Evaluating the role of ACTL6A and ACTL6B in the Pathogenesis of Polyomavirus driven Merkel Cell Carcinoma

Sunil Lala

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Abstract

Merkel Cell Carcinoma (MCC) is a rare (0.2 - 0.45 cases per 100,000) but extremely aggressive form of dermal neoplasm with a very poor prognosis. Approximately 53% MCCs occur in the head and neck, while 35% occur in the extremities. MCC is highly metastatic - about 75% to 83% patients develop metastases. The carcinoma commonly spreads to the lymph nodes first, and eventually metastasizes to the brain, bones, liver or lungs. The nonspecific characteristics of MCC make exact diagnosis extremely difficult. Most diagnoses occur only in very late stages, upon performance of a biopsy.

Merkel Cells are thought to be involved in mechanoreception and sensory perception. The exact origin of Merkel Cells is still controversial and unknown. It is thought that it might have an epidermal stem cell origin or a neural crest origin. Similarly, while it is known that old age, long-term exposure to sun, as well as a weak immune system greatly increase the risk of MCC, the exact causes of this neoplasm are unknown. The Merkel Cell Polyomavirus (MCPyV) is found in a significantly large percentage of MCCs. However, since this virus is relatively common and MCC extremely rare, the exact role of the virus in the pathogenesis of MCC is still unknown.

Recent research has identified possible roles of various proteins and their interaction with MCPyV in the transformation of Merkel Cells. One such protein – Actin like 6A (ACTL6A) – is known to bestow stem cell like properties to various cells and has a role in many cancers. The goal of my research is to focus on this protein, as well on its highly related but distinct homolog, Actin like 6B (ACTL6B), and evaluate their role in MCC. The research specifically looks at the levels of ACTL6A and ACTL6B in MCC and compares cell viability and apoptosis levels between ACTL6A positive versus ACTL6A knockout MCC cells.
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Chapter I

Introduction

Merkel Cell Carcinoma Background

Merkel Cell Carcinoma (MCC) is a malignancy of the skin that is very rare but highly aggressive. Prior research suggested that the Merkel Cell Polyomavirus was involved in about 80% of all MCCs, but more recent research using improved detection techniques shows that the prevalence might be much higher (Rodig et al, 2012). It is also known that the virus-negative form of MCC is prevalent in areas that have high sun – and therefore high UV – exposure. Research has shown that unlike virus-positive MCC which had very low mutation rates, virus-negative MCC has a large number of mutations associated with UV induced gene damage (Wong et al, 2015).

The Merkel Cell Polyomavirus is a double-stranded DNA virus and belongs to the *Polyomaviridae* family. Its genome is made up of 5387 base pairs. It has a non-coding control region (NCCR) that separates the early and late stage coding regions. The NCCR includes the 71 base pair long origin of replication that has an AT rich region, an early enhancer domain, and ten Guanine rich pentanucleotide sequences that act as the binding site for the large T antigen (Figure 1).

The approximately 3000bp long MCPyV T antigen locus belongs to the early coding region, and is transcribed as a single transcript. It is then alternatively spliced into the Small T Antigen (ST), the Large T Antigen (LT), and the 57kD Antigen (57kT). The mapping of these antigens is shown in Figure 2.

All three antigens – LT, ST and 57kT – have the DnaJ region (which binds cellular Heat Shock Protein Hsc70) and the conserved Cr1 region in common. The LT antigen is 816 amino
acids in length and is made up of two exons. This region includes the pRb binding domain, a Nuclear Localization Signal (NLS) domain and the Origin Binding Domain (OBD). It also has viral DNA binding and helicase regions in its carboxy half – these regions recruit numerous proteins that are required for viral replication. The LT antigen has roles in the initiation of viral replication and manipulation of the host cell cycle.

The ST region is 186 amino acids long and also has roles – possibly through its PP2A Aα subunit binding site - in viral replication and cellular transformation. A recently discovered PP4C site may have a role in protecting MCPyV from the immune system, while another recently discovered LBD (LT Binding Domain) stabilizes LT and assists in viral replication.

The 57kT region is a product of linkage between three alternatively spliced exons. It is 432 amino acids in length and while not a whole lot is known about its function, it is homologous with the SV40 17kT which promotes host cell proliferation.

The late coding region produces two structural capsid proteins – VP1 and VP2. The VP3 protein, which is present in other polyomaviruses, is absent in the MCC polyomavirus (Schowalter et al, 2013).

While clonal integration of the MCC polyomavirus genome as well as mutation in LT is necessary for tumorigenesis, LT itself is not sufficient to cause cell transformation. The Small T Antigen has a more direct role in host cell transformation.

Recent studies (Cheng J. and DeCaprio J., manuscript in production) have shown that MCPyV ST binds to the p400 protein complex to activate gene expression (Figure 3). The p400 complex contains EP400 (p400), TRRAP, KAT5 (Tip60) as well as ACTL6A and ACTL6B.

The TRRAP subunit seems to have a role in bringing these subunits together – deletion of this gene causes the sub-complex to collapse (Cheng J. and DeCaprio J., manuscript in production). The interaction between these subunits is shown in Figure 4.
However, the exact nature of the role of some of the other proteins that co-precipitate with MCPyV ST is unknown. Specifically, the role of ACTL6A and ACTL6B – two subunits of the BAF protein complex - could be important as these proteins play a part in chromatin remodeling. Note that ACTL6A and ACTL6B are also sometimes referred to as BAF53A and BAF53B. For the rest of this thesis, I will use the ACTL6A/ACTL6B nomenclature.

It is known that ACTL6A and ACTL6B are involved in the terminal differentiation process of cells with a neural crest origin, and that ACTL6A gets switched with ACTL6B as neuronal cells move from a stem cell like state to a more differentiated state. Similarly, research has shown that ACTL6A forces epidermal cells into a more progenitor like state by modulating the SWI/SNF complex (Bao X et al, 2013) – knocking out ACTL6A results in terminal differentiation, whereas ectopic expression of ACTL6A results in epidermal cells acquiring a progenitor like state. A number of genes that are regulated by ACTL6A were also found to be targets of KLF4, an activator of epidermal differentiation. Other research has shown a role for ACTL6A in promoting both metastasis and epithelial to mