Basse helped with confocal microscopy imaging. T. Toptan performed MBP pull-down experiment. X. Liu performed all other experiments described in this section. X. Liu, P. S. Moore, O. V. Gjoerup and Y. Chang conceived the project, analyzed the results and wrote the manuscript.

To investigate the mechanisms into virus lifecycle and host-virus interaction, we sought to identify novel cellular partners for MCV LT antigen.

To this end, we performed tandem affinity purification (TAP) pull-down assay using LT fragments containing a 200-amino acid unique region, which is not present in other polyomavirus T antigens. Purification of these stably expressed LT fragments allows us to isolate LT and its associating proteins. The purified protein complexes were disrupted and separated on a SDS-PAGE gel, followed by silver staining to visualize individual protein bands. We isolated specific bands that only present in MCV LT samples and sent them for mass spectrometric analysis.

One of the interactor candidates is human Vam6 protein (hVam6p), a component of lysosomal machinery. To confirm this interaction, we performed immunoprecipitation and found that hVam6p is an authentic binding partner for MCV LT *in vivo*. Since the interaction between hVam6p, a cytoplasmic protein, and MCV LT, a nuclear protein is unexpected, we performed immunofluorescence experiments to examine protein localizations. We found that in the presence of MCV LTs harboring nuclear localization signals, hVam6p translocates to the nucleus and co-localizes with LT. In MCV positive MCC cell lines, a portion of hVam6p also localizes in the nucleus. Further confocal microscopy showed that hVam6p is present in the nucleus, colocalizes with Lamin B1 to the nuclear membrane, and focally in a perinuclear pattern. These findings suggest an unusal nuclear sequestration of a lysosomal protein by MCV LT.

To determine the hVam6p binding domain on LT, we carried out mutational analysis and GST pull-down assay. We found that a single mutation (W209A) completely abolished the LT binding to hVam6p. This hVam6p binding motif is adjacent to pRB binding LXCXE domain, but LT-hVam6p and LT-pRB are two independent interactions. We also mapped the LT binding site on hVam6p and showed that the clathrin homology domain is essential for this association.

To explore the functional significance of this interaction, we examined TGF-β signaling, mTOR pathway as well as vesicular trafficking. We observed no effect on TGF-β and mTOR signaling functions. Interestingly, we found that MCV LT disrupts hVam6p-induced lysosome clustering by relocalizing hVam6p to the nucleus. A naturally occurring, tumor-derived mutant LT (MCV350) lacking a nuclear localization signal fails to inhibit hVam6p-induced lysosome clustering, suggesting that nuclear sequestration is required to antagonize this effect.

Collectively, we show that MCV LT associates with hVam6p through a motif adjacent to the pRB-binding domain and sequesters cytoplasmic hVam6p to the nucleus. This interaction is unique to MCV and has not been reported to any other viral proteins. Mislocalization of hVam6p by MCV LT abolishes hVam6p-induced lysosome clustering but does not have readily identifiable effects on TGF-β or mTOR signaling. Our study demonstrates that MCV modulates lysosomal machinery through a novel nuclear sequestration mechanism.

2.1 INTRODUCTION

Merkel cell polyomavirus (MCV) is a newly discovered human polyomavirus detected in ~80% of Merkel cell carcinomas (MCC) (Feng et al., 2008; Shuda et al., 2008). MCV encodes large T (LT), small T (ST), and 57kT transcripts similar to those of SV40 (Shuda et al., 2008). These MCV T antigen sequences retain motifs found in other polyomavirus T antigens known to cause oncogenic transformation of rodent cells, such as an LXCXE (LFCDE) motif that binds to the retinoblastoma family of proteins and a DnaJ domain that interacts with heat-shock proteins (Campbell et al., 1997a; DeCaprio et al., 1988; Srinivasan et al., 1997). Significant mechanistic insights have been gained by identifying and characterizing cellular proteins that associate with polyomavirus T antigens. In addition to pRB and Hsc70, other cellular proteins linked to neoplastic transformation that interact with various T antigens include p53, Cul7, Bub1, PP2A, PI3-kinase, and Shc (Ali et al., 2004; Campbell et al., 1997b; Campbell et al., 1994; Cotsiki et al., 2004; DeCaprio et al., 1988; Dilworth et al., 1994; Lane and Crawford, 1979; Linzer and Levine, 1979; Pallas et al., 1990; Whitman et al., 1985). Identifying novel MCV T antigen binding partners is likely to provide critical insights into the mechanisms underlying MCV-induced tumorigenesis or viral replication.

MCV tumor-derived LTs have stop codon mutations that eliminate the helicase/ATPase domain but spare N-terminal LXCXE and DnaJ domains (Shuda et al., 2008). Compared with SV40 T antigen, however, MCV T antigen contains a distinct 200-amino acid region, that we term the MCV T antigen unique region (MUR). MUR is located between the first exon and the

origin-binding domain, and is conserved among tumor-derived MCV strains. Although MUR contains a partially conserved Bub1 binding motif similar to that of SV40 LT (Cotsiki et al., 2004), Bub1 does not appear to interact with MCV LT (O.Gjoerup, unpublished observations). There are no other obvious features of MUR that might indicate its function for the virus.

We identified human Vam6p (hVam6p) by tandem-affinity pull-down of cellular proteins with a tagged MUR protein sequence. hVam6p is a cytoplasmic protein that promotes lysosome clustering and fusion in vivo through citron homology (CNH) and clathrin heavy chain repeat (CLH) domains (Caplan et al., 2001). hVam6p also exhibits homology to the Saccharomyces cerevisiae vacuolar protein sorting 39 protein (Vps39). In yeast, membrane tethering is orchestrated at endosomes by the class C core vacuole/endosome transport (CORVET) complex and at lysosomes by the homotypic fusion and vacuole protein sorting (HOPS) complex. The HOPS complex is composed of the class C Vps complex (Vps11, Vps33, Vps18, Vps16) present in CORVET as well as Vps39 and Vps41 (Price et al., 2000). Individual subunits appear to be conserved in mammalian cells. In addition to lyso- somal metabolism, isoforms of hVam6p play roles in trans- forming growth factor-β (TGF-β) and mTOR signaling. hVam6p has been reported to be identical to an isoform of the TRAP-1-like protein that regulates the balance between Smad2 and Smad3 signaling through Smad4 interactions (Felici et al., 2003). Vam6/Vps39 in yeast has been shown to be a guanine nucleotide exchange factor for Gtr1, a Rag family GTPase that promotes TORC1 activation in response to amino acid availability (Li and Guan, 2009). In mammalian cells, hVam6p has been proposed to regulate mTORC1 signaling (Flinn et al., 2010).

In this study we show that MCV LT binds hVam6p through a domain adjacent to the Rb-binding LXCXE motif and sequesters cytoplasmic hVam6p to the nucleus. This interaction is unique to MCV and does not occur with SV40 LT. Mislocalization of hVam6p by MCV LT

inhibits hVam6p-induced lysosome clustering but does not have readily identifiable effects on TGF-β or mTOR signaling. This study shows that MCV modulates lysosomal clustering through a novel nuclear sequestration mechanism.

2.2 MATERIALS AND METHODS

2.2.1 Plasmids

For generation of pNTAP-T4 and pNTAP-T5, MCV sequence was amplified from 5' RACE product of MCV genomic LT (pcDNA3.1.MCV350) using primers MCV.861.*BglII*.S: 5'-GG AGA TCT AGT TGA CGA GGC CCC TAT ATA TGG G-3', Exon2.1565.*XhoI*.AS: 5'-CG CTC GAG AGT AGG AAC AGG AGT TTC TCT G-3'; MCV.196.*BglII*.S: 5'-C GGG AGA TCT GGA TTT AGT CCT AAA TAG GAA AGA AAG-3', Exon2.1565.*XhoI*.AS: 5'-CG CTC GAG AGT AGG AAC AGG AGT TTC TCT G-3'. The *BglII*/*XhoI* digested fragments were cloned into pNTAP-B (Stratagene) using *BamHI* and *XhoI* restriction sites. pcDNA3.1.MCV350 was generated using MCC350 tissue DNA as template and primers 350.FLT.*KpnI*.F2.S: 5'-GG GGT ACC CAG CTC ATT TGC TCC TCT GCT G TT TCT-3' and 350.FLT.*XhoI*.R: 5'- CCG CTC GAG CGG TGG GTC TAT TCA GAC AGG CTC T-3'. PCR product was digested with *KpnI/XhoI* and cloned into pcDNA3.1/Zeo vector (Invitrogen). To generate pNTAP-SV40.T1-136, pCMV.SV40.LT was amplified using primers T1-136.S: 5'-CCT TTA GGA TCC GCC

ATG GAT AAA GTT TTA AAC AGA-3' and T1-136.AS: 5'-CCT TTA GGA TCC TTA CTT GGG GTC TTC TAC C-3'. PCR product was digested with *BamHI* and cloned into pNTAP-B (Strategene). Construction of gLT-V5, LT-V5, cLT339-V5 and cLT350-V5 expression plasmids were described previously (Shuda et al., 2008). LT-EGFP, cLT339-EGFP and cLT350-EGFP were constructed using inserts from LT-V5, cLT339-V5 and cLT350-V5 digested with *NheI/SacII* and cloned into pEGFP-N1 (Clontech). SV40.LT-EGFP was amplified from pCMV.SV40.LT and cloned into pEGFP-N1 (Clontech).

To generate GST fusion MCV LT truncations GST-LT(1-258), GST-LT(79-170), GST-LT(171-258), GST-LT(171-218) and GST-LT(219-258), pcDNA6.LT-V5 (2) was amplied using primers: pGEX.LT.1-78.*BglII*(S): 5'-CCT TTA AGA TCT GCC ATG GAT TTA GTC CTA AAT AGG- 3', pGEX.LT.1-258. *BglII*(AS): 5'-CCT TTA AGA TCT TTA ATC TGT AAA CTG AGA TGA CG-3'; pGEX.LT.79-258.*BglII*.S: 5'-CCT TTA AGA TCT GCC GTT GAC GAG GCC CCT ATA TAT GGG-3', pGEX.LT.79-170.*BglII*.AS: 5'-CCT TTA AGA TCT TTA TTC CTC ATG GTG TTC GGG AGG-3'; pGEX.LT.171-258.*BglII*.S: 5'-CCT TTA AGA TCT GCC CCC ACC TCA TCC TCT GGA TCC-3', pGEX.LT.1-258.*BglII*.AS: 5'-CCT TTA AGA TCT TTA ATC TGT AAA CTG AGA TGA CG-3'; pGEX.LT.171-258.*BglII*.S: 5'-CCT TTA AGA TCT GCC CCC ACC TCA TCC TCT GGA TCC-3', pGEX.LT.171-218.*BglII*.AS: 5'-CCT TTA AGA TCT TTA AAG TGA TTC ATC GCA GAA GAG-3'; pGEX.LT.219-258.*BamHI*.S: 5'-CCT TTA GGA TCC GCC TCC TCC CCT GAG CCT CCC TCG-3', pGEX.LT.1-258.*BglII*.AS: 5'-CCT TTA AGA TCT TTA ATC TGT AAA CTG AGA TGA CG-3'. The *BglII* digested 1-258, 79-170, 171-258, 171-218 and *BglII/BamHI* digested 219-258 fragments were cloned into pGEX 4T-2 using *BamHI* restriction site. For GST fusion LT deletion mutant, GST.dl171-181, GST.dl182-192, GST.dl193-203 and GST.dl204-218 were generated by QuikChange Lighting Site-Directed Mutagenesis Kit (Agilent) using GST.LT(1-258) as a

template and the following primer pairs: dl171-181.S: 5'-CCT CCC GAA CAC CAT GAG GAA GAG ACC ACC AAT TCA GGA AGA-3', dl171-181.AS: 5'-TCT TCC TGA ATT GGT GGT CTC TTC CTC ATG GTG TTC GGG AGG-3'; dl182-192.S: 5'-CCT CTG GAT CCA GTA GCA GAG AGC CCA ATG GAA CCA GTG TAC CTA G-3', dl182-192.AS: 5'-CTA GGT ACA CTG GTT CCA TTG GGC TCT CTG CTA CTG GAT CCA GAG G-3'; dl193-203.S: 5'-CAG GAA GAG AAT CCA GCA CAA GAA CGT ATG GCA CCT GGG A-3', dl193-203.AS: 5'-TCC CAG GTG CCA TAC GTT CTT GTG CTG GAT TCT CTT CCT G-3'; dl204-218.S: 5'-CCA GTG TAC CTA GAA ATT CTT CCT CCT CCC CTG AGC CTC CCT CGT C-3', dl204-218.AS: 5'-GAC GAG GGA GGC TCA GGG GAG GAG GAA GAA TTT CTA GGT ACA CTG G-3'.

MCV LT alanine substitution mutants, GST.LT(1-258).R204A, T205A, Y206A, G207A, T208A and W209A were generated using QuikChange Lighting Site-Directed Mutagenesis Kit (Agilent) using GST.LT(1-258) as a template and the following primer pairs: R204A.S: 5'-CCT AGA AAT TCT TCC GCA ACG GAT GGC ACC TGG-3', R204A.AS: 5'-CCA GGT GCC ATC CGT TGC GGA AGA ATT TCT AGG-3'; T205A.S: 5'-GAA ATT CTT CCA GAG CGG ATG GCA CCT GGG-3'; T205A.AS: 5'-CCC AGG TGC CAT CCG CTC TGG AAG AAT TTC-3'; Y206A.S: 5'-GAA ATT CTT CCA GAA CGG CTG GCA CCT GGG AGG ATC-3', Y206A.AS: 5'-GAT CCT CCC AGG TGC CAG CCG TTC TGG AAG AAT TTC-3'; G207A.S: 5'-CTT CCA GAA CGG ATG CCA CCT GGG AGG ATC TC-3', G207A.AS: 5'-GAG ATC CTC CCA GGT GGC ATC CGT TCT GGA AG-3'; T208A.S: 5'-CCA GAA CGG ATG GCG CCT GGG AGG ATC TCT TC-3', T208A.AS: 5'-GAA GAG ATC CTC CCA GGC GCC ATC CGT TCT GG-3'; W209A.S: 5'-GAA CGG ATG GCA CCG CGG AGG ATC TCT TCT GC-3', W209A.AS: 5'-GCA GAA GAG ATC CTC CGC GGT GCC ATC CGT TC-3'. LT.W209A-V5 mutant was made by site-directed mutagenesis of pcDNA6.LT-V5 (Shuda et al., 2008) using

PCR primers: W209A.S: 5'-GAA CGG ATG GCA CCG CGG AGG ATC TCT TCT GC-3', W209A.AS: 5'-GCA GAA GAG ATC CTC CGC GGT GCC ATC CGT TC-3'. The Rb binding domain mutant, gLT.LXCXK-V5 was described previously (Shuda et al., 2008).

To generate pDsRed.LT, LT-GFP was used as template using primers: pDsRed.LT.*XhoI*.S: 5'-CCG CTC GAG ATG GAT TTA GTC CTA AAT AGG-3' and pDsRed.LT.*XmaI*.AS: 5'-CCC CCC GGG GTT GAG AAA AAG TAC CAG AAT C-3'. The *XhoI/XmaI* digested fragments were cloned into pDsRed-Monomer-Hyg-N1 (Clontech) using *XhoI* and *XmaI* restriction sites. pDsRed.LT.W209A was made by site-directed mutagenesis of pDsRed.LT using PCR primers: W209A.S: 5'-GAA CGG ATG GCA CCG CGG AGG ATC TCT TCT GC-3', W209A.AS: 5'-GCA GAA GAG ATC CTC CGC GGT GCC ATC CGT TC-3'.

To generate GST fusions of full-length LT (GST-LT) and LT hVam6p binding mutant (GST-LT.W209A), pcDNA6.LT-V5 (Shuda et al., 2008) and pcDNA6.LT.W209A-V5 (described above) were digested with *EcoRV* and *XhoI*. These fragments were cloned into pALEX vector, which was first digested with *NotI*, subsequently treated with mung bean exonuclease and further digested with *XhoI*. To generate maltose binding protein tagged hVam6p (MBP-hVam6p), hVam6p was amplified using 5'-CG GGA TCC CAC GAC GCT TTC GAG CC-3' and 5'-GC TCT AGA TCA AGT GTC AGC TGG GTT TAC-3' primer pairs. The *BamHI* and *XbaI* digested PCR product was then inserted into the corresponding restriction sites of the pMAL-2c vector (New England BioLabs).

pXS-HA-hVam6p and pXS-Myc-hVam6p were kindly provided by Dr. Juan S. Bonifacino (Caplan et al., 2001). mVps39.FL-GFP, mVps39.Nter-GFP, mVps39.Cter-GFP and mVps39.CNH-GFP constructs were kindly provided by Dr. J. Paul Luzio (Poupon et al., 2003). To generate mVps39.Δ(CNH+CLH)-GFP, mVps39.FL-GFP was used as template using primers:

pEGFP.mVps39.dl.*KpnI*.S: 5'-CGG GGT ACC GTG CTG AGA GAC TTC-3' and pEGFP.mVps39.dl.*BamHI*.AS: 5'-CGC GGA TCC TCA GGT GTC GGC TGA-3'. The KpnI/BamHI digested fragment was cloned into pEGFP-C1 (Clontech).

2.2.2 Cell Culture and Transfection

U2OS (ATCC), 293 (ATCC), 293H (Invitrogen), 293FT (Invitrogen), HT1080 (ATCC) and HeLa (ATCC) cells were maintained in DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. Cells in 100 mm plates or 12-well plates (60% confluence) were transfected 24 hours after plating with 6 μg or 2 μg of plasmid DNA using Fugene-6 (Roche) following manufacturer's instructions. UISO (Shuda et al., 2008), MCC13 (Shuda et al., 2008), MCC26 (Shuda et al., 2008), MKL-1 (Shuda et al., 2008), MS-1 (Houben et al., 2010) and WaGa (Houben et al., 2010) were grown in RPMI 1640 (Sigma) supplemented with 10% FBS.

2.2.3 Tandem Affinity Puification (TAP)

293H cells transfected with pNTAP.SV40.T1-136, pNTAP.MCV.T4 and pNTAP.MCV.T5 were resuspended in lysis buffer containing protease inhibitor cocktail (Roche) and 0.1 mM PMSF. Cells were subjected to three successive rounds of freeze-thaw by incubating on dry ice for 10 min then in cold water for 10 min. Supernatant was collected after centrifugation at 16,000 × g for 10 min. 0.5 M EDTA and 14.4 M β-mercaptoethanol

(Stratagene InterPlay Mammalian TAP System) was added. Washed streptavidin resin (50% slurry) was added to the lysate, followed by rotation at 4 °C for 2 hrs to allow tagged proteins to bind. Resin was collected by centrifugation at 1,500 × g for 5 min and washed twice in 1 ml of streptavidin binding buffer (SBB). Streptavidin elution buffer (SEB) was added to the resin, followed by rotation at 4 °C for 30 min to elute protein complexes. The resin was centrifuged at 1,500 × g for 5 min and supernatant was transferred to a fresh tube. Streptavidin supernatant supplement, calmodulin binding buffer (CBB) and the washed calmodulin resin (50% slurry) was added to the supernatant, followed by rotation at 4 °C for 2 hrs to allow protein complexes to bind to the calmodulin resin. Then the resin was washed twice in CBB and collected by centrifugation at 1,500 × g for 5 min. Calmodulin elution buffer (CEB) was added to the calmodulin resin and rotated at 4 °C for 30 min to elute protein complexes. Supernatant containing purified protein complexes were collected after centrifugation.

2.2.4 Mass Spectrometric Analysis

Individual protein bands were excised from polyacrylamide gels and washed twice with 50% acetonitrile in HPLC grade water and sent to the Mass Spectrometry Core Facility at Beth Israel Deaconess Medical Center, Boston, for characterization.

2.2.5 Antibodies

Anti-MCV LT antibody CM2B4 was generated as previously described (Shuda et al., 2009b). hVam6p rabbit polyclonal antibody was kindly provided by Dr. Robert C. Piper (Richardson et al., 2004). Anti-V5 rabbit antibody (Bethyl Laboratories), anti-HA.11 clone 16B12 monoclonal antibody (Covance), rabbit polyclonal recognizing GFP (Abcam), anti-myc tag clone 4A6 antibody (Millipore), purified mouse anti-human retinoblastoma protein antibody (BD Pharmingen), anti-Human CD107a (LAMP1) Alexa Fluor 488 (eBioscience), phospho-4EBP-1 Thr 37/46 (Cell signaling), phospho-4EBP-1 Ser 65 (Cell signaling), anti-mouse secondary antibody (GE Healthcare), anti-rabbit secondary antibody (Sigma-Aldrich) were purchased commercially. hVam6p/Vps39 antibodies tested include rabbit polyclonal Cat. #16219-1-AP (Proteintech); goat polyclonals Cat. # sc-104759 and sc-104761 (Santa Cruz); mouse polyclonal Cat. #ab69669 (Abcam) and rabbit polyclonal Cat. #ab90516 (Abcam).

2.2.6 Immunoprecipitation

U2OS cells were co-transfected with pXS-HA-hVam6p and either pcDNA6/V5-HisB plasmid (Invitrogen) or LT plasmid (pcDNA6.gLT-V5, cLT-V5, cLT339-V5, cLT350-V5) using Fugene-6 (Invitrogen). Cells were harvested 48 h after transfection and suspended in lysis buffer (50mM Tris-HCl, 0.15M NaCl, 1.5% NP-40, pH 7.4) supplemented with protease inhibitors. Precleared lysates were immunoprecipitated with rabbit anti-V5 (Bethyl) for 2 h at 4 °C. Lysates were incubated with Protein A/G sepharose beads (SantaCruz) for 1 h at 4 °C, collected and

washed with lysis buffer. Beads were resuspended in 3 × SDS loading buffer and proteins were separated by SDS/PAGE. Immunoblotting was performed with anti-HA (Covance).

2.2.7 Western Blotting

Transfected cells were lysed in buffer (40mM Tris-HCl pH7.4, 120mM NaCl, 0.5% Triton, 0.3% SDS) containing protease inhibitor cocktail (Roche). Sonicated lysates were electrophoresed in 10% SDS-PAGE, transferred to nitrocellulose membrane (GE Healthcare) and reacted with anti-HA (1:1,000 dilution, Covance) for overnight at 4 °C, followed by anti-mouse IgG-HRP conjugates (1:3,000 dilution, GE Healthcare) for 1 hr at room temperature. Detection of peroxidase activity was performed by Western Lightning Plus-ECL reagent (Perkin Elmer).

2.2.8 Immunofluorescence Analyses

For high resolution microscopy, cells were fixed with 4% paraformaldehyde for 20 min, permeablized with phosphate-buffered saline (PBS) with 0.2% Triton X-100, blocked with 10% BSA and then incubated with anti-Myc (1:100 dilution, Millipore) overnight at 4°C. Secondary antibody (Alexa Fluor 568-conjugated anti-mouse or Alexa Fluor 488-conjugated anti-mouse, 1:1,000 dilution, Invitrogen) was incubated for 1 h at room temperature. All antisera were diluted in 1% BSA in PBS, and intervening washes of slides were carried out three times with PBS for 15 min. For LAMP1 clustering experiment, transfected HeLa cells were fixed and

permeabilized in PBS with 10% FCS, 2% paraformaldehyde, 0.3% saponin for 15 min at room temperature as previously described (Peralta et al., 2010). Cells were washed twice in PBS with 0.03% saponin before and after staining with anti-Human CD107a (LAMP1) Alexa Fluor 488 (eBioscience). Cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) and examined under a fluorescence microscope (AX70, Olympus).

2.2.9 Confocal Microscopy

MKL-1 cells were fixed with 4% paraformaldehyde for 20 min at 4 °C, permeablized with phosphate-buffered saline (PBS) with 0.2% Triton X-100, blocked with 10% BSA, incubated with rabbit polyclonal anti-hVam6p (1:100 dilution, from Dr. Robert C. Piper (Richardson et al., 2004)) for 4 h at room temperature followed by incubation with mouse anti-Lamin B1 (1:50 dilution, ZYMED) overnight at 4 °C. Secondary antibody (Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 568-conjugated anti-rabbit, 1:1,000 dilution, Invitrogen) was applied for 1 h at room temperature. Cells were stained with DRAQ5 (1:1,000 in PBS, Biostatus) for 10 min at room temperature, washed in PBS for 10 min and mounted with VECTASHIELD mounting medium for fluorescence (Vector Laboratories, Inc.). Confocal images were acquired using a Leica TCSSL confocal microscope.

2.2.10 GST Fusion Protein Purification and GST Pull-down Assay

Cultures of JM109 bacterial host cells expressing GST fusion plasmids were grown overnight, followed by subculture (1:10) into 250 ml LB with ampicillin. After 1 h, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 0.5 mM final concentration. Induced cultures were grown for another 3-4 h and harvested by centrifugation at 3,000 rpm for 10 min. Cell pellets were resuspended in cold NETN (0.5% NP-40, 1 mM EDTA, 50 mM Tris-pH 8.0, 120 mM NaCl) containing protease inhibitors, followed by sonication. Supernatants were collected by centrifugation and 300 µl glutathione-Sepharose 4B beads (GSH beads, 50% slurry in NETN) were added followed by incubation at 4 °C for 30 min. GSH beads were washed 5 times with NETN buffer. For the binding assay, 293FT cells were transiently transfected with HA-hVam6p and harvested 48 hrs after transfection in NETN lysis buffer in the presence of protease inhibitors. Lysates were precleared using 20 µl GSH beads loaded with GST (pGEX4T-2) for 1 h at 4 °C. Complexes were collected by addition of 30 µl GSH beads loaded with appropriate fusion protein, rotation for 2 h at 4 °C followed by centrifugation. The isolated beads were washed three times for 30 min at 4 °C with NETN and eluted with 50 µl freshly made elution buffer (25 mM glutathione, 50 mM Tris-HCl pH 8.8, 200 mM NaCl, pH 8.8). Elution samples were resuspended in 3X SDS loading buffer and proteins were separated by SDS/PAGE using 10% polyacrylamide gels. Immunoblotting was performed with anti-HA (Covance).

2.2.11 MBP Fusion Purification and MBP Pull-Down Assay

GST-LT, GST-LT.W209A and MBP-hVam6p were expressed in *E.coli* Rosetta (DE3) pLysS and BLR(DE3) (Novagen), respectively, and induced with 0.5 mM IPTG for 2 h at 37°C. Cells were harvested by centrifugation at 6,000 x *g* for 15 min at 4°C. GST-fusion proteins were extracted in lysis buffer (0.5% NP40, 250 mM NaCl, 50 mM Hepes/KOH pH7.6, 1 mM EDTA, pH 7.4) supplemented with 0.2 mg/ml lysozyme and protease inhibitor cocktail (Roche). MBP-hVam6p was extracted in ice-cold column buffer (200 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.02% NaN_3) supplemented with 0.2 mg/ml lysozyme, 1 mM DTT and 1 mM PMSF. The suspensions were incubated for 20 min on ice. Following repeated freeze-thaw cycles, cells were sonicated on ice and centrifuged at 10,000 x *g* for 30 min at 4°C. For purification of fusion proteins, cleared supernatants were incubated with 1/100th volume of equilibrated glutathione-Sepharose 4B beads (GE Healthcare) or amylose resin (New England Biolabs) for 30 min at room temperature or 2 h at 4°C, respectively. Beads were collected by centrifugation at 1,000 x *g* for 1 min and washed four times with lysis buffer. Fusion proteins were eluted by addition of 10 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0 or 30 mM maltose, 50 mM Tris-HCl, pH 8.0, followed by silver staining analysis.

For the pull down experiment, MBP-hVam6p bound amylose resin beads were incubated with eluted GST, GST-LT or GST-LT.W209A proteins for 2 h at 4°C in binding buffer (0.5% NP-40, 120 mM NaCl, 50 mM Tris-pH 8.0 and 1 mM EDTA) supplemented with protease inhibitor cocktail (Roche). Beads were washed three times with binding buffer for 30 min at 4°C, resuspended in SDS loading buffer, boiled for 10 min and proteins were separated on a 8% SDS/PAGE gel. Immunoblotting was performed with anti-MCV LT antibody CM2B4 (Shuda et al., 2009b) and anti-MBP (New England Biolabs).

2.2.12 TGF-β Inducible Luciferase Reporter Assay

HT1080 cells were transfected with the TGF-β inducible reporter p3TP-Lux (Addgene) and empty vector, wild-type LT, hVam6p binding mutant LT, alone or together with hVam6p. The p3TP-Lux reporter contains three 12-O-tetradecanoylphorbol 13-acetate (TPA) response elements (TRE) from the human *collagenase* promoter and the TGF-β responsive element from the *plasminogen activator inhibitor-1* promoter ligated upstream to the adenovirus E4 minimal promoter (Wrana et al., 1992). 24 h after transfection, cells were replaced with fresh DMEM with 0.2% FBS, left non-treated or treated with recombinant human TGF-β1 (GIBCO) at 5 ng/ml for 18 h. Transfection efficiency was normalized with pRL-null vector (Promega). Renilla and Firefly luminescence activities were measured using the Dual-Luciferase Reporter Assay System (Promega) on a microplate luminescence counter (TopCount-NXT^{TM}, Packard).

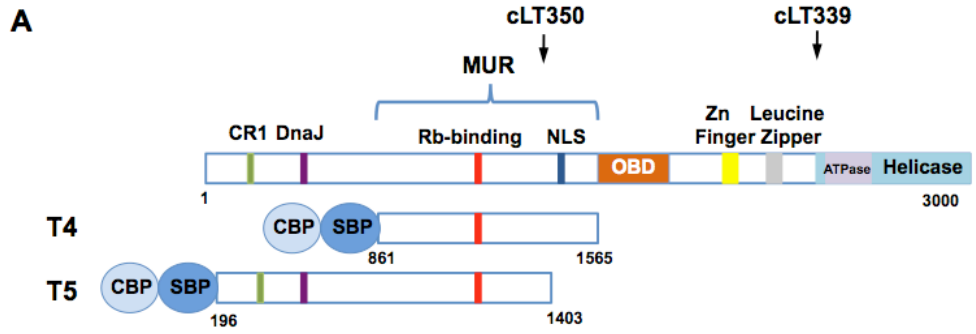
2.3 RESULTS

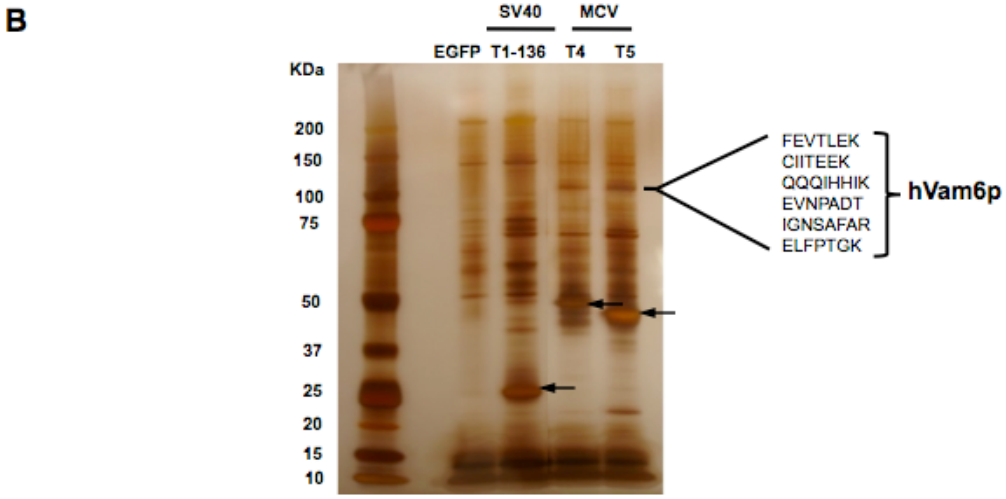
2.3.1 MCV Large T Antigen Associates with hVam6p

To identify novel cellular proteins associated with MCV LT, we performed tandem affinity purification (TAP) using two LT MUR fragments fused with N-terminal calmodulin binding protein (CBP) and streptavidin binding protein (SBP) tags (Fig. 14A). After stably expressing the dual-tag constructs in 293H cells, LT MUR interacting proteins were separated on SDS/PAGE gel. EGFP and SV40 T1-136 expressing controls were used to identify unique proteins only present in MCV LT samples. Following silver staining, a specific band at 120 kDa (Fig. 14B) was isolated and identified by mass spectrometry as hVam6p (hVps39). Rb was also recovered from this fraction, consistent with our previous report (Shuda et al., 2008).

To confirm LT-hVam6p interaction, we performed a co-immunoprecipitation assay using U2OS cells over-expressing HA-hVam6p together with V5-tagged genomic LT (also expressing small T and 57 kT proteins), cDNA LT (cLT, expressing LT alone) or two tumor-derived LTs (cLT339 and cLT350) proteins (Fig. 14C). All forms of MCV LT co-immunoprecipitated hVam6p, whereas no specific immunoprecipitation was found with the empty vector. To investigate endogenous interaction between hVam6p and MCV LT, LT protein was immunoprecipitated using anti-MCV LT antibody CM2B4 (Shuda et al., 2009b). MCC cell lines harboring MCV (MKL-1, MS-1 and WaGa) were compared to MCV negative cell lines (UISO, MCC13 and MCC26). Immunoprecipitated proteins were immunoblotted with anti-hVam6p

antibody (Fig. 14D). Robust immunoprecipitation was detected in MKL-1 and WaGa, and to a lesser extent in MS-1 cells but not in MCV-negative cell lines. These results indicate that hVam6p is an authentic binding partner for LT *in vivo*. Interaction between hVam6p, a cytoplasmic protein, and MCV LT, a nuclear protein is unexpected.





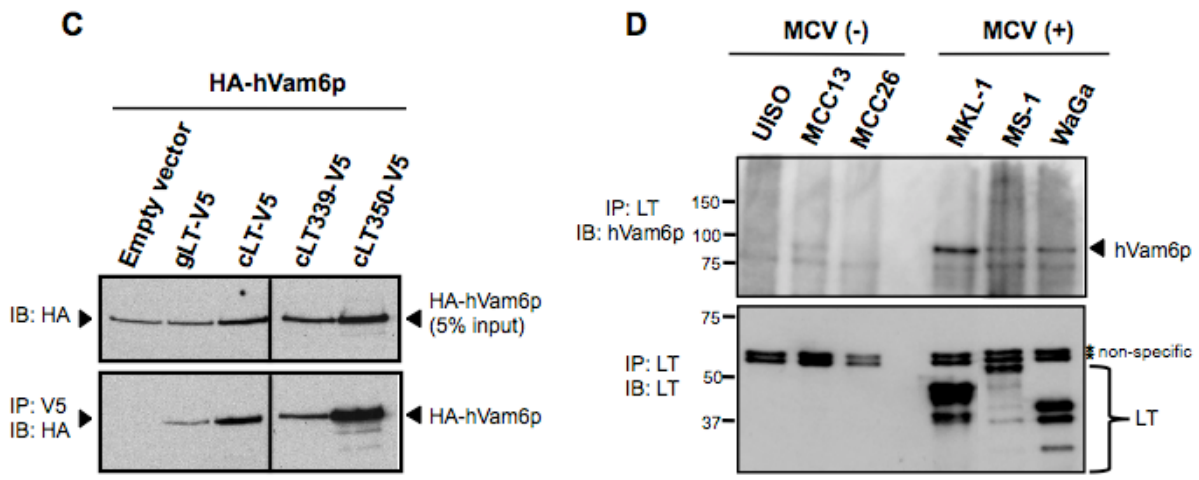
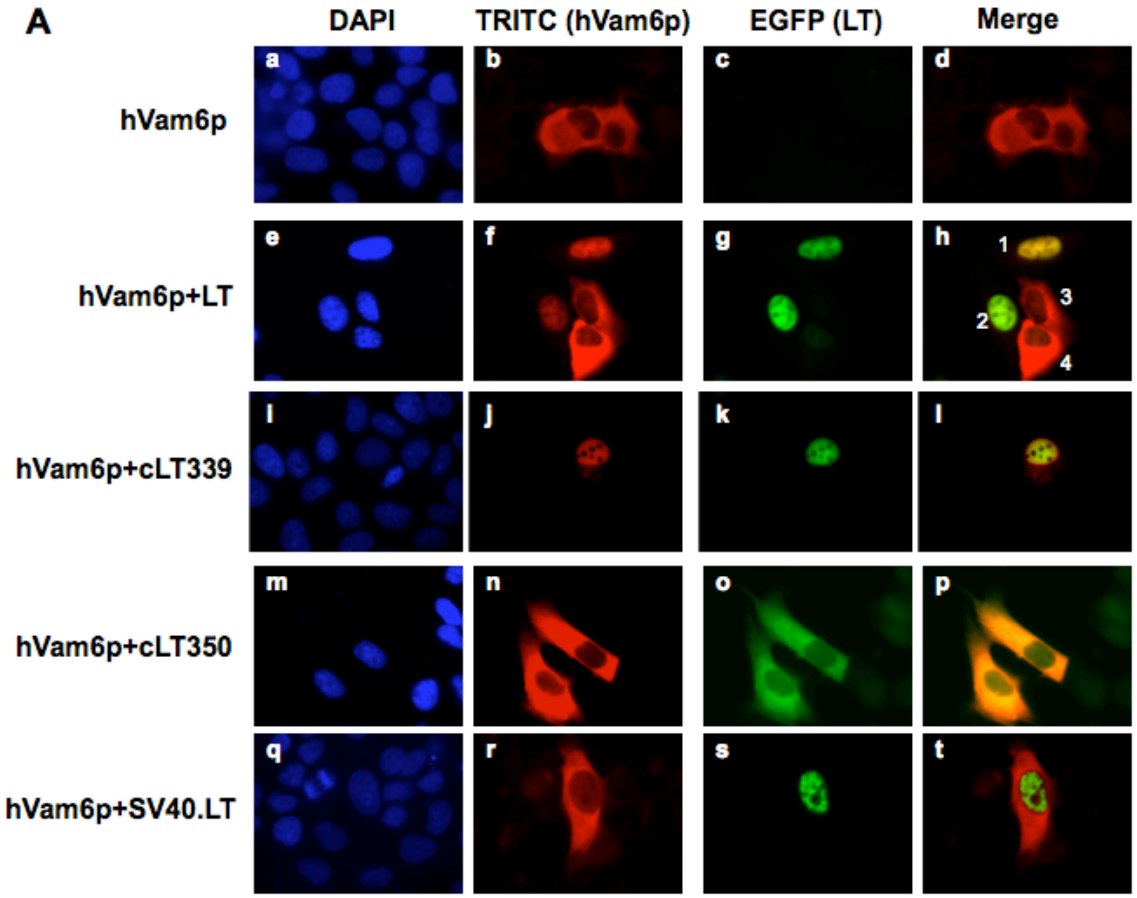


Figure 14. Identification of hVam6p as an MCV LT Interactor. ***A*****. Schematic diagram of MCV LT showing the site of the MCV unique region (MUR) and location of T4 and T5 LTs fused to N-terminal dual tags and used for tandem affinity purification (TAP) experiments. Positions of tumor-derived T antigen truncations cLT350 and cLT339 are shown (arrows). NLS: nuclear localization signal (Nakamura et al., 2010); CBP: calmodulin-binding-protein; SBP: streptavidin-binding-protein.** ***B*****. Silver stain of SDS/PAGE gel separating protein lysates after TAP isolation. Arrows indicate various TAP-tagged LT proteins stably-expressed in 293H cell lines. A 120 kDa band, present only in MCV LT TAP from T4 and T5, was excised for mass spectrometric analysis. hVam6p peptide sequences identified from these bands are shown.** ***C*****. Lysates from U2OS cells transiently over-expressing HA-hVam6p and V5-tagged LTs were immunoprecipitated for LT with anti-V5 antibody and immunoblotted for hVam6p using anti-HA antibody.** ***D*****. Endogenous LT-hVam6p interaction in MCV-negative and MCV-positive cell lines. MCV LT is detected only in MCV-positive cell lines (MKL-1, MS-1 and WaGa) and LT immunoprecipitation pulls down endogenous hVam6p protein.**

2.3.2 Co-localization of MCV LT and hVam6p

To determine that the interaction occurs in cells, we performed immunofluorescence localization of MCV LT and hVam6p. LT-EGFP fusion proteins and Myc-hVam6p were

transiently over-expressed in U2OS cells followed by anti-Myc immunostaining. Immunofluorescence reveals that in the absence of MCV LT, hVam6p is distributed in the cytoplasm (Fig. 15A, *a-d*) as previously noted (Caplan et al., 2001; Peralta et al., 2010). However, in the presence of MCV LT or cLT339, which both harbor nuclear localization signals (NLS), hVam6p translocates to the nucleus and co-localizes with LT (Fig. 15A, *e-h*; *i-l*). The naturally occurring tumor MCV cLT350 lacking an NLS, however, co-localizes with hVam6p in the cytoplasm (Fig. 15A, *m-p*). SV40 LT does not co-localize or mislocalize hVam6p to the nucleus (Fig.15A, *q-t*), consistent with hVam6p being an interactor specific for the MCV LT. We also examined endogenous hVam6p localization in MCV positive MCC cell lines (MKL-1 and MS-1). Immunofluorescence shows that a portion of hVam6p is present in the nucleus (Fig. 15, *c, g, solid arrow*). Additionally, in naturally infected MCV T antigen expressing Merkel cell carcinoma derived cell lines, hVam6p also displays a perinuclear dot-like localization in subpopulation of tumor cells (Fig. 15B, *c*, *dashed arrow*). In comparison, hVam6p is not relocalized to the nucleus in an MCV negative cell line, UISO (Fig. 15B, *i-l*). To confirm this, we performed confocal microscopy which showed similar results as conventional fluorescence microscopy. hVam6p is present in the nucleus, colocalizes with Lamin B1 to the nuclear membrane, and focally in a perinuclear pattern. Analysis of single cell fluorescence profiles along two dimensional axis shows intranuclear, nuclear membrane, as well as a perinuclear concentration of signal for hVam6p (Fig. 15C).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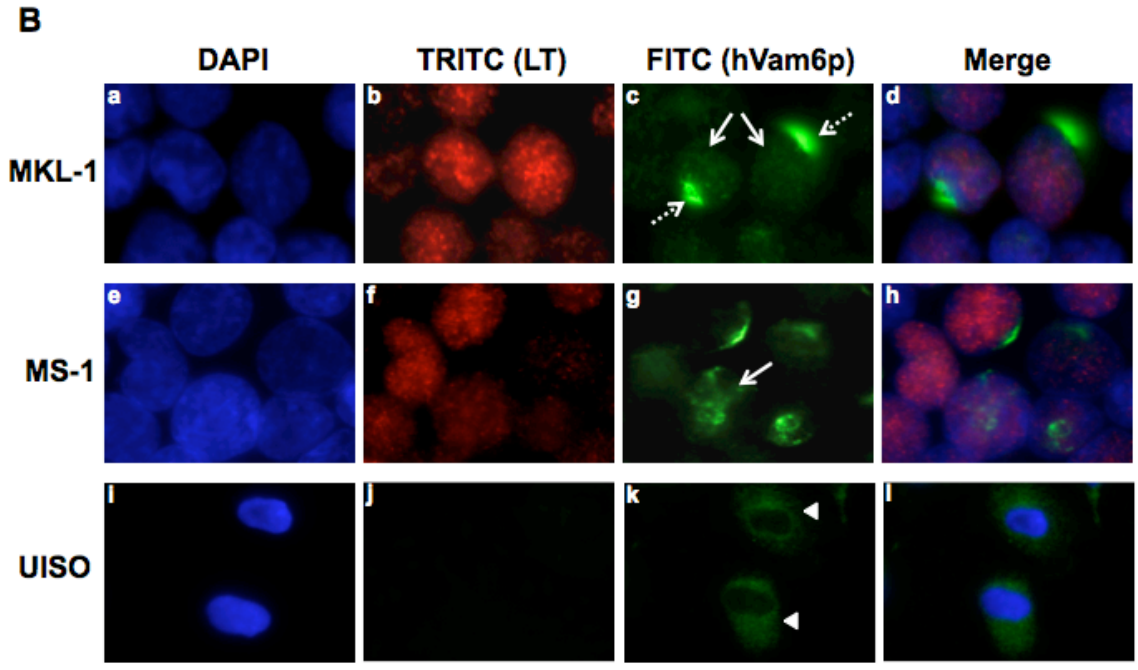
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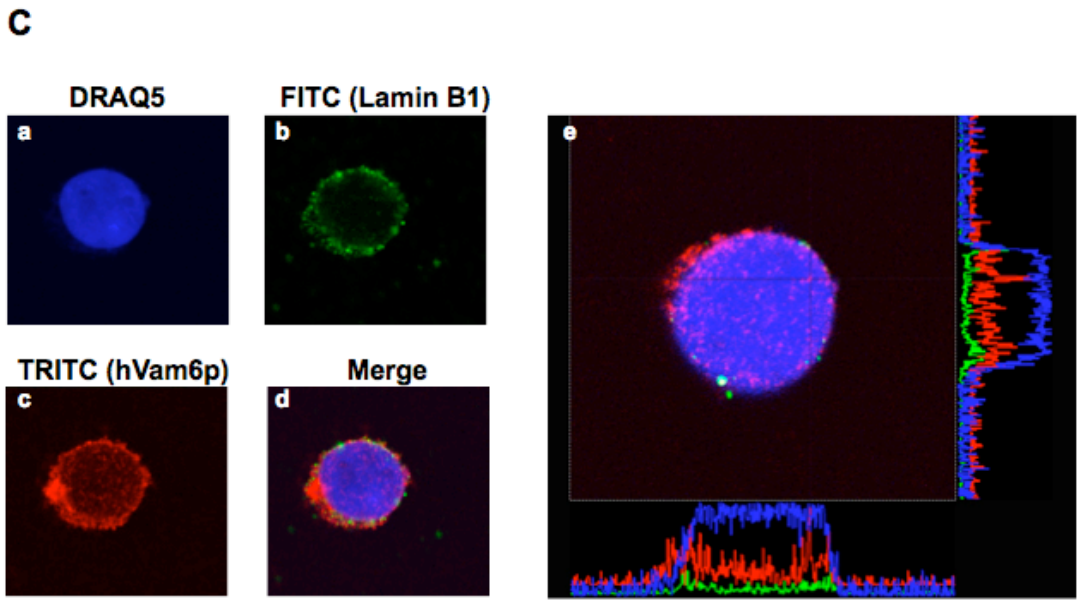
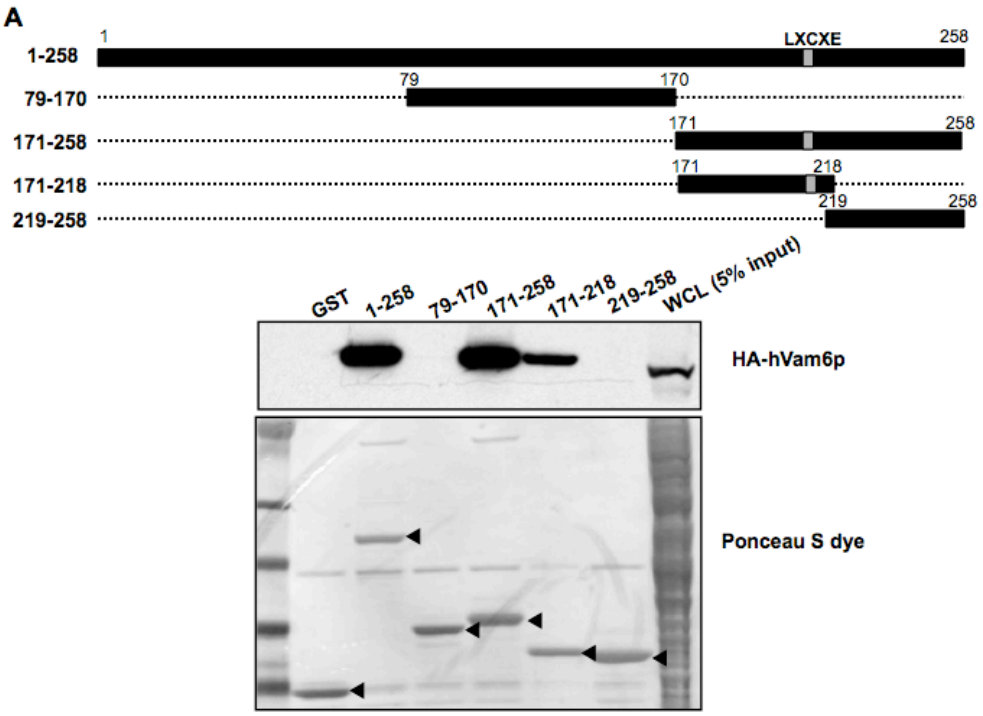


Figure 15. Localization of hVam6p. ***A.*** **Nuclear relocalization of hVam6p in U2OS cells during MCV LT expression. U2OS cells were transfected with Myc-hVam6p alone (*a-d*) or together with EGFP-LT (*e-h*), EGFP-cLT339 (*i-l*), EGFP-cLT350 (*m-p*) and EGFP-SV40.LT (*q-t*). The expression of hVam6p is visualized with anti-Myc antibody and Alexa Fluor 568-conjugated anti-mouse (TRITC), while the LTs are visualized by tagged EGFP fluorescence. hVam6p is present in the cytoplasm (*d*), but relocalized to the nucleus with wild-type LT expression *(h*). In a representative view of four cells (1-4), 1 and 2 contain both over-expressed wild type, full length LT and hVam6p; whereas, cells 3 and 4 show only over-expressed hVam6p. hVam6p relocalizes to the nucleus only when LT is also present. Tumor-derived, truncated MCV LT protein (cLT339) also relocalize hVam6p (*l*), however, a tumor-derived MCV LT lacking a nuclear localization signal (cLT350) fails to relocalize hVam6p to the nucleus (*p*). No hVam6p relocalization is seen in conjunction with SV40 LT protein expression (*t*).** ***B.*** **Immunofluorescence detection of endogenous hVam6p in naturally infected, MCV positive MKL-1 (*a-d*) and MS-1 (*e-h*) cell lines. Two patterns are seen. A portion of endogenous hVam6p shows an intranuclear localization similar to MCV LT (solid arrow), another portion demonstrates prominent perinuclear localization (dashed arrow) distinct from LT. In MCV negative UISO cell lines (*i-l*), no LT is expressed and hVam6p remains cytoplasmic (arrowhead).** ***C.*** **Confocal microscopy of LT expressing MKL-1 cells shows hVamp6p *(c)* distributed largely in the same pattern as Lamin B1 *(b),* a marker for the nuclear membrane. In addition, hVam6p is also present in a diffuse pattern within the nuclear compartment *(c).* In panel *e* showing another typical cell, analyzed with cross-sectional graphing fluorescence intensity from DNA (blue), Lamin B1(green), and hVamp6 (red) show increased hVam6p signal within the nuclear compartment defined by DNA and demarcated by Lamin B1.**

2.3.3 Fine Mapping of the MCV LT Domain Interacting with hVam6p

To better characterize the association of MCV LT and hVam6p, we mapped the domain of MCV LT necessary for its association with hVam6p. Using a series of LT MUR deletion mutants (1-258, 79-170, 171-258, 171-218 and 219-258) fused to GST (Fig. 16A), we performed GST pull-downs on extracts from 293FT cells over-expressing hVam6p and found 171-218 to be the minimal fragment that binds hVam6p (Fig. 16A). Further mapping was performed using LT deletions created from this minimal region (dl171-181, dl182-192, dl193-203 and dl204-218); only the dl204-218 mutant was found to be defective in binding hVam6p (Fig. 16B). Six adjacent amino acid alanine substitution mutations (R204A, T205A, Y206A, G207A, T208A and W209A) were then generated in the dl204-218 region (Fig. 16C, *top panel*). W209 is positioned 2 residues N-terminal to the LXCXE domain and was found to be essential for hVam6p binding (Fig. 16C, *bottom panel*).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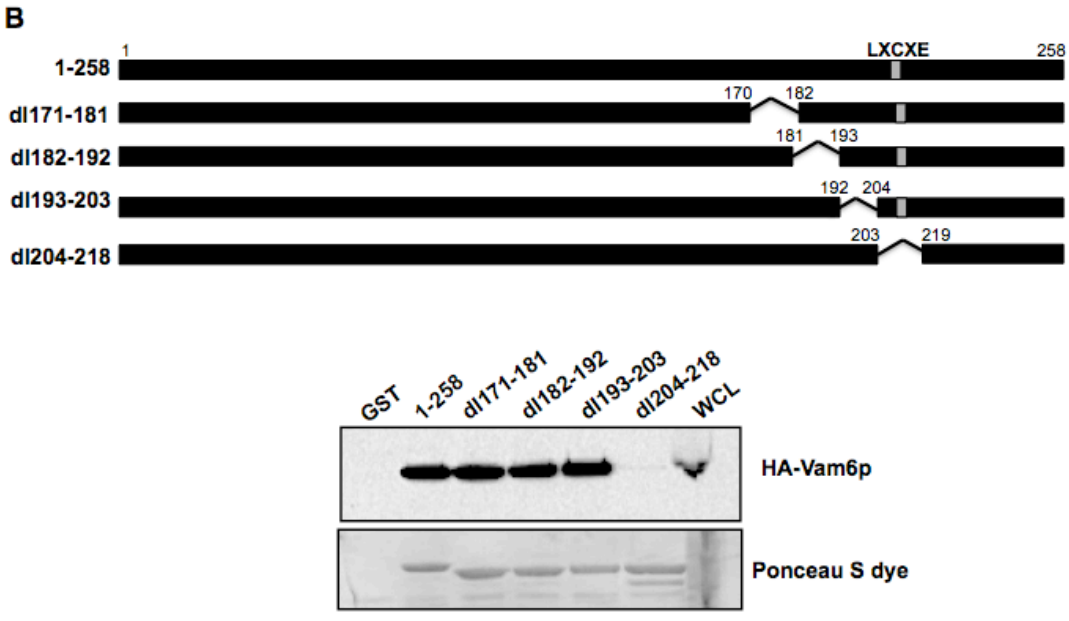
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HA-Vam6p
Ponceau S dye

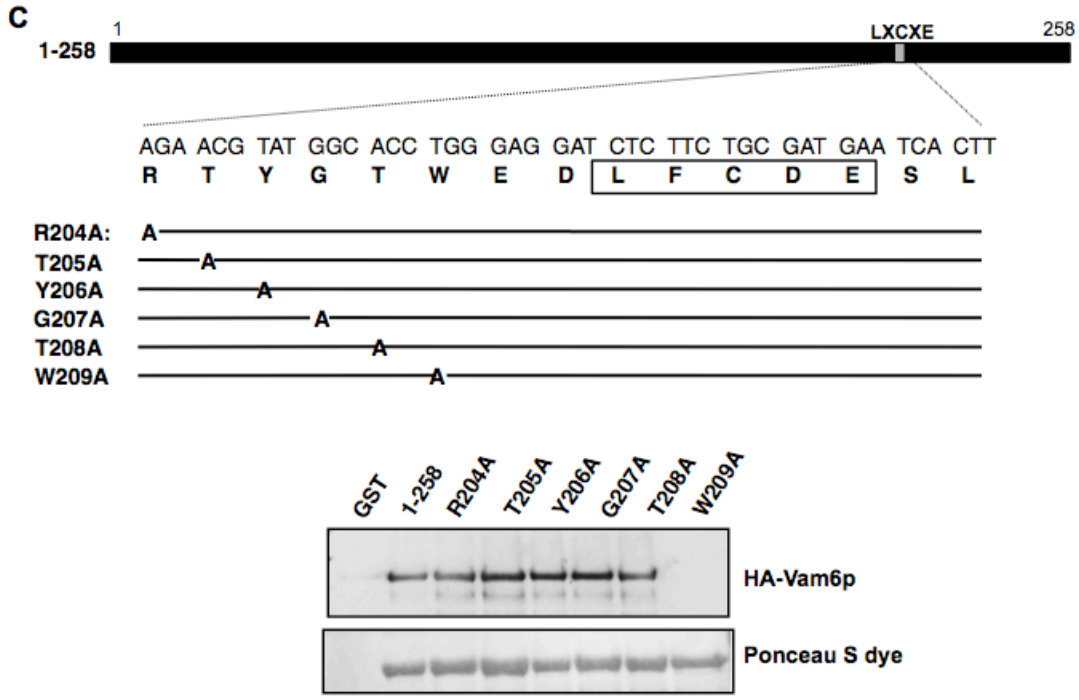


Figure 16. Mapping the hVam6p Binding Site on MCV LT Antigen. ***A.*** **Diagram of GST-tagged LT deletion constructs. GST-LT fusion proteins were incubated with protein extracts of 293FT cells transfected with HA-hVam6p. Bound proteins were revealed by immunoblotting with anti-HA antibody (*top panel*). The GST fusion LT proteins were visualized by Ponceau S staining (*bottom panel*).** ***B.*** **Further deletion analysis of the 171-218 subregion in the context of 1-258 LT. Ponceau S shows comparable expression of LT constructs and 1-258 has appropriately retarded migration *(bottom panel)*. Co-immnoprecipitation shows that only dl204-218 loses the ability to interact with hVam6p *(top panel)*.** ***C.*** **Schematic diagram of MCV LT alanine substitution mutants based on localization of hVam6p binding to residues 204-218 (*A & B*). LFCDE: Rb-binding domain.**

The binding domain was confirmed by engineering the W209A substitution into full-length V5-tagged LT and performing immunoprecipitation in U2OS cells with HA-tagged hVam6p (Fig. 17A). The alanine substitution at LT W209 completely abolishes LT interaction

with hVam6p. When this alanine substitution is cloned into an EGFP-tagged LT (LT.W209A-EGFP), co-localization and nuclear translocation of hVam6p is lost (Fig. 17B).

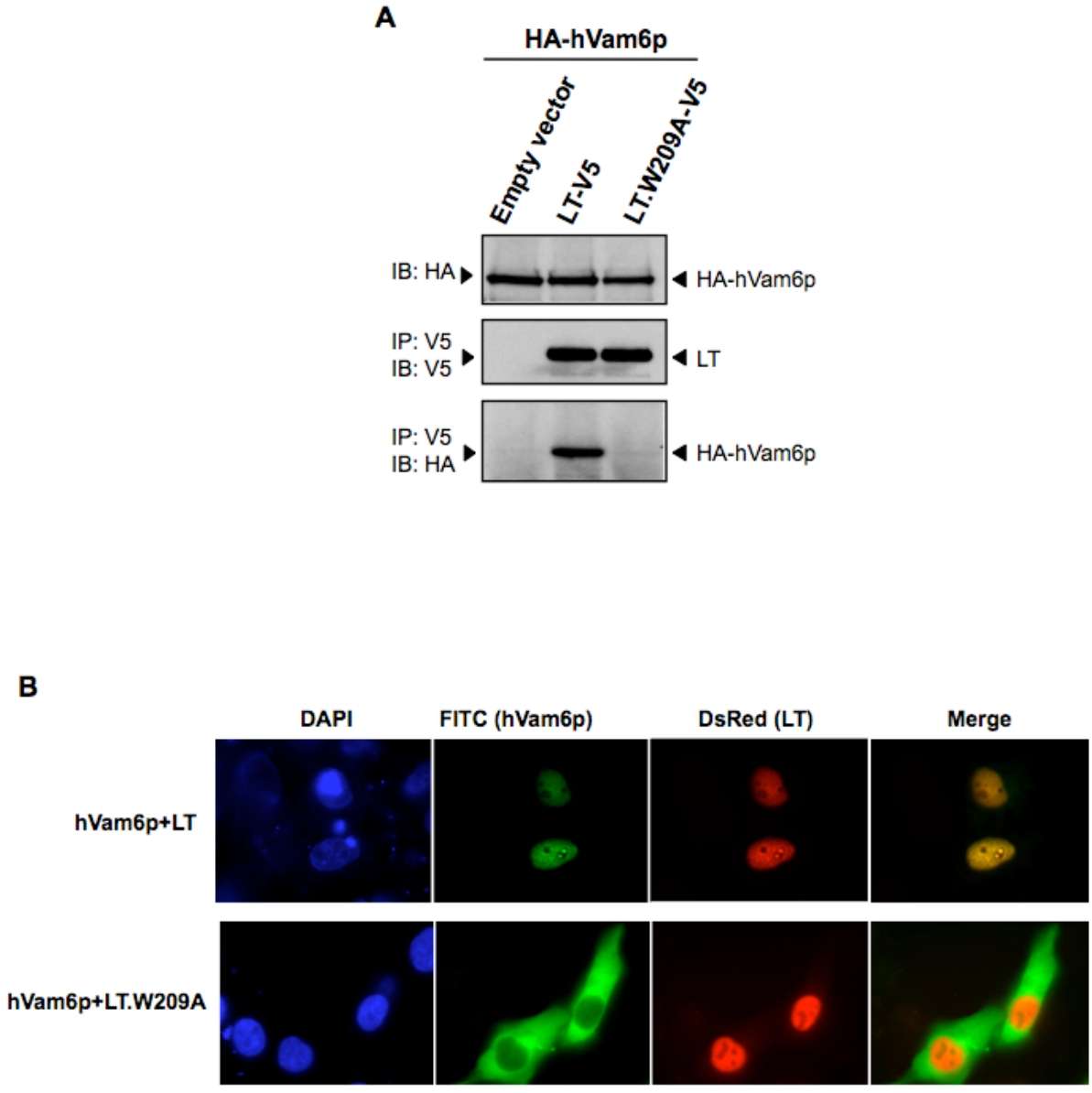


Figure 17. MCV LT.W209A Mutant Is Defective in Interacting with and Relocalizing hVam6p. ***A.*** **Mutation at W209 on full length LT completely abolished LT-hVam6p binding. Lysates from U2OS cells transiently co-expressing HA-hVam6p with empty vector, LT-V5 or LT.W209A-V5 were immunoprecipitated for LT with anti-V5 antibody and immunoblotted for hVam6p by anti-HA antibody.** ***B.*** **LT.W209A fails to relocalize hVam6p to the nucleus. U2OS cells were co-transfected with Myc-hVam6p and LT-DsRed or LT.W209A-DsRed. hVam6p is visualized with anti-Myc antibody and Alexa Fluor 488-conjugated anti-mouse antibody (FITC), while the wild type and mutant LTs are visualized by DsRed fluorescence.**

2.3.4 Direct Binding of MCV LT to hVam6p

To test if LT-hVam6p association is direct, we produced maltose binding protein tagged hVam6p (MBP-hVam6p), GST-LT and GST-LT.W209A in bacteria, followed by a MBP pull-down assay. As shown in Figure 18A, MBP-hVam6p specifically pulled down GST-LT, but not GST alone or the GST-LT.W209A mutant, indicating a direct binding of MCV LT to hVam6p. As shown in the input lanes, similar amounts of wild-type MCV LT and the mutant were used. Reprobing of the blot with MBP antibody revealed equivalent loading of MBP-hVam6p for all of the pull-downs (Fig. 18B).

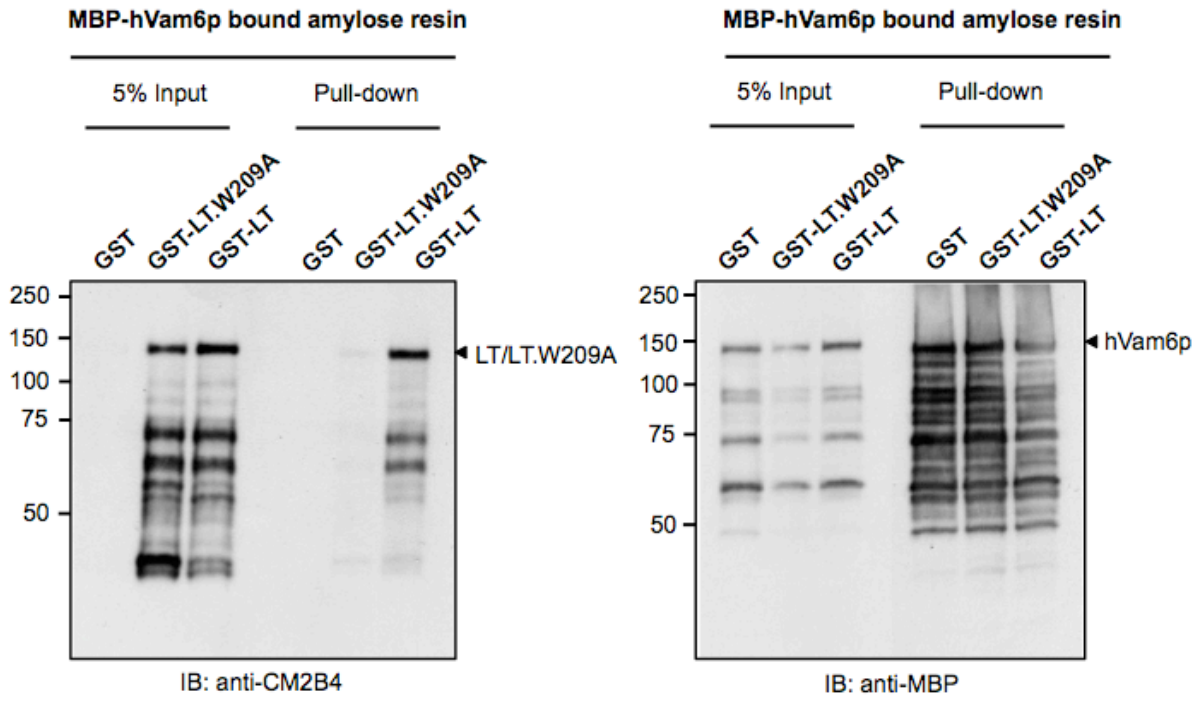
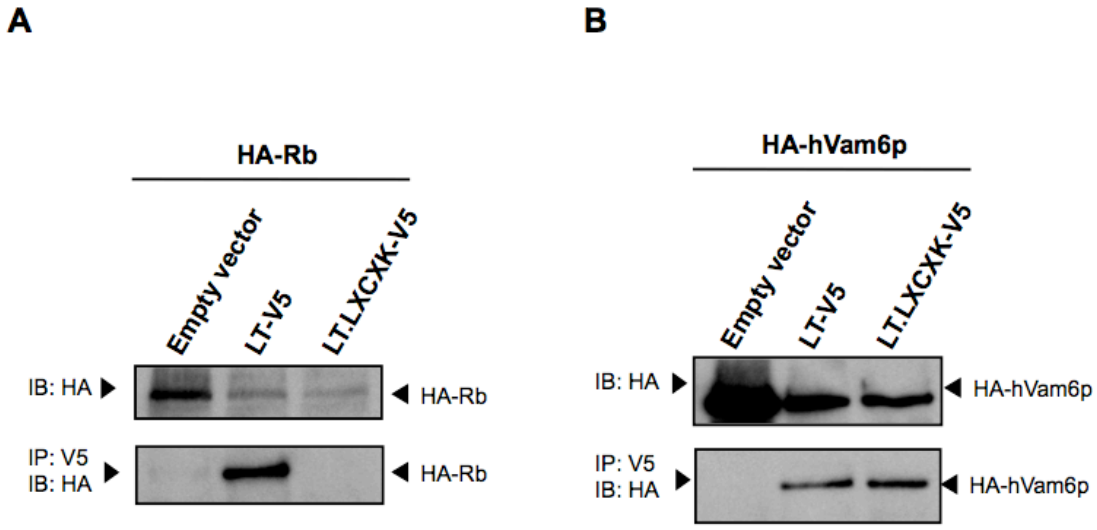


Figure 18. Direct Binding of MCV LT to hVam6p. MBP-hVam6p bound amylose resin beads were incubated with eluted GST, GST-LT.W209A or GST-LT proteins for a pull-down assay. 5% input and bound proteins were separated on an 8% SDS/PAGE gel. Immunoblotting was performed with anti-MCV LT CM2B4 antibody (*A*) and anti-MBP antibody (*B*).

2.3.5 Rb and hVam6p Interaction Domains Are Discrete Sites on LT

Since W209 is adjacent to the L*X*C*X*E Rb-binding motif, we tested whether a previously described mutation in the Rb binding motif (Shuda et al., 2008) also affects hVam6p interaction

with MCV LT. As seen in Figure 19, substitution of lysine for glutamate at position 216 (LT.L*X*C*X*K), disrupts Rb interactions with LT (Fig. 19A) but has no effect on hVam6p interaction with LT (Fig. 19B). Additionally, we tested the LT-hVam6p binding defective mutant LT.W209A and found it retains interaction with Rb (Fig. 19C). These findings suggest that Rb and hVam6p interaction domains are discrete sites. Since these two interaction sites are in close proximity, we next sought to determine whether hVam6p associates with Rb in the absence or presence of LT. As seen in Figure 19D, hVam6p neither interacts with Rb directly, nor is bridged by LT to Rb. Similar results are found for the Rb family members, p107 and p130 (data not shown).



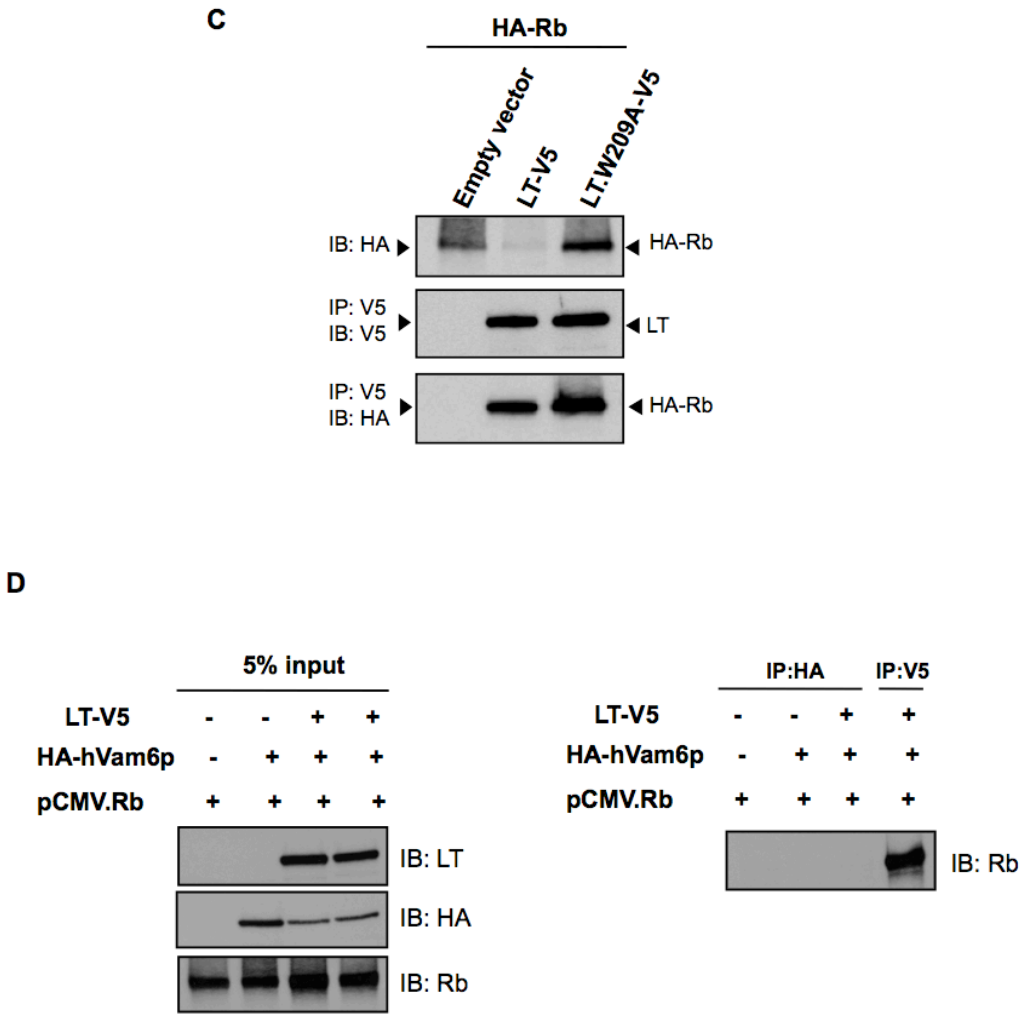
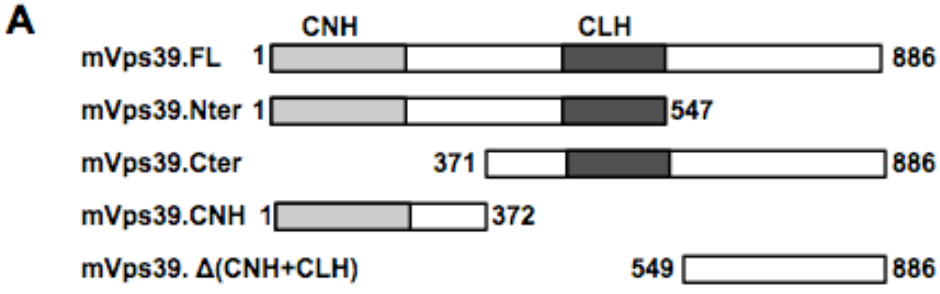


Figure 19. Rb and hVam6p Interaction Domains Are Discrete Sites on LT. ***A.*** **LT.L*X*C*X*K mutant fails to bind Rb. U2OS cell lysates co-expressing HA-Rb with empty vector, LT or Rb-binding mutant LT- L*X*C*X*K were immunoprecipitated for LT by anti-V5 antibody and immunoblotted for HA-Rb.** ***B.*** **LT. L*X*C*X*K mutant retains hVam6p interaction. U2OS cell lysates co-expressing HA-hVam6p with empty vector, LT or Rb-binding mutant LT- L*X*C*X*K were immunoprecipitated for LT by anti-V5 antibody and immunoblotted for HA-hVam6p.** ***C.*** **LT.W209A mutant has no effect on Rb-binding. U2OS cell lysates co-transfected with empty vector, LT or LT.W209A were immunoprecipitated for LT by anti-V5 antibody and immunoblotted for HA-Rb.** ***D.*** **hVam6p does not associate with Rb in the presence or absence of LT.** ***Left panel*****: 5% lysate input for LT, Rb and hVam6p.** ***Right panel*****: immunoprecipitation and immunoblotting for Rb.**

2.3.6 Vam6p CLH domain is responsible for binding to MCV LT

hVam6p has a NH2-terminal citron homology (CNH) domain and a central clathrin homology (CLH) repeat domain, both of which are required for lysosome clustering and fusion (Caplan et al., 2001). We used a series of EGFP-tagged murine Vps39 (mVps39/mVam6p is near identical to hVps39/hVam6p) deletions (Poupon et al., 2003) to map the LT binding site on mVps39 (Fig. 20A). U2OS cells were transfected with LT and mVps39 deletion mutants, followed by LT immunoprecipitation and immunoblotting for EGFP-mVps39. As seen in Figure 20B, full-length mVps39 and mVps39 having the centrally located CLH domain and either N-terminal or C-terminal deletions all strongly interact with LT. In contrast, constructs lacking the CLH domain (CNH and Δ(CNH+CLH)) fail to interact with LT, suggesting that the Vam6p CLH domain is critical in binding LT.



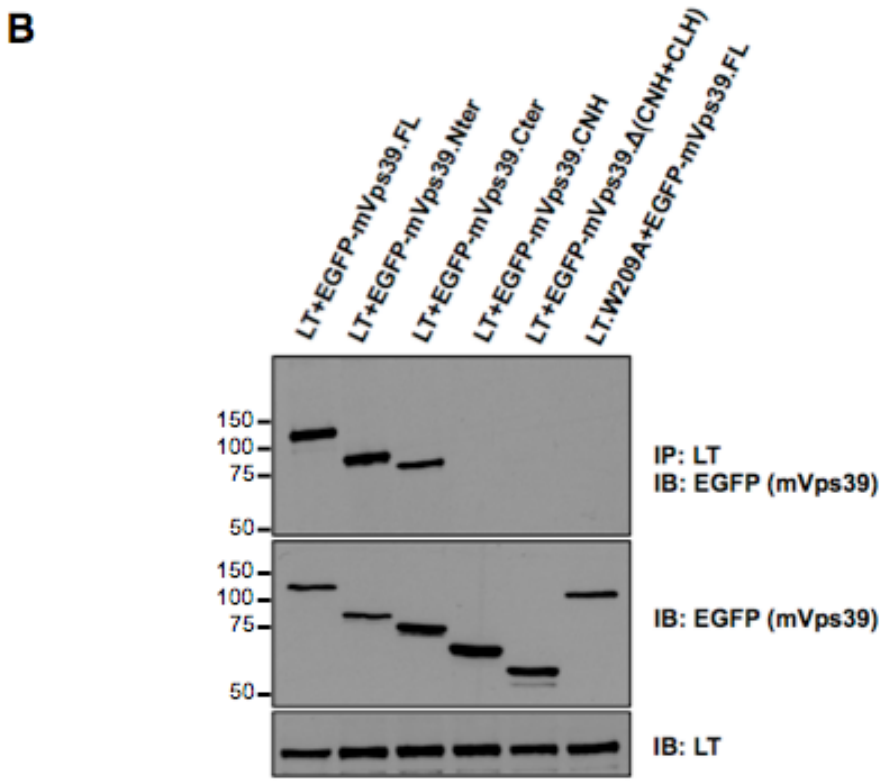
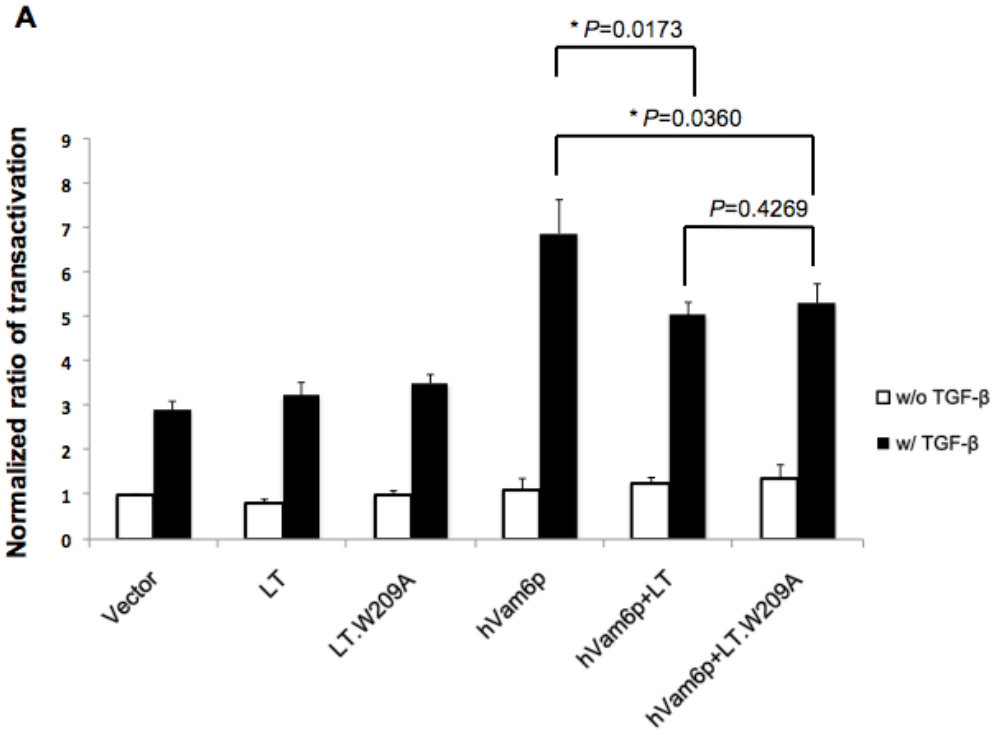


Figure 20. Mapping of the LT Binding Site on Vam6p to Regions Containing the Central Clathrin Homology Repeat Domain. ***A.*** **Schematic representation of murine Vps39 (mVps39) and various deletion constructs. CNH: NH2-terminal citron homology domain; CLH: central clathrin homology repeat domain.** ***B.*** **Immunoprecipitation in U2OS cells of mVps39 deletions with MCV LT, using LT mutant as negative control** ***(last lane). Top panel*****: immunoprecipitation of LT with CM2B4 antibody and immunoblotting of mVps39 with anti-EGFP antibody.** ***Middle panel*****: 5% lysate input of each deletion construct.** ***Bottom panel*****: 5% lysate input of LT or LT mutant.**

2.3.7 Lack of effect on TGF-β and mTOR signaling

Since hVam6p has been reported to be identical with TLP except for an 11 amino acid insertion and TLP functions to balance Smad2 and Smad3 signaling (Felici et al., 2003), we examined MCV LT effect on TGF-β signaling in HT1080 cells using the TGF-β-responsive luciferase reporter 3TP-Lux. Without TGF-β treatment, hVam6p expression has miminal effect on basal 3TP-Lux activity but reporter activity nearly doubles with hVam6p expression after TGF-β treatment (Fig. 21A). LT expression modestly reduces this activity but this effect is independent of the LT.W209A substitution, suggesting that LT does not directly target TGF-β signaling through hVam6p (Fig. 21A).

Vam6p (Vps39 in yeast) also functions as a Rag family GTPase nucleotide exchange factor (GEF) to promote TORC1 activation in response to increased amino acid concentrations in the environment of yeast, leading to mTOR-dependent phosphorylation of the cap-binding protein 4EBP-1 (Binda et al., 2009). To assess the effect of LT on mTOR signaling, we transfected 293 cells with HA-hVam6p together with wild-type LT or LT.W209A alone or together with hVam6p. After overnight serum starvation and 2 hrs amino acid starvation, 4EBP-1 phosphorylation was assessed by Western blotting. Phospho-4EBP-1 Thr37/46 and Ser65 levels are low under starvation conditions compared to cells grown with serum and amino acids (baseline, Fig. 21B). hVam6p had no obvious activity on 4EBP-1 phosphorylation either during starvation or under replenished conditions. Further, no significant or consistent change in 4EBP-1 phosphorylation was noted after expression of either the wild-type LT or LT.W209A (Fig. 21B).


A
* P=0.0173
* P=0.0360
P=0.4269
Normalized ratio of transactivation
0
1
2
3
4
5
6
7
8
9
w/o TGF-β
w/ TGF-β
Vector
LT
LT.W209A
hVam6p
hVam6p+LT
hVam6p+LT.W209A

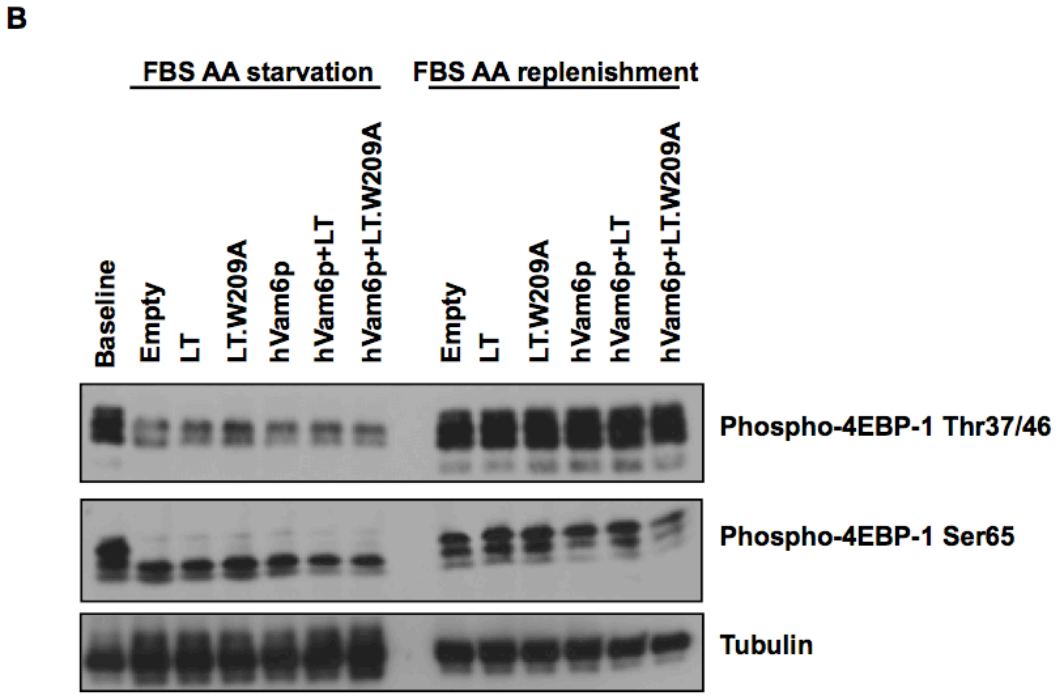


Figure 21. Lack of Effect on TGF-β and mTOR Signaling. ***A*****. LT-hVam6p binding has no significant effect on TGF-β pathway. HT1080 cells were transfected with TGF-β inducible reporter p3TP-Lux and empty vector, wild-type LT, hVam6p binding mutant LT, alone or together with hVam6p. 24 h after transfection, cells were replaced with fresh DMEM with 0.2% FBS, left non-treated or stimulated with recombinant human TGF-β1 at 5 ng/ml for 18 h. Reporter activities were measured using Dual-Luciferase Reporter Assay System (Promega) and normalized to non-treated empty vector transfected condition. Independent experiments were performed in triplicate three times. Error bars and *P*-values are shown.** ***B*****. LT-hVam6p binding has no effect on mTOR pathway. 293 cells were transfected with empty vector, wild-type LT, hVam6p binding LT mutant alone or together with hVam6p, left in serum starvation (0.3% FBS) overnight. On second day cells were starved in amino acid free medium for 2 h and replenished with amino acid solution for 1 h. Non-stimulated and stimulated lysates were subjected to a western blot analysis to probe for mTOR down stream target 4EBP-1 phosphorylation.**

2.3.8 MCV LT Disrupts hVam6p-Induced Lysosome Clustering

A third function described for hVam6p over-expression is promotion of lysosome clustering (Caplan et al., 2001; Peralta et al., 2010). HeLa cells were transiently transfected with myc-hVam6p and wild-type LT or hVam6p binding LT mutant, and lysosome clustering was assessed by cytoplasmic clustering of lysosomal-associated membrane protein 1 (LAMP1). Under standard growth conditions, LAMP1 shows a diffusely punctate distribution in the cytoplasm (Fig. 22, *a-d*). Over-expression of hVam6p induces perinuclear lysosome clustering as previously reported (Caplan et al., 2001; Peralta et al., 2010) (Fig. 22, *e-h*). This clustering is abolished by expression of wild-type LT (Fig. 22, *i-l*) but not LT.W209A (Fig. 22, *m-p*). To determine if nuclear sequestration of hVam6p is required for inhibition of lysosomal clustering, hVam6p was co-expressed with MCV350 LT which lacks a nuclear localization signal but still interacts with hVam6p. Clustering was present with MCV350 LT expression (Fig. 22, *q-t*) suggesting that LT binding alone to hVam6p is insufficient to antagonize hVam6p-induced lysosomal clustering and nuclear sequestration is required to antagonize this effect.

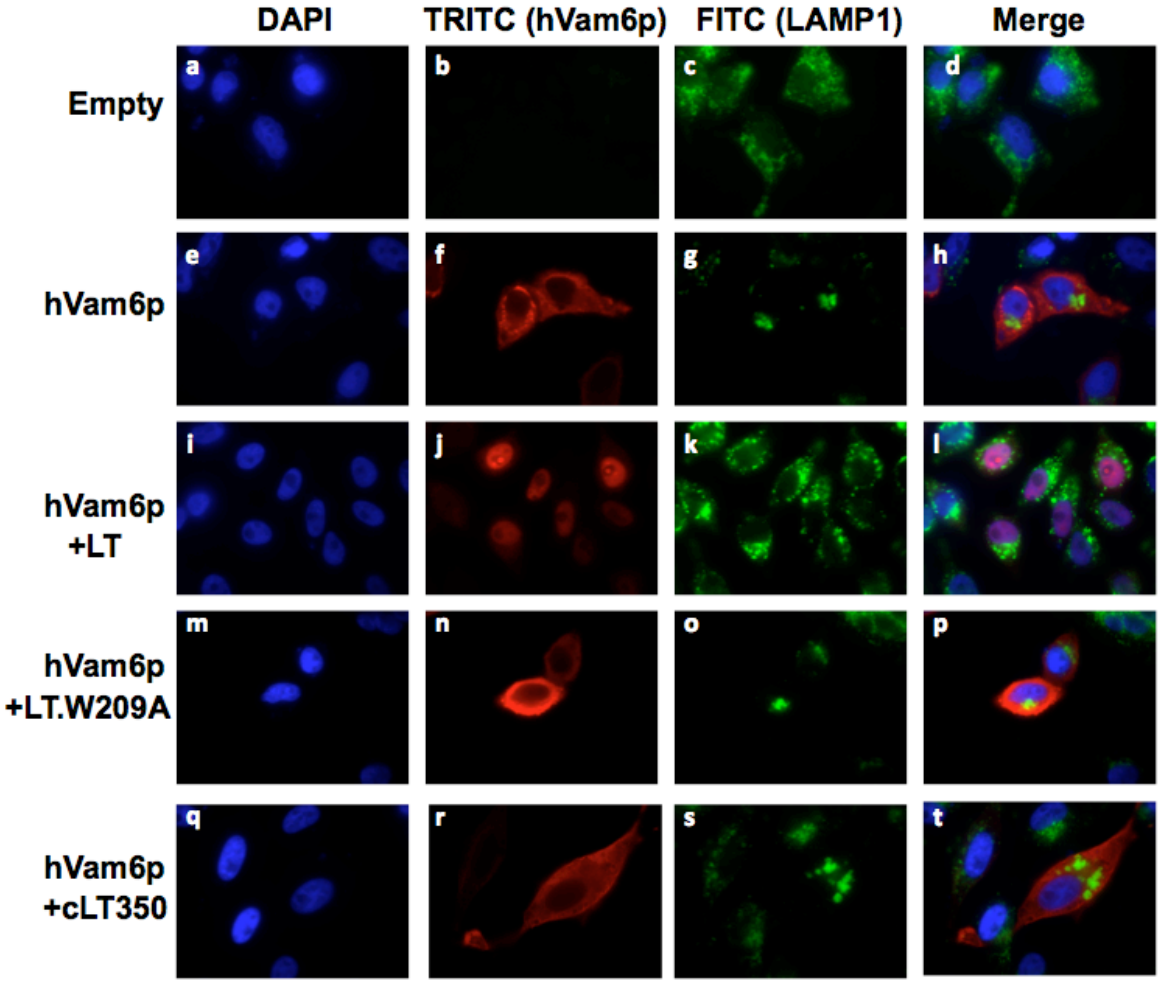


Figure 22. LT Inhibits hVam6p-induced Lysosome Clustering in HeLa Cells. HeLa cells were transfected with myc-hVam6p alone (*e-h*), together with LT (*i-l*), LT.W209A (*m-p*) or cLT350 (*q-t*). The expression of hVam6p was visualized with anti-myc antibody and Alexa Fluor 568-conjugated anti-mouse antibody (TRITC). LAMP1 was visualized with anti-Human CD107a (LAMP1) Alexa Fluor 488. hVam6p-induced clustering of lysosomes (*h*) is lost when full-length LT (*l*) but not LT.W209A (*p*) or cLT350 (*t*) is co-expressed.

2.4 DISCUSSION

We identified cytoplasmic human Vam6p as a unique interactor for nuclear MCV LT. The hVam6p interaction domain is not present in LT from other polyomaviruses and we find that direct targeting of hVam6p does not occur with SV40 LT. To our knowledge, hVam6p has not been reported as a binding partner for any other viral protein. MCV LT associates with endogenous hVam6p in MCV positive cell lines making it likely that this interaction has physiological consequences. Interestingly, LT relocalizes hVam6p from the cytoplasm to the nucleus. Nuclear sequestration of a cytoplasmic protein is an unusual regulatory phenomenon that has been described for cytomegalovirus TRS-1 protein sequestration of protein kinase R (Hakki et al., 2006), poliovirus-induced nuclear relocalization of eIF4E through eIF4G cleavage (Sukarieh et al., 2010), and HPV induced nuclear relocalization of p80 by the E1 protein (Cote-Martin et al., 2008). While MCV T antigen causes a loss-of-function for hVam6p involvement in lysosomal trafficking, we do not know if there is a subsequent gain-of-function via nuclear relocalization of hVam6p.

Current evidence indicates that MCV is a bona fide human cancer virus causing most cases of Merkel cell carcinoma (Houben et al., 2010). Like other human polyomaviruses, MCV is a common if not ubiquitous infection that does not normally integrate into the host chromosome as part of its life cycle. Virus mutation leads to genomic integration that contributes to tumor formation (Feng et al., 2008; Shuda et al., 2008). Integrated MCV genomes are clonal within tumors and most tumor cells express abundant LT protein (Shuda et al., 2009b). Expression of the full-length LT protein in cells harboring an integrated MCV genome, however, will lead to unlicensed replication from the integrated viral origin that is predicted to cause DNA

fragmentation (Shuda et al., 2008). Thus, additional virus mutations truncating the LT to eliminate its DNA replication capacity are commonly found in tumor-derived viruses. MCV species found in tumors are replication defective and do not represent free-living and transmissible viruses.

Our studies suggest the possibility that hVam6p sequestration might play a role in MCV replication rather than tumorigenesis. Unlike most MCV LT proteins, the tumor-derived MCV350 strain LT protein possesses a truncation that eliminates its nuclear localization signal (Nakamura et al., 2010). Most MCV LTs are localized to the nucleus whereas MCV350 LT has a diffuse nuclear and cytoplasmic distribution. While MCV350 LT still interacts with hVam6p, it does not inhibit lysosomal clustering. This circumstantial evidence suggests hVam6p antagonism by MCV LT involves nuclear sequestration rather than simple binding, and that this sequestration may not be critical to MCV-induced tumorigenesis. In a limited survey of TGF-β and mTOR signaling functions, we did not observe an effect for LT dependent on hVam6p interaction. We cannot rule out the possibility that more extensive studies might reveal contributions to cell growth and proliferation by MCV LT targeting of hVam6p. In fact, interference with hVam6p function was found to enhance growth factor independent cell survival and potentially modulate autophagy (Liang et al., 2008; Peralta et al., 2010).

At present, there is no MCV replication system available to test for specific functions of LT in virus assembly, transmission and replication but we anticipate that these questions can eventually be addressed using MCV molecular clone viruses. Because MCV is only found in high copy number in tumors, naturally-occurring replication-competent MCV genomes have not been cloned until recently (Laude et al., 2010; Schowalter et al., 2010).

Clues to the functions for MCV LT binding to hVam6p may come from infection studies of other polyomaviruses. Polyomaviruses make use of endosomal and lysosomal trafficking to

transit from the cell membrane to the nucleus, although precise mechanisms differ between polyomaviruses (Mannova and Forstova, 2003). Murine polyomavirus, for example, binds the ganglioside GD1a and is transported to endolysosomes where pH-dependent conformational changes allow transport of the virus into the endoplasmic reticulum and then the nucleus for uncoating and replication (Qian et al., 2009). SV40 transit to the endoplasmic reticulum is dependent on viral coat protein binding initially to caveosomes and subsequently to caveolin-free membrane structures (Pelkmans et al., 2005). Little is known about the cellular functions of hVam6p in lysosomal trafficking and many of its functions are inferred from studies of the yeast homolog, Vps39p (Nakamura et al., 1997). Disruption of Vps39 interaction with the C-Vps complex mislocalizes Vps39 from membrane-enriched subcellular fractions to the cytosolic fraction and causes hydrolase missorting defects, suggesting that localization of Vps39 to the vacuole is essential for vacuolar protein transport (Wurmser et al., 2000). Nuclear sequestration of hVam6p can be expected to affect its regulatory interactions with C-Vps complex in human cells but it is unlikely to completely disrupt this complex. A class C-Vps component, hVps11, is not mislocalized by MCV LT protein expression (data not shown). Since LT is an early viral protein, it is more likely that targeting of hVam6p plays an important role in viral egress rather than entry.

Our studies provide the first evidence for cytoplasmic targeting of endolysosomal components by a polyomavirus using a novel nuclear sequestration mechanism. MCV LT provides a unique reagent to study Vam6/Vps39 functions in cytoplasmic lysosomal trafficking. Development of robust MCV replication systems will allow examination of the consequences of hVam6p sequestration by LT to MCV infection.

3.0 CELLULAR AND VIRAL FACTORS REGULATING MERKEL CELL POLYOMAVIRUS REPLICATION

Work described in this section is accepted by *PLoS ONE* with authors Huichen Feng, Hyun Jin Kwun, Xi Liu, Ole V. Gjoerup, Donna B. Stolz Yuan Chang and Patrick S. Moore.

X. Liu performed Nuclease-protection assay and Quantitative RT-PCR. H. J. Kwun performed MCV origin replication assays. Donna B. Stolz helped with the electron microscopy imaging. H. Feng performed all other experiments described in this section. H. Feng, Y. Chang and P. S. Moore conceived the project, analyzed the results and wrote the manuscript.

MCV was found to harbor deletions or mutations eliminating viral replication capacity. Since no full-length viral genomes from nontumor sources have been isolated, we sought to construct a consensus MCV genome that can replicate in cells to examine MCV DNA replication and encapsidation.

MCV consensus genome (MCV-HF) was designed based on seven full-length MCV genomes. MCV-HF was synthesized and cloned into an expression vector. Using site-directed mutagenesis, we also generated a replication-deficient clone possessing the MCV350 mutation in the replication origin (MCV-Rep$^-$).

To measure the virion production of MCV molecular clones, we performed nuclease-protection assay and quantitative real-time PCR. We found that MCV-HF is able to produce virions in cells but MCV-Rep$^-$ is defective in virion production. These findings were confirmed by Southern blotting for viral DNA, suggesting that MCV-Rep$^-$ loses the capacity for DNA replication. Additionally, we showed that MCV replication and encapsidation is increased by overexpression of MCV sT, but not LT, consistent with sT being a limiting factor during virus replication.

To assess the effect of LT-hVam6p interaction (see Chapter 2) in MCV virion production, we introduce the W209A mutation into the wild-type MCV-HF virus to abolish LT binding to hVam6p (MCV-hVam6p$^-$). This mutation leads to a 4-6 fold increase in nuclease-resistant virion production compared to MCV-HF. Increased virion and subgenomic DNA production for MCV-hVam6p$^-$ was confirmed by Southern blotting. Immunoblotting for encapsidated VP1 after gradient purification also reveals markedly increased viral particle production for MCV-hVam6p$^-$, suggesting a previously unrecognized role for hVam6p in regulating virus replication.

Taken together, our study generated a replicating MCV molecular clone that can be manipulated to evaluate effects on viral replication and encapsidation. We also identified viral sT and cellular hVam6p as important factors regulating MCV replication.

3.1 INTRODUCTION

Merkel cell polyomavirus (MCV) was identified by digital transcriptome subtraction from Merkel cell carcinoma (MCC), a rare but aggressive human skin cancer (Feng et al., 2008; Feng et al., 2007). MCV is a double-stranded DNA virus belonging to the *Polyomaviridae* family, members of which share conserved early, late, and regulatory regions. The polyomavirus early viral tumor (T) antigens play key roles in viral genome replication as well as tumorigenesis. Large T (LT) antigen-encoded helicase activity, for example, unwinds the viral replication origin (Fanning and Zhao, 2009; Kwun et al., 2009) and enhances the polyomavirus late promoter leading to an early-to-late switch in gene expression. For murine polyomavirus, this switch has been shown to depend on LT-initiated viral DNA replication (Liu and Carmichael, 1993). The late region encodes viral capsid proteins (VP1 and VP2) that self-assemble into virus-like particles (VLP) when expressed in cells (Chen et al., 2011; Pastrana et al., 2010; Pastrana et al., 2009; Tolstov et al., 2009; Touze et al., 2010). MCV VLP have been used to infect cells and can be used in neutralization experiments (Pastrana et al., 2009) but

replication of full MCV genome has not been described. The concerted regulation and interaction of both early and late polyomavirus proteins are necessary to produce viral particles.

Loss of viral replication capacity, or permissivity, is a common feature of virus-initiated tumors (Moore and Chang, 2010; zur Hausen, 2008). Approximately 80% of MCC are infected with MCV in which the viral genome is clonally-integrated into the host tumor cell genome, preventing viral replication (Feng et al., 2008; Sastre-Garau et al., 2009; Shuda et al., 2008). MCV obtained from tumors also possess LT gene mutations that are a central feature of MCV-driven human tumor formation (Shuda et al., 2008). LT normally binds a specific site in the viral replication origin and initiates DNA replication through its C-terminal helicase domain. Tumor-specific mutations prevent LT-initiated DNA replication at the integrated genome thus preventing independent and unlicensed DNA replication from the viral genome that could lead to catastrophic replication fork collisions and DNA breakage when multiple virus-initiated replication forks proceed onto the cellular DNA template (Shuda et al., 2008).

The minimal MCV replication origin has been mapped to a 71 bp fragment in a non-coding region that LT protein binds in order to initiate viral DNA replication. Among MCV proteins, LT protein alone is sufficient for this process but MCV small T (sT) protein acts as an accessory factor that greatly increases the efficiency MCV origin firing (Kwun et al., 2009). In one MCV tumor strain (MCV350), a point mutation in its replication origin prevents proper assembly of the LT helicase complex, also rendering the tumor-derived virus nonpermissive (Kwun et al., 2009). Additional virus mutations in capsid genes, including in the MCV350 strain, have been described that are predicted to prevent virion self-assembly and replication (Kassem et al., 2008b). The sT and the N-terminal portions of LT, however, are unaffected by tumor-specific mutations, suggesting that they may play a key role in MCC tumorigenesis. The importance of viral early gene contributions to this cancer is shown by knockdown of the

common T antigen exon 1 sequence, which leads to cell cycle arrest and cell death of MCV-positive MCC cells (Houben et al., 2010).

More than 50% of the healthy adult population is serologically positive for MCV antibodies (Carter et al., 2009; Kean et al., 2009; Pastrana et al., 2009; Tolstov et al., 2009) and most adult MCV infections are asymptomatic (Y. Tostov, L. Kingsley, Y. Chang and P.S. Moore Manuscript under review). In contrast to MCC tumors, only very low level MCV genomic DNA is present in healthy tissues, including skin, peripheral blood mononuclear cells, gastrointestinal tract (Feng et al., 2008; Loyo et al., 2010; Schowalter et al., 2010), human respiratory tract secretions (Goh et al., 2009), and other tissues (Loyo et al., 2010). Using rolling circle amplification, Schowalter et al. have recently isolated several encapsidated strains of wild-type MCV from healthy skin (Schowalter et al., 2010). Nonetheless, the limiting amounts of wild-type MCV flora present in healthy tissues have been a significant barrier to isolation of replication-competent MCV.

To search for novel cellular factors binding to MCV early proteins, we performed tandem-affinity pulldown assays with a unique region of the MCV LT (Liu et al., 2011). An MCV LT domain that is conserved in both tumor-derived and wild-type MCV strains interacts with the cytoplasmic vacuolar sorting protein, hVam6p (also known as Vps39), a component of the HOPS (homotypic fusion and protein sorting) complex involved in late endosomal and lysosomal fusion (Caplan et al., 2001). Coexpression of MCV LT with hVam6p causes relocalization of hVam6p from the cytoplasm, where it is normally found, to the nucleus and to perinuclear bodies. This interaction can be abrogated by a single alanine substition at tryptophan 209 (LT.W209A) in the hVam6p binding domain of LT. No differences in cell viability or cell cycling have been detected for the wild-type LT and LT.W209A expression and so the role for this LT interaction remains unknown. MCV LT antagonizes the ability of

hVam6p to induce lysosomal clustering, raising the possibility that hVam6p might modulate MCV replication or egress.

Development of an MCV molecular clone allows engineering the viral genome, such as introducing a mutation at the hVam6p interaction site in LT, to examine effects on virus replication and assembly. To generate an MCV clone, we aligned MCV genomes from MCC tumors as well as non-tumor tissues and designed a consensus MCV molecular genome (MCV-HF). Subsequent to construction of this consensus clone, the same viral sequence was identified in several naturally occurring MCV strains obtained from healthy human skin samples (Schowalter et al., 2010), supporting the notion that MCV-HF is a permissive viral clone.

We show here that the MCV-HF clone is replication competent and sequentially expresses early and late viral proteins to generate packaged virions after transfection of the molecular clone DNA into 293 cells. MCV-HF has modest but reproducible replication capacity in a variety of tissue culture cell lines that is enhanced by MCV sT coexpression. In contrast, MCV engineered with the MCV350 origin point mutation is replication-deficient, only transiently expresses LT protein, has diminished or absent expression of other early spliced transcript protein forms and does not express late MCV protein. Overexpression of hVam6p is potent in inhibiting MCV replication whereas engineering MCV-HF with the LT.W209A mutation amplifies virus replication suggesting a key role for this vacuolar sorting protein in MCV virion production.

3.2 MATERIALS AND METHODS

3.2.1 Cell Lines and Clinical Samples

Cell lines (293, 293FT, 293TT, 3T3, A549, COS7 and BSC40) were maintained in DMEM medium supplemented with 10% FBS, penicillin and streptomycin (pen/strep). Cell lines (MKL-1, UISO, BJAB, Raji) and peripheral blood mononuclear cell (PBMC) were cultured in RPMI 1640 medium with 10% FBS and pen/strep. MCC clinical specimens (MCC350, MCC337, MCC339, MCC344, MCC345, MCC347, MCC349 and MCC352) and PBMC sample have been described (Feng et al., 2008).

3.2.2 Construction of Consensus MCV Genomes

MCV genomes in MCC cases were directly sequenced with 13 pairs of contig primer sets as previously described (Feng et al., 2008). Long PCR was performed to amplify the whole genome in a MCV positive PBMC sample with two primer sets (contig.1f-8r and contig.9f-1r). The consensus genome (MCV-HF) was generated from 6 tumor-type MCV genomes and 1 wild-type MCV genome using MacVector program (MacVector Inc.). The whole genome was synthesized by the DNA 2.0 Inc (Menlo Park, CA) and cloned into a Kanamycin selectable vector. The consensus MCV genome was linearized at a *BsrF*I restriction site (RCCGGY) in the VP region. A replication-defective MCV genome (MCV-Rep$^-$) was mutagenesized from

consensus MCV-HF with 5'-GAA AAA AAA GAG AGA GGA CTC TGA GGC TTA AGA G-3' and 5'-CTC TTA AGC CTC AGA GTC CTC TCT CTT TTT TTT C-3' primers, using the QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene). Consequently, MCV-Rep$^-$ posses MCV350 origin sequences and loses replication activity. MCV-hVam6p$^-$ was generated with the primer set (W209A.S: 5'-GAA CGG ATG GCA CCG CGG AGG ATC TCT TCT GC-3', W209A.AS: 5'- GCA GAA GAG ATC CTC CGC GGT GCC ATC CGT TC-3') to eliminate the hVam6p binding to T antigen. Plasmids containing MCV-HF, MCV-Rep$^-$ or MCV-hVam6p$^-$ were propagated in *E. coli* JM109 and purified using the Qiagen maxiprep kit (Valencia, CA). Linear MCV was digested out with *BsrF*I and re-ligated into circular form under low concentration of T4 ligase (1 U/ml, New England Biolabs) overnight at 16^{o}C. Circular DNA was further digested with *Ava*I to linearize non-MCV DNA, and treated with Plasmid-Safe™ Exonuclease (Epicentre, Madison, WI) to isolate circular MCV genomes. All three genomes were sequenced confirmed.

3.2.3 Lentivirus Production and Infection

293FT cells (Invitrogen) were transfected with lentiviral construct containing MCV LT or sT antigen together with packaging plasmids, psPAX2 and pMD2.G (Addgene) in 100 mm dish by Lipofectamine 2000 (Invitrogen). 293 cells were infected with lentivirus in the presence of 1 μg/ml polybrene. At day 3 after infection, puromycin (3 μg/ml) was added, and infected cells were selected in bulk for 7 days for stably expression of MCV LT and sT antigen. Expression was confirmed by immunoblotting.

3.2.4 Nuclease-Protection Assay

Cells transfected with MCV-HF or MCV-Rep$^-$ or MCV-hVam6p$^-$ were collected and lysed with 3 freeze-thaw cycles in DPBS-Mg^{2+} buffer. After centrifugation, supernatants were treated with 250 units of benzonase (Promega) and 5 units of RNase A (Ambion) at 37^{o}C for 4 hrs. EDTA was then added to inactivate nuclease. Proteinase K was used to digest capsid proteins at 56^{o}C for 1 hr. The capsid-protected MCV DNA was prepared by phenol-chloroform extraction and dissolved in 50 μl TE buffer. One microliter of DNA was used for PCR quantification with VP2 primers and TaqMan probe as previously described (Shuda et al., 2009a).

3.2.5 Immunoblotting

HEK 293 cells and stably infected 293 cells lines with LT or sT lentiviral vector (293-LT or 293-sT) were seeded in 6-well plates and transfected with 1 μg circularized MCV genomes using Lipofectamine 2000 (Invitrogen). Radio immunoprecipitation assay buffer (RIPA) was used to lyse cells. Monoclonal antibody CM2B4 (Shuda et al., 2009a) was used to examine LT antigen and 57kT antigen expression at 1:2000 dilution. Monoclonal antibody CM8E6 (Houben et al., 2010) was used to detect sT antigen expression at a dilution of 1:250. Late gene expression of VP1 was examined with monoclonal antibody CM9B2 at dilution of 1:2000. The CM9B2 antibody (IgG2b isotype) was generated by immunizing mice with KLH-derivatized (DKGKAPLKGPQKASQKES) peptide from MCV VP1 using standard methods (Epitope Recognition Immunoreagent Core Facility, University of Alabama) and tested for

reactivity to the MCV VP1 protein. Tubulin (Sigma) was used to quantitate sample input at dilution of 1:2000.

3.2.6 MCV Origin Replication Assay

For MCV origin replication assay, 293 cells were transfected with a plasmid containing MCV origin (Ori339(589)) (Kwun et al., 2009), together with wild type (genomic T (TAg), LT) or mutant type (TAg.W209A, LT.W209A) of T antigens as well as hVam6p gene (Caplan et al., 2001). These cells were harvested at day 2 after transfection. Low molecular weight DNA was extracted with modified Hirt-extraction and Southern blot analysis was performed by method previously described (Kwun et al., 2009). To measure MCV-HF virus replication, 2.5 μg of extracted DNA was digested overnight with 5 units of *Dpn*I and *BamH*I and subjected to Southern blotting. DNA fragments containing the MCV origin were used for probe labeled with ^{32}P to measure replication efficiency. The blot was analyzed by using a PhosphorImager (Typhoon 9400, GE Healthcare) and ImageJ software (National Institute of Health, USA).

3.2.7 Virion Purification and Cell Infection

At day 4 after post-transfection, cells were harvested and matured overnight with benzonase, RNase A and Plasmid-safe™ nuclease as previously described (Tolstov et al., 2009). Cell lysate was separated on a 27-33-39% Optiprep (Sigma, St. Louis, MO) gradient

after ultracentrifugation for 3.5 hrs at 234,000 g. Fractions were collected after puncturing the bottom of ultracentrifuge tube using a 25-G needle and stored at -80^{o}C. Viral particles were stained with 1% uranyl acetate negative staining and observed on JEOL JEM-1011 (Tokyo, Japan) electron microscope at 80 kV and compared to self-assembling VP1-VP2 MCV virus-like particles generated as previously described (Tolstov et al., 2009). In MCV infection assay, various cells (293, 293TT, UISO, A549, BJAB, Raji, BSC40 and PBMC purified from whole blood) were cultured with 4 μl ultracentrifuged fractions containing MCV virions together with or without 1 μg/ml polybrene (Sigma) treatment. For transwell experiments, 6-well transwell plates were used (Corning, New York, USA) with a 0.2 or 0.45-mm pore size polycarbonate membrane. Transfected 293 cells with MCV-HF genomes were labeled with intracellular fluorescent dye 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor any cell contamination in transwell experiments. 293, A549 and UISO were seed in the lower chamber to co-culture with transfected 293 cells for 6 days.

3.3 RESULTS

3.3.1 Design and Construction of a Consensus MCV Genome

At the initiation of this study, no full-length viral genomes from nontumor sources had been isolated. We originally found eight of 10 (80%) MCC tumors to be positive for MCV

DNA (Feng et al., 2008). We sequenced full-length MCV genomes by PCR-direct sequencing from 5 of the 8 virus-positive tumors (MCV350, MCV339, MCV344, MCV349, MCV352), as well as from 1 MCV positive cell line (MKL-1), which were compared to a single whole virus sequence (MCV85) obtained from a peripheral blood sample (Fig. 23A, *GenBank IDs to be supplied*). All tumor-derived sequences have T antigen truncations, including mutations or short genomic deletions. For some strains, tumor-derived mutations are also observed in late gene regions, as reported by A. Zur Hausen's group (Kassem et al., 2008b). For example, a 200-bp deletion is present in the VP1 locus of MCC352, generates a truncated VP1 protein that is likely to lead to incomplete viral assembly for this strain (Fig. 23A).

To address the issue of polymorphisms between and within individual cases, we designed a consensus genome (MCV-HF, *GenBank IDs to be supplied)*. This cloned genomic DNA (available through our website, www.tumorvirology.pitt.edu/mcvtools.html) is based on the 7 full-length MCV genomes. Compared to these genomes, the MCV-HF genome is located centrally in the phylogenetic tree (Fig. 23B), closest to wild-type R17a strain and has the same nucleotide sequence as the 17b, 18b and 20b strains identified by Schowalter et al. from human normal skin (Schowalter et al., 2010). The consensus MCV-HF was synthesized and cloned into a kanamycin selectable vector (Fig. 23C). An MCV genome variant possessing the MCV350 mutation in the replication origin (MCV-Rep$^-$) was generated by site-directed mutagenesis to serve as a negative control for viral replication (Kwun et al., 2009).

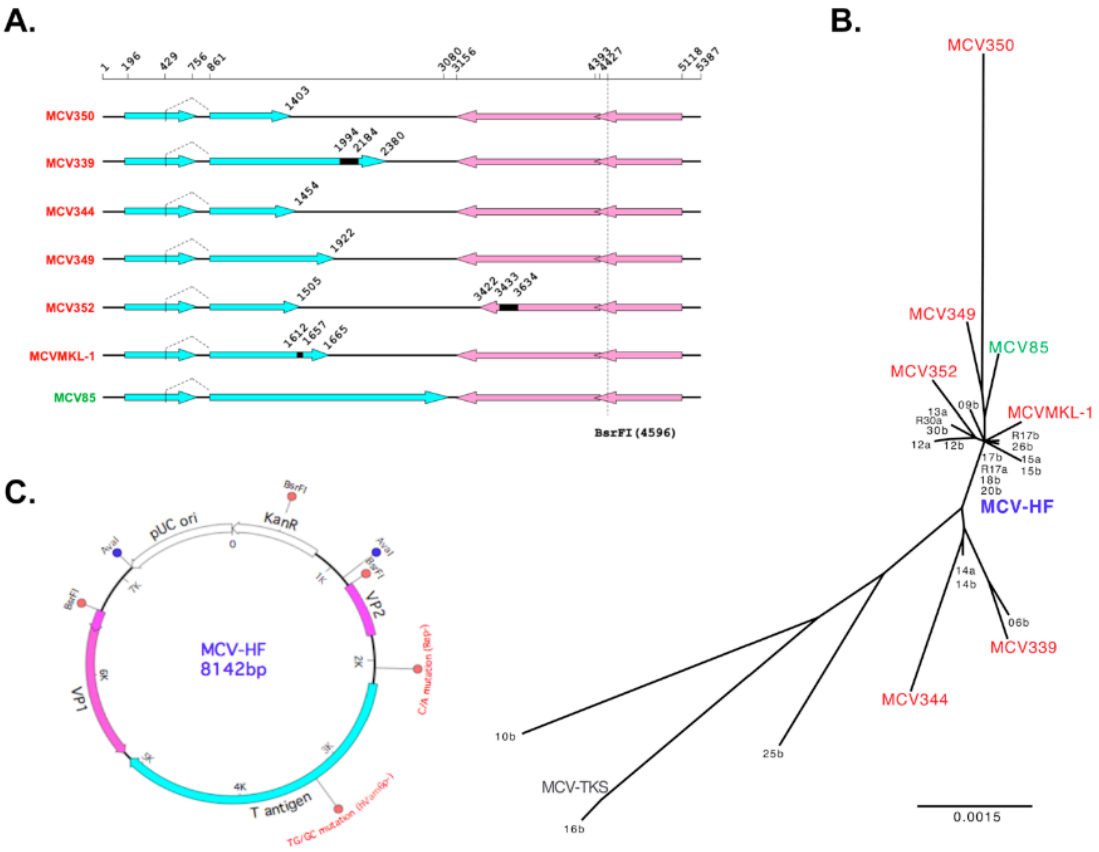


Figure 23. MCV Genome. (A) Full-length of MCV genomes identified from 5 MCC tumors (MCV350, MCV339, MCV344, MCV349, MCV352), 1 MCC cell line (MCVMKL-1) and 1 PBMC sample (MCV85). T antigen ORFs are shown in blue arrows, VP ORFs in pink arrows. Numbers stand for positions in MCV genome. Black solid boxes indicate genomic deletions in MCV genome. (B) Phylogenetic tree of MCV genomes. The consensus genome (MCV-HF, JF813003) is located in the centerof a tree including MCV350 (EU375803), MCV339 (EU375804), MCV344 (JF812999), MCV349 (JF813000), MCV352 (JF813001), MCVMKL-1 (FJ173815), MCV85 (JF813002) and other MCV sequences obtained from human skin (Schowalter et al., 2010) and Kaposi's sarcoma (Katano et al., 2009). (C) The consensus MCV-HF genome can be linearized at *BsrF*I site (4,596 nt) and cloned for propagation in *E. coli*. Sites for mutations engineered into two MCV-HF genomes (MCV-Rep$^-$ (C/A) and MCV-hVam6p$^-$ (TG/GC)) are shown.

3.3.2 MCV-HF and MCV-Rep$^-$ Viral Protein Expression in 293 Cells

MCV-HF or MCV-Rep$^-$ circular genome DNAs were transfected into 293 cells and viral protein expression determined by immunoblotting for LT, 57kT, sT antigen and VP1 proteins (Fig. 24). The defective replication origin in MCV-Rep$^-$ genome does not affect initial LT antigen expression, indicating that this mutation does not directly alter early gene transcription. LT protein is readily detected 24 hrs after transfection for both MCV-HF and MCV-Rep$^-$ and equal amounts of LT expressed by day 2 for both clones. This reveals that the early promoter regulating LT is intact in both viruses and similarly active at time points prior to active viral replication and amplification. Surprisingly, expression of the alternatively spliced 57kT form is reduced with MCV Rep$^-$ at day 2. This is not due to differences in detection since both LT and 57kT are determined on the same blots with the same CM2B4 antibody. LT protein expression continues over 5 days for the MCV-HF virus. LT protein levels peak at 48 hrs for MCV-Rep$^-$ and then subsequently decline. Notably, the spliced 57kT antigen protein is diminished at all time points for the MCV-Rep$^-$ clone. In contrast to LT and 57kT, no sT (an alternate spliced form from the early Tag locus) expression is detected after transfection of MCV-Rep$^-$, but sT is readily detected by day 3 after transfection with the replication-competent genome. These findings suggest that splicing efficiency among early MCV genes may be dependent on viral genome replication.

VP1 structural protein also increases in abundance from day 3 through 5 post MCV-HF transfection (Fig. 24), consistent with a switch from early to late gene expression to generate spontaneously assembling virus particles. VP1 expression, however, is not detected after MCV-Rep$^-$ transfection. Thus, in 293 cells, transient transfection of only the wild-type MCV-HF genome produces both early and late proteins required for virus replication and assembly.

Alternatively-spliced early proteins (sT and 57kT) are diminished or absent, as is the late VP1 protein, for the MCV-Rep$^-$ virus despite similar initial levels of LT expression with both permissive and nonpermissive viruses.

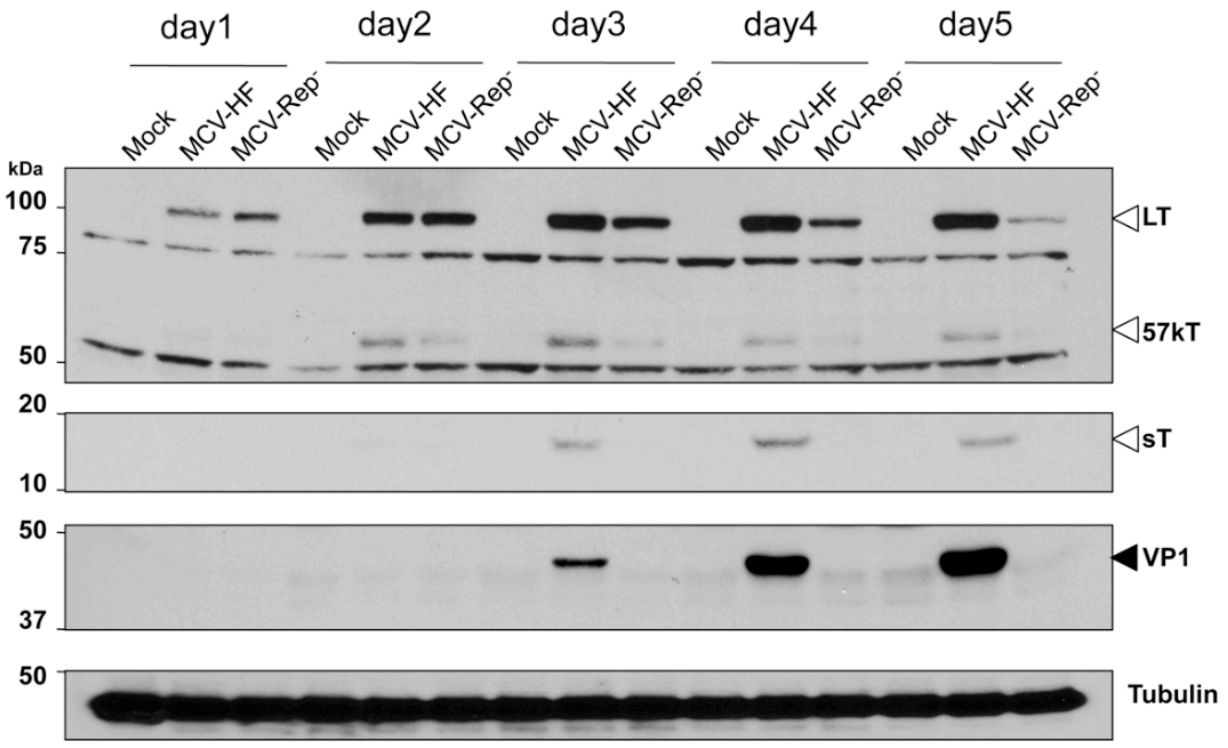


Figure 24. Gene Expression of MCV in 293 Cells. One microgram of recircularized MCV genomes (wild-type MCV-HF or replication-defective MCV-Rep$^-$) were transfected into 293 cells in a six-well plate well. Immunoblotting was performed to examine T antigen expression (indicated by hollow arrows) and VP1 protein (indicated by solid arrow) using CM2B4 (LT, 57kT), CM8E6 (sT) and CM9B2 (VP1) antibodies, respectively. Alpha-tubulin detection was used as a protein loading control. LT protein is expressed equally at day 2 for both viruses but decreases for MCV-Rep$^-$ on days 3-5. VP1 increases on days 3-5 for MCV-HF, corresponding to viral DNA replication. Other early proteins are diminished (57kT) or absent (sT) in the replication deficient MCV-Rep$^-$.

3.3.3 Detection of MCV-HF Virus in 293 Cells

We next examined the replication capacity for virion production in 293 cells tranfected with MCV-HF. At day 4 post MCV-HF transfection, cells were harvested, lysed, matured overnight and treated with benzonase, RNase A and Plasmid-safe™ nuclease. Fractions were collected from an ultracentrifuged Optiprep™ (iodixanol) gradient and immunoblotted for VP1 protein (Fig. 25A). High molecular weight aggregates of the ~45 kDa VP1 protein are present in fraction 4 having a 1.24 g/ml buoyant density. A high molecular weight VP1 form (~90 kDa) is also present polyacrylamide gels that may represent covalently-crosslinked dimeric VP1 protein. VP1 is also present in the lowest density fractions 9 and 10, that represent unassembled, free VP1 protein. Fraction 4 contains typical 38-43 nm diameter polyomavirus particles detected by transmission electron microscopy with uranyl acetate negative staining (Fig. 25B). Quantitative real-time PCR for DNA isolated from Optiprep gradients reveals highest copies of nuclease-resistant DNA in fraction 4 (Fig. 25C). In contrast, nuclease-resistant MCV genome is not detected in fractions 9 and 10. Encapsidated viral DNA increases up to day 4 after MCV-HF transfection and then plateaus (representative results are shown in Fig. 25D), correlating with VP1 protein expression levels. Viral DNA is present immediately after MCV-Rep$^-$ transfection but diminishes below the level of detectability by day 5.

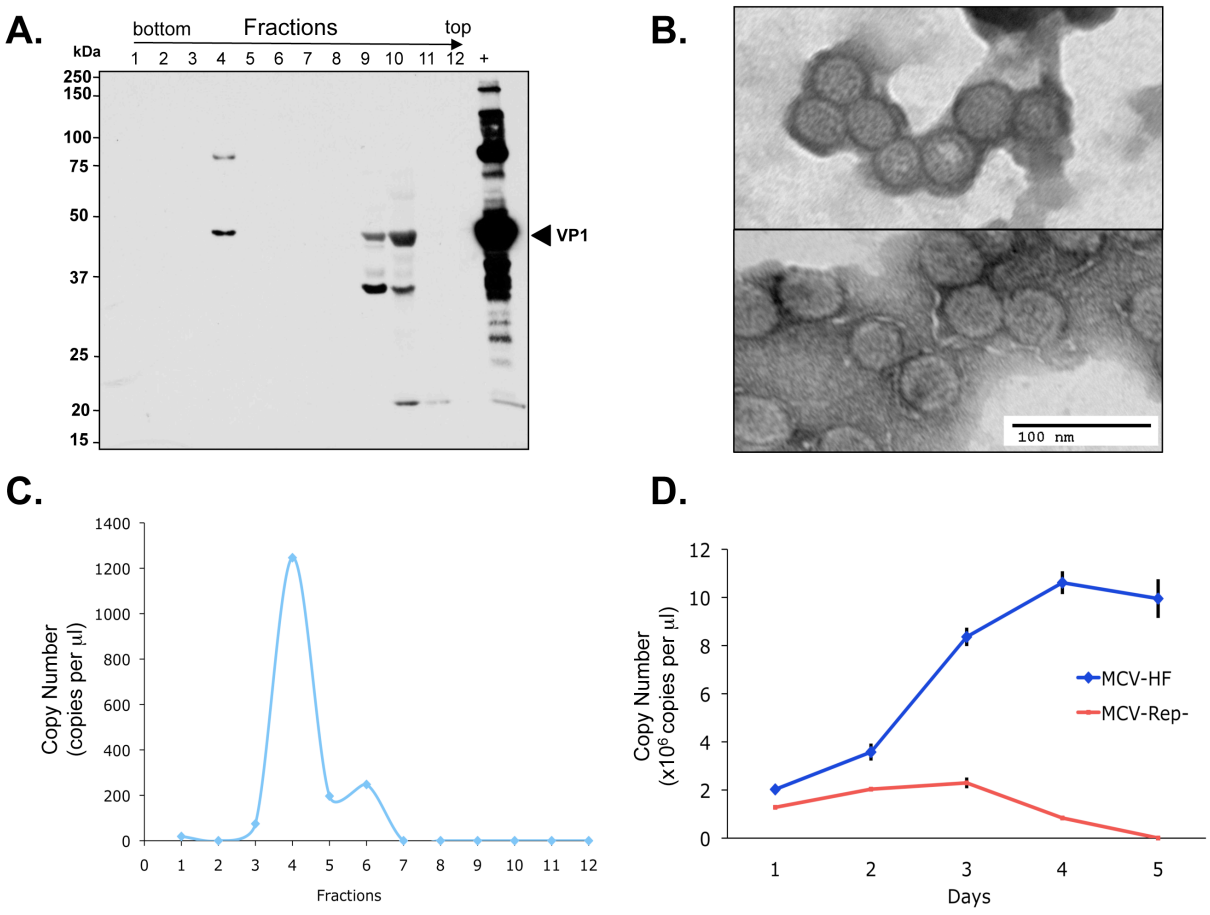


Figure 25. Fractionation of Viral Capsid Protein VP1 by Optiprep™ Density Gradient Ultracentrifugation. (A) Twelve fractions were collected from highest to lowest density, and analyzed by immunobloting with CM9B2 antibody to detect VP1 capsid protein. Assembled 45 kDa VP1 protein is isolated in Fraction 4. Unassembled, free VP1 protein is present in Fractions 9 and 10. The positive control (+) is virus-like particle (VLP) prepared from 293TT cells by MCV VP1 and VP2 transfection. (B) Typical 40 nm diameter icosahedral Merkel cell polyomavirus particles present in Fraction 4. (upper panel). The bottom panel shows MCV particles for comparison. (C) Nuclease-resistant MCV DNA in various gradient fractions quantitated by real time PCR. Highest levels of encapsidated DNA are present in Fraction 4, correspond the fraction having high levels of complexed VP1 and MCV virions. (D) Time-course for MCV virion production after transfection of 1 mg replication competent (MCV-HF) or incompetent (MCV-Rep$^-$) genomes into 293 cells was determined on lysed cells by quantitative PCR after nuclease treatment. Genome replication and packaging of MCV-HF is evident by day 3.

These findings are confirmed by Southern blotting for viral DNA (Fig. 27). In this experiment, DNA from 293 cells 4 days post-transfection, with either MCV-HF (lane 1) or MCV-Rep$^-$ (lane 2), were treated with *Dpn*I to digest unreplicated DNA and with *BamH*I to linearize MCV genome. No bands are present for MCV-Rep, while a weak 5.4 kb *Dpn*I-resistant band representing full-length genome (Tolstov et al., 2009) is present in extracts from MCV-HF transfected 293 cells. Significantly, a subgenomic 0.3-2 kb smear of *Dpn*I-resistant MCV DNA is also present, consistent with a large fraction replicated MCV DNA being composed of either abortively-replicated or partially-digested genome fragments.

3.3.4 Optimization of MCV Clone Replication

To determine whether coexpression of MCV early genes might enhance MCV replication, 293 cells were stably transduced with MCV sT, LT or empty vector expression constructs. MCV-HF and MCV-Rep$^-$ genomes were then transfected into these cells and MCV virion production was quantitated by real-time PCR. Cells coexpressing MCV sT generate approximately 5-fold increased nuclease-protected MCV DNA compared to cells without sT coexpression (Fig. 26). In contrast, only a small increase in MCV DNA is present in cells stably expressing LT protein, suggesting that sT but not LT protein levels are limiting for genome replication after transfection. Several other cell lines (UISO, 293TT, 3T3 and COS7) were also examined, with and without various MCV gene coexpressions, in an attempt to

optimize MCV virion production (Table 2). None of these conditions led to appreciably greater MCV virion production compared to 293 cells alone.

Table 2. Optimization of MCV Production in Various Cell Lines and Effect of Co-expression of Viral Proteins. *Note: Relative expression was determined in individual experiments by PCR(§) , MCV protein expression (¶), or both.

Cell lines	Co-transfected MCV plasmid(s)	Production level *
293	None§¶	++
	Large T antigen§¶	++
	Small T antigen§¶	+++
UISO	None§	+
	Genomic T antigen§	+
	VP1 and VP2§	+
293TT	None§	+/-
	Genomic T antigen§	+
	VP1 and VP2§	+/-
3T3	None§	+/-
	Genomic T antigen§	+
COS7	None§	+/-
	Genomic T antigen§	+

3.3.5 MCV LT-hVam6p Interaction Diminishes MCV Replication

As described in Chapter 2, our tandem-affinity pull-down studies have found that MCV LT binds to hVam6p and relocalizes it from the cytoplasm to the nucleus, but the biological importance of this interaction is unclear (Liu et al., 2011). To evaluate the effect of LT-hVam6p interaction on viral replication and assembly, a mutant MCV-HF, designated as MCV-hVam6p$^-$, was engineered to encode a W209A substitution in the LT gene to prevent hVam6p interaction. As seen in Figure 26, mutation of the hVam6p-binding site leads to a 4-6 fold increase in nuclease-resistant virion production compared to the wild-type MCV-HF virus. Enhanced virion replication is additive with sT coexpression, suggesting that LT.W209A and sT coexpression are independent of each other and act at different stages of replication.

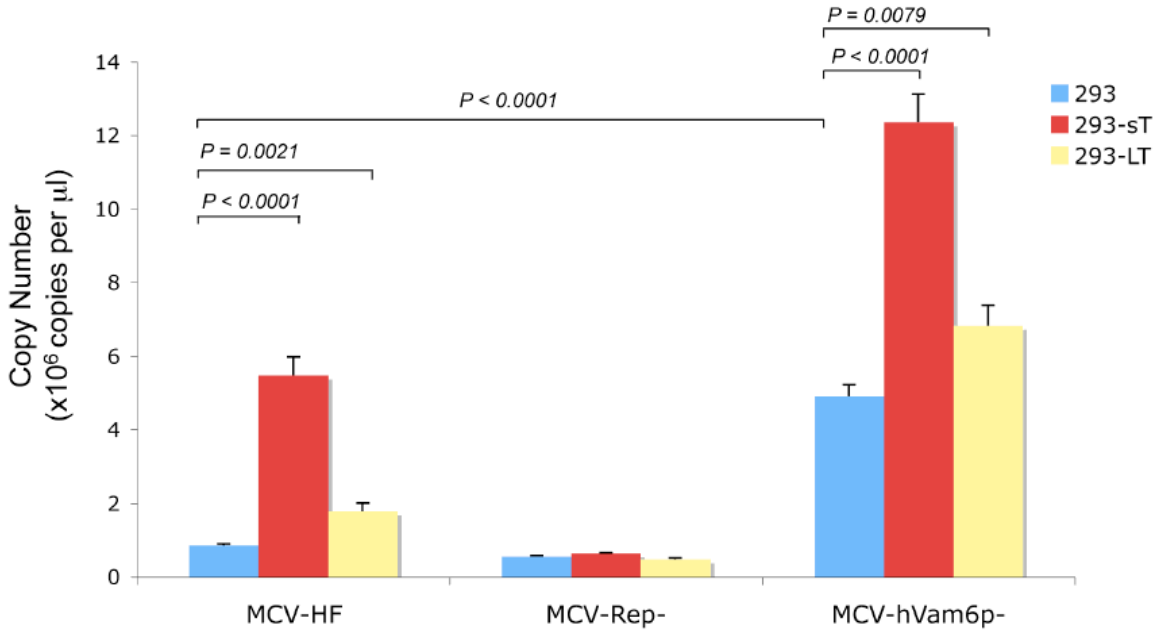


Figure 26. Quantitative PCR for MCV Virion Production. One microgram MCV clone DNAs were transfected into 293 cells stably transduced to express MCV sT or LT proteins. DNA was extracted and treated with benzonase and RNase to discriminate packaged viral DNA. The nuclease-resistant MCV genome was precipitated and measured by quantitative PCR after proteinase K treatment. Cellular sT expression increases virion production for both MCV-HF and MCV-Vam6p- viruses. Comparison of MCV-HF and MCV-Vam6p- shows that loss of the hVam6p binding site also increases virus production. Coexpression of sT and mutation of the hVam6p binding site in the MCV genome are additive in virion production compared to MCV-HF without sT coexpression.

Increased virion and subgenomic DNA production for MCV-hVam6p$^-$ was confirmed by Southern blotting (Fig. 27, lane 3). The comparable levels of *Dpn*I-sensitive bands in all lanes on the Southern blot demonstrate that this effect is unlikely to be due to differences in transfection efficiency.

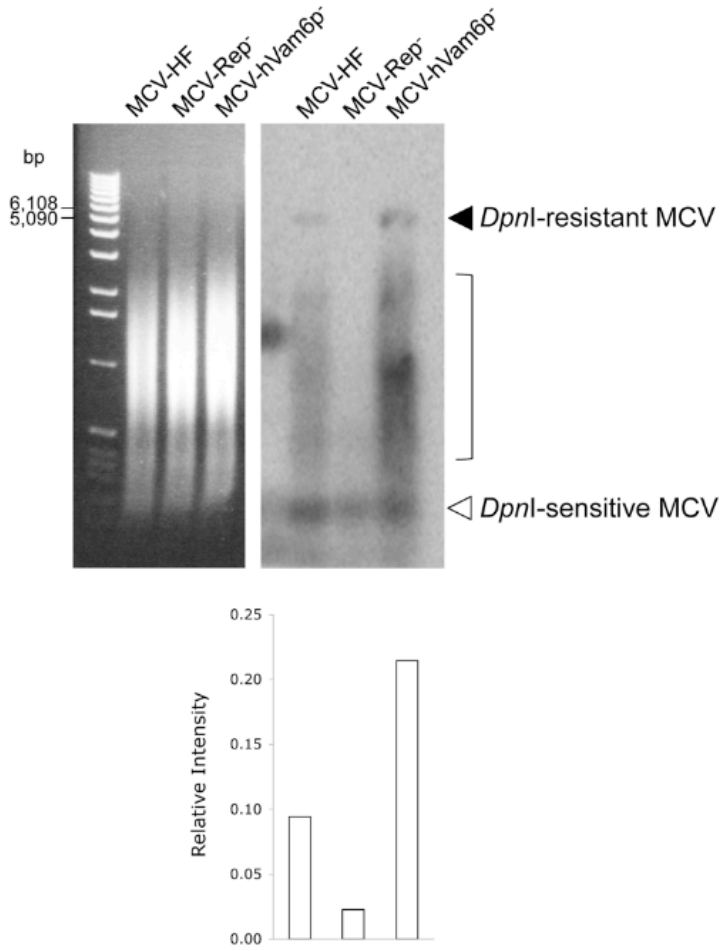


Figure 27. MCV Genome Replication. Southern blot (top panel) for MCV for MCV-HF (lane 1), MCV-Rep$^-$ (lane 2) and MCV-hVam6p$^-$ (lane 3) viruses four days after transfection of 1 mg circular genomic DNA into 293 cells. Panel on left shows the ethidium bromide-stained gel prior to transfer indicating equal DNA loading. Bands for the full-length 5.4 kb MCV genome are present as *Dpn*I-resistant bands in MCV-HF and MCV-hVam6p$^-$ viruses (lanes 1 and 3) but not in the replication deficient MCV-Rep$^-$ virus (lane 2). The replication efficiency was measured by the ratio between the DpnI-resistant 5.4 kb band and the DpnI-sensitive band. The MCV-hVam6p- virus generates ~2-fold more full length genome compared to wild-type MCV-HF virus. Replicated viral DNAs also show the presence of extensive subgenomic fragments.

Immunoblotting for encapsidated VP1 after gradient purification also reveals markedly increased viral particle production with MCV-hVam6p$^-$ virus (Fig. 28).

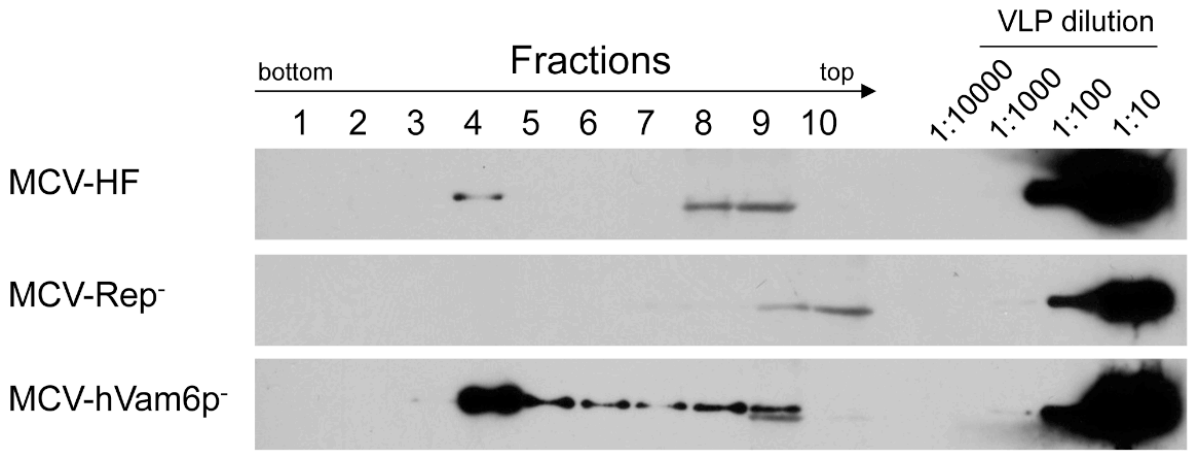


Figure 28. Immunoblot Analysis of Viral Capsid Protein VP1. Optiprep™ density gradient fractions from wild type (MCV-HF) and mutant viruses (MCV-Rep$^-$, MCV-hVam6p$^-$) generated from transfected 293 cells were used for Western blotting. Dilutions of MCV virus-like particles (VLP) provide a marker for the relative abundance of VP1 protein in each fraction. Assembled MCV-hVam6p$^-$ virus VP1 expression is ~10 fold increased in fraction 4 compared to MCV-HF.

In contrast, hVam6p coexpression inhibits nuclease-resistant, encapsidated DNA production from MCV-HF to levels comparable to the nonpermissive MCV-Rep$^-$ virus (Fig. 29). The MCV-hVam6p$^-$ clone replication is relatively resistant to the effects of hVam6p coexpression compared to MCV-HF but also declines in a dose-dependent fashion. hVam6p overexpression does not decrease 293 cell viability or alter cell cycling, nor does it alter transfection efficiency (data not shown).

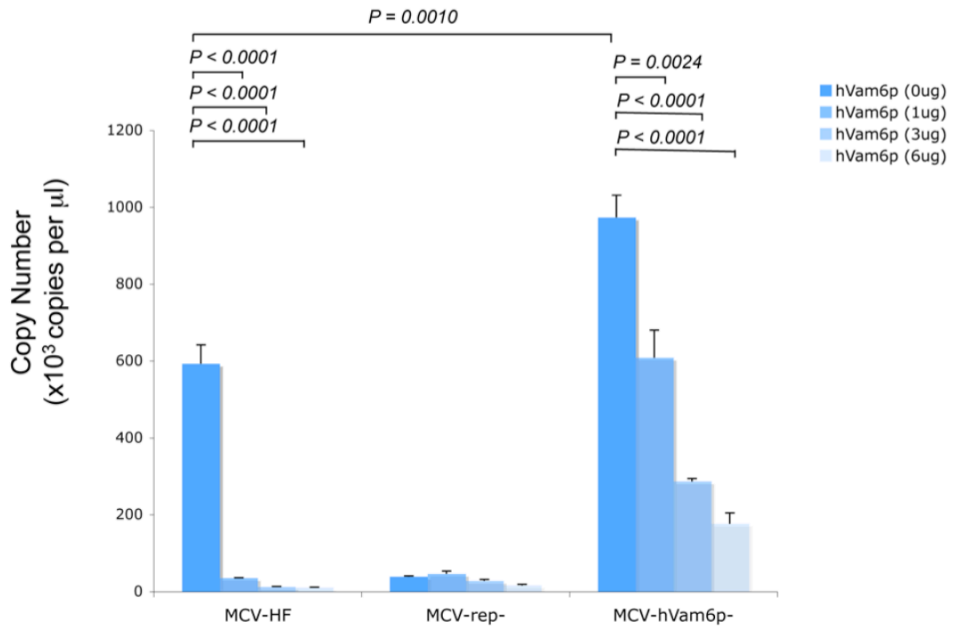


Figure 29. Effect of hVam6p Coexpression on MCV Virion Production. 293 cells cotransfected with hVam6p and MCV genomes at day 4 as measured by nuclease-resistant DNA by quantitative PCR. Circularized viral plasmids (1 mg) together with varying amounts of hVam6p expression plasmid were simultaneously transfected during this experiment.

Since hVam6p is normally a cytoplasmic protein that relocalizes to nuclear and perinulclear sites when MCV LT is co-expressed, we sought to determine whether hVam6p might directly inhibit viral DNA synthesis in the presence of LT (Fig. 30). An *in vitro* origin replication assay was performed with either the entire T antigen gene locus, Tag (expressing LT, 57kT and sT), or the LT cDNA alone. These plasmids were transfected together with the MCV origin cloned into the pCR2.1 vector, with or without hVam6p expression. Southern blotting for the origin plasmid measures T antigen-initiated DNA replication (*Dpn*I-resistant band) relative to unreplicated, transfected origin DNA (*Dpn*I-sensitive band) (Kwun et al., 2009). As previously reported (Kwun et al., 2009), the genomic TAg locus (Fig. 30, lane 3)

expressing all early proteins is markedly more efficient at initiating origin replication than the LT cDNA alone (Fig. 30, lane 7), confirming an accessory role for sT in LT-mediated DNA replication. Unlike virion production, however, coexpression of hVam6p does not significantly change *in vitro* MCV origin replication by TAg (Fig. 30, lane 5) or LT cDNA (Fig. 30, lane 9). Further, the LT.W209A substitution in either TAg or LT cDNA does not appreciably affect origin replication efficiency (Fig. 30, lanes 4, 6, 8 and 10).

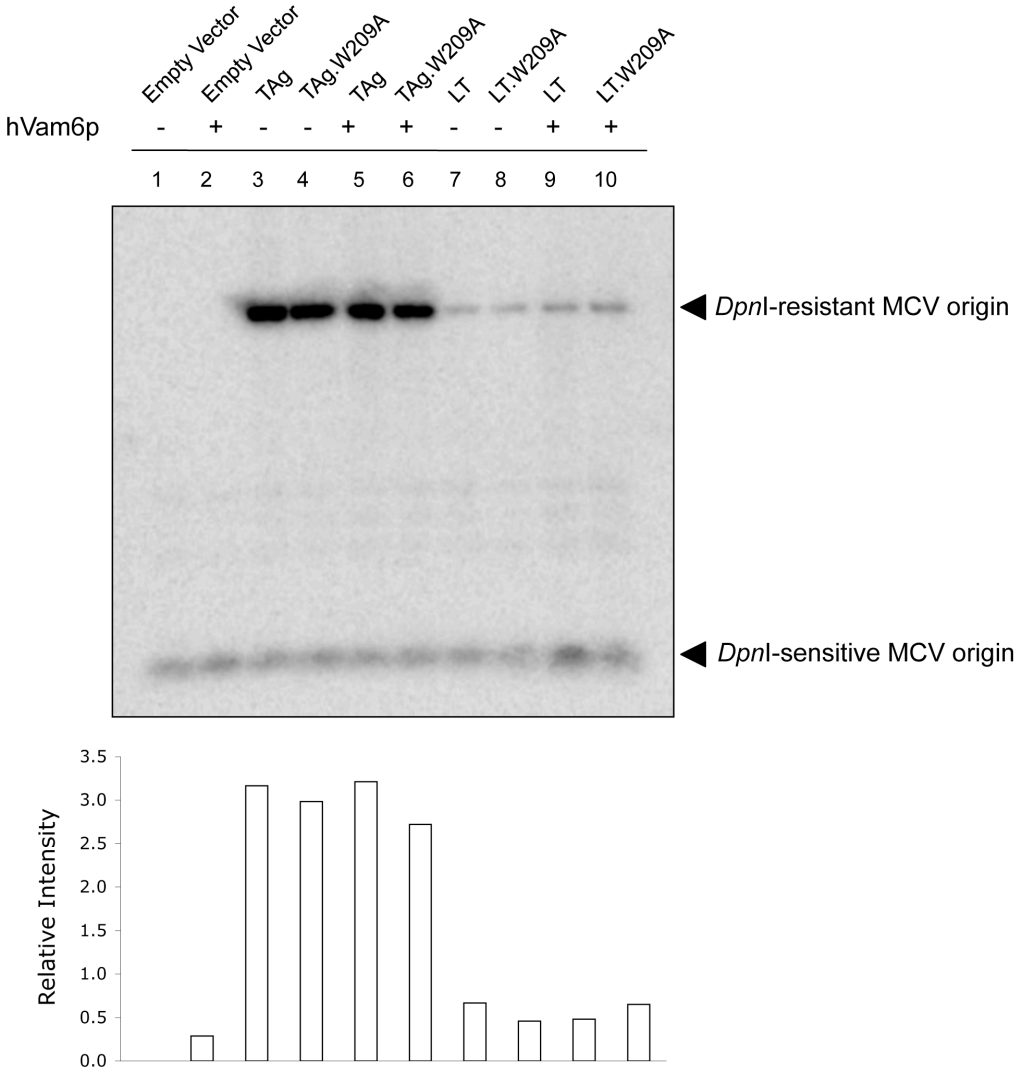


Figure 30. Effect of hVam6p on *In Vitro* MCV Origin Replication. 293 cells were transfected with plasmids containing MCV origin and equal amounts of either wild type of genomic T antigen (TAg), LT cDNA, or the

corresponding constructs containing the hVam6p-binding site mutations (TAg.W209A and LT.W209A). Origin replication was assessed through Southern blotting by comparing the ratio of *Dpn*I-resistant (replicated) to *Dpn*I-sensitive (unreplicated) DNA. For each condition, replication in the absence or presence of simultaneously cotransfected hVam6p expression plasmid was determined. Expression of the genomic TAg containing both sT and LT showed increased replication of the MCV origin compared to the LT cDNA regardless of hVam6p coexpression. Neither mutation of the hVam6p binding site on LT nor coexpression of exogenous hVam6p significantly altered MCV origin replication.

3.3.6 Failure to Achieve Secondary MCV-HF Transmission

We used fraction 4 (Fig. 28A), which contains encapsidated MCV virions to infect a variety of cell types including 293, 293TT, UISO, A549, BJAB, Raji, BSC40 and PBMC purified from whole blood with or without polybrene treatment. No cytopathic effect (CPE) was observed in long-term culture (4 weeks). Immunoblotting and immunofluorescence staining for T antigen and VP1 proteins were not positive for cells exposed to virus (data not shown). We also did not detect viral transcripts by RT-PCR (not shown), suggesting no secondary detectable infection occurred. Co-culture of MCV-HF transfected 293 cells with 293, A549 and UISO cells separated by 0.2/0.45-mm membranes on a transwell plate also failed to demonstrate secondary virus infection. These results may be due in part to the limited amount of infectious virus generated after transfection.

3.4 DISCUSSION

We generated a replicating MCV molecular clone that can be manipulated to assess effects on virus DNA replication and encapsidation. As previously seen in origin-replication studies (Kwun et al., 2009), introduction of the MCV350 strain point mutation into the MCV-HF replication origin abolishes the clone's ability to replicate. We also confirm that MCV sT protein expression together with LT expression is required for optimal MCV replication, a feature of viral genome replication that MCV shares with the human JC polyomavirus (Prins and Frisque, 2001).

Fully-encapsidated MCV virions were isolated in Fraction 4 using the replicating clone at 1.24 g/ml on iodixanol (Optiprep™) gradients. . Evidence for this includes peak isolation of nuclease-protected DNA and ultra-high molecular weight aggregates of VP1 protein specific to this fraction, as well as polyomavirus particles. In the 1970s, hyperosmolar CsCl isopycnic gradients were used to isolate polyomaviruses having higher apparent buoyant densities (e.g., 1.34 g/ml) (Crawford et al., 1962). It is now well-established that hyperosmolar CsCl gradients overestimate the densities of large macromolecular structures, such as viruses, since CsCl gradients dehydrate virions, replacing water with heavy salts, which reduces viability and artifactually increases the buoyant density. JC virus has a buoyant density of 1.20 g/ml on linear sucrose gradients and 1.35 g/ml in CsCl gradients. Similarly, goose hemorrhagic polyomavirus virion has a 1.20 g/ml density in sucrose and an apparent 1.34-1.35 g/ml density with CsCl gradients (Guerin et al., 2000). MCV DNA has been isolated from skin at 1.22 g/ml density using iodixanol gradients, which is in agreement with our findings (Schowalter et al., 2010). The encapsidated MCV we identify in fraction 4 by uranyl acetate negative staining

have the same size (~40 nm) as similarly prepared MCV VLP but are smaller than MCV VLP (55-58 nm) visualized with phosphotungstic acid staining.

MCV, like other polyomaviruses, has a strictly regulated order of viral gene expression that leads to encapsidation of the viral genome. MCV LT and 57kT proteins are expressed early after MCV-HF transfection. Subsequently sT and VP1 (and presumably other virion protein components) are detectably expressed, leading to generation of self-assembling viral particles. Our study reveals an unexpected and interesting complexity for MCV gene expression. MCV LT, sT and 57kT are generated from alternatively-spliced, overlapping genes driven by the same early promoter. MCV-Rep$^-$ expression for LT is initially identical to MCV-HF, indicating that the early promoter is intact, but other early proteins are diminished (57kT) or absent (sT) suggesting the possibility that viral DNA replication regulates early Tag splicing. Late VP1 protein expression also is not detected using the MCV-Rep$^-$ genome indicating that it is likely the early-to-late promoter switch also depends on viral DNA replication. This is similar to mouse polyomvirus, a close relative to MCV in the murine polyomavirus clade, in which late viral gene expression depends on viral DNA replication (Liu and Carmichael, 1993). Well-established late lytic expression and packaging cascades for other viruses, such as herpesviruses, are also dependent on active viral DNA replication (Sarid et al., 1998). Although our results are consistent with DNA replication-dependent viral transcription, MCV-Rep$^-$ is mutated with a single pentamer sequence (PS) 7/8 substitution in the MCV origin and so we cannot exclude the possibility that this point mutation also affects late promoter activity in addition to genome replication.

One key factor regulating MCV-HF virion production is the abundance of sT protein, which our data suggests is in turn regulated in a positive feedback loop during MCV replication. When sT is coexpressed with the transfected MCV-HF genome, there is 5-6 fold

increased virus production. For mouse polyomavirus, sT can signal to AP-1 and PEA3 factors to promote viral replication and late gene expression (Chen et al., 2006). Based on SV40 studies, the most prominent role of sT involves inhibition or retargeting of substrates for the major cellular phosphatase, PP2A (Pallas et al., 1990). We previously identified sT as an important accessory factor for efficient viral DNA replication (Kwun et al., 2009), and our current results indicates that sT expression is limiting in virus production. Southern blotting reveals that most of the replicated viral DNA is fragmented. It is possible that this is due to DNA digestion but it is reasonable to speculate that MCV replication, at least under conditions of plasmid transfection, is inefficient and small increases in successful MCV genome replication may have a large impact on virion replication and encapsidation. Whether the same is true during natural infection will only be determined through development of a successful MCV transmission system.

Our findings on hVam6p inhibition of MCV production are unexpected and, to a degree, paradoxical. MCV LT retains a conserved hVam6p-binding domain that represses virion production. Other polyomaviruses also encode gene sequences that repress viral replication (Yamaguchi and Matsukage, 1989), which might reflect the need for these small DNA viruses to suppress virus replication to sustain a chronic, persistent infection without virion production (e.g., latency). Although the effects on MCV replication are clear, the mechanism by which hVam6p suppresses replication is not. Our origin replication studies in the presence and absence of hVam6p overexpression, together with LT.W209A mutations in LT coding region, showed no direct effect of hVam6p on initiation of viral DNA replication. Additional studies are needed to investigate this effect, however, since Southern blotting reveals amplified viral DNA replication for MCV-hVam6p$^-$ virus compared to MCV-HF,

suggesting hVam6p might be inhibitory to viral DNA replication in the context of the full viral genome.

It is noteworthy that overexpression of this vacuolar sorting protein has a profound antiviral effect on MCV replication. Little is known about hVam6p that can help explain these effects. It possesses citron and clathrin homology domains, the latter being involved the MCV LT binding (Liu et al., 2011) that are important to its functions in the HOPS-CORVET complex as an accessory factor for endosomal fusion (Price et al., 2000). In yeast, the hVam6p homolog also has been reported to act as a guanine nucleotide exchange factor for Gtr1 that contributes to TORC1 activation (Li and Guan, 2009). An isoform of hVam6p, TRAP-1-like protein (TLP) regulates the balance between Smad2 and Smad3 in TGF-β signaling (Felici et al., 2003). We have not found either mTOR activation or TGF-β signaling, however, to be appreciably altered by MCV LT expression (Chapter 2). Even low levels of hVam6p transfection reduce MCV-HF virus production to those occurring the replication-deficient clone. For the MCV-hVam6p$^-$ virus, hVam6p inhibits replication in a dose-dependent manner, suggesting that either the W209A substitution incompletely disables the hVam6p-binding site so that higher concentrations of hVam6p can still act on the LT site or that hVam6p also acts in other steps of MCV replication than those depending on LT. If hVam6p plays a role in inhibiting egress of MCV, this might represent a novel component of innate immunity. Tetherin, for example, is a recently-discovered innate immune component that prevents enveloped viral budding from cells (Kaletsky et al., 2009). Detailed analysis of hVam6p's role in antiviral responses is beyond our current study but development of a replicating MCV clone that can be genetically manipulated provides a critical reagent for use in these follow-on investigations.

Secondary MCV transmission was not detected in our study suggesting that MCV may have a tissue tropism that is not easily modeled in undifferentiated tissue culture. In this way, MCV resembles other small DNA viruses such as JCV, as well as other human tumor viruses including human papillomaviruses (HPV), hepatitis B and C viruses and KSHV. Poor MCV transmissibility may in part be due to the low virus yield generated by transfection and, even under optimal conditions, we were able to achieve viral genome replication that only could be detected after prolonged exposure on Southern blotting. Cloning of a replication-competent MCV genome, however, provides a key tool for testing conditions to optimize virus yield that favor laboratory MCV transmission.

4.0 GENERAL DISCUSSION

4.1 SUMMARY

In search of novel cellular interactors binding to MCV T antigen unique region, we identified hVam6p as a binding partner by tandem-affinity purification. hVam6p is a cytoplasmic protein that promotes lysosome clustering and fusion *in vivo* through citron homology (CNH) and clathrin heavy chain repeat (CLH) domains (Caplan et al., 2001). Co-immunoprecipitation assays revealed that hVam6p is an authentic binding partner for LT *in vivo*. Indirect immunofluorescence showed that both wild-type LT and tumor-derived LTs co-localize with hVam6p. Interestingly, hVam6p is re-localized to the nucleus by LT that harbors nuclear localization signal. This finding was substantiated by confocal microscopy performed in MCV positive MCC cell lines showing that a portion of endogenous hVam6p is present in a diffuse pattern within the nuclear compartment while another portion of hVam6p demonstrates prominent perinuclear localization.

Using a GST pull-down assay, the hVam6p-binding site on LT was mapped to a unique region adjacent to the retinoblastoma protein (pRB)-binding motif. However, hVam6p neither interacts with pRB directly, nor is bridged by LT to pRB. Similar results were found for the pRB family members, p107 and p130 (data not shown). CLH domain in hVam6p is critical for

binding to LT.

hVam6p and its isoform, TRAP-1-like protein (TLP), have been reported to have at least two non-vesicular functions, modulation of TGF-β signaling (Felici et al., 2003) and guanine nucleotide exchange factor (GEF) activity in TORC1 activation (Li and Guan, 2009). We assessed the effect of LT-hVam6p interaction in these two pathways, but no identifiable effect was seen.

A third function described for hVam6p overexpression is promotion of lysosome clustering (Caplan et al., 2001). We examined the effect of LT coexpression on the localization of a lysosome marker, LAMP1 protein. We find that hVam6p-induced clustering is abolished by expression of wild-type LT, but not by the hVam6p binding mutant LT.W209A. To determine whether nuclear sequestration of hVam6p is required for inhibition of lysosome clustering, hVam6p was co-expressed with MCV350 LT, which lacks a nuclear localization signal but still interacts with hVam6p. Clustering was present with MCV350 LT expression suggesting that nuclear sequestration is required to antagonize lysosome trafficking.

We next engineered the W209A substitution into a replication-competent MCV molecular clone to assay the role of LT binding to hVam6p in virion production. Using nuclease-protection quantitative real-time PCR, we show that mutation of the hVam6p-binding site leads to a significant increase in encapsidated virion production compared to the wild-type virus, which was confirmed by Southern blotting. Unexpectedly, hVam6p overexpression potently inhibits nuclease-resistant DNA production from wild-type virus to levels comparable to the nonpermissive replication-deficient virus. The hVam6p-binding mutant virus is relatively resistant to the effects of hVam6p coexpression but also decreases in a dose-dependent manner. Unlike virion production, however, coexpression of hVam6p does not significantly change *in*

vitro MCV origin replication by T antigen. In addition, the LT.W209A substitution does not appreciably affect origin replication efficiency, suggesting that hVam6p might be inhibitory to viral DNA replication in the context of the full viral genome. Collectively, we have revealed a previously unrecognized role for hVam6p in viral replication and hVam6p interaction with MCV LT diminishes MCV replication.

4.2 DISCUSSION

Our results demonstrate that MCV LT prevents hVam6p-induced lysosome fusion and clustering by a nuclear sequestration mechanism. There are two possible biological consequences of this effect. First, since lysosomes are the terminal degradation compartments for endocytic pathways, it could be speculated that internalized MCV potentially has evolved a strategy to avoid the subsequent degradation. Support of this notion comes from a previous study showing that the K1 capsule modulates trafficking of E. coli-containing vacuoles and enhances intracellular bacterial survival by preventing lysosome fusion (Kim et al., 2003). Additionally, it was shown that a Beclin1-binding autophagic tumor suppressor, UVRAG (UV radiation resistance-associated gene) protein, interacts with the class C Vps complex to stimulate endosome fusion, resulting in rapid endocytic trafficking and degradation of cargo molecules in lysosomes (Liang et al., 2008). An alternative explanation is that MCV might hijack the endocytic pathway to facilitate viral replication and virus release. Interestingly, the

vacuolar protein sorting (VPS) pathway has been suggested to play an important role in the release of Marburg virus and Ebola virus (Kolesnikova et al., 2009; Silvestri et al., 2007). It was revealed that the inhibition of the VPS pathway by expression of a dominant-negative form of Vps4 inhibited the release of Marburg virus filamentous particles (Kolesnikova et al., 2009). Furthermore, inhibition of Vps4 gene expression protected mice from lethal Ebola virus infection (Silvestri et al., 2007). To a certain extent, these findings are consistent with our results illustrating that when hVam6p is inhibited by LT interaction, the virion production is decreased, implicating a critical role of hVam6p in MCV lifecycle. Our discovery, however, raises the question, "why does MCV preferentially reduces virus yield by interacting with a component of the VPS pathway?" A possible explanation is that, as a common feature of human tumor viruses, MCV persists as a latent infection as an immune evasion strategy to escape from the recognition of host cells (Moore and Chang, 2010).

4.3 FUTURE DIRECTIONS

Our study suggests that MCV antagnizes the cellular lysosomal machinery and that LT-hVam6p interaction modulates viral replication. However, little is known about hVam6p and its functions in MCC cells. Our future work will focus on the following questions: (1) What is the exact role of hVam6p in anti-viral response during MCV infection? To address this issue, knockdown of cellular hVam6p is necessary to assess its effect on MCV virion production as

well as MCC cell growth. To examine whether hVam6p plays a role in MCV turnover in the lysosomes, further experiments need to be performed to compare the degradation of wild-type MCV with the hVam6p-binding mutant virus. (2) Do other viruses counteract hVam6p as well? Direct evidence would come from protein binding assays of hVam6p with other polyomavirus T antigens. It is worthwhile to investigate whether or not inhibition of hVam6p is a conserved function among viruses. (3) What are the relationships between LT-hVam6p and LT-pRB interactions? Further experiments to determine whether pRB and hVam6p competes with each other in binding to LT are currently underway, which help to better understand whether LT-hVam6p interaction affects cell cycle progression. (4) What are the hVam6p functions in the nucleus? Whether relocalization of hVam6p causes a gain-of-function in the nucleus is unknown. One could further study the effect by engineering hVam6p with a nuclear localization signal (NLS) sequence, followed by microarray analysis to reveal previously unrecognized functions. (5) What does the localization of hVam6p contribute to MCC tumorigenesis? In MCV positive MCC cells, a perinuclear body of hVam6p colocalizes with CK20, a lower molecular weight marker for MCC. It would be interesting to investigate whether this localization reflects any mechanisms underlying carcinogenesis of MCC. Further examinations of hVam6p structure by electron microscopy will be helpful to fully understand its function. Collectively, understanding hVam6p's roles will provide significant insights into virus lifecycle, tumorigenesis and host anti-viral response.

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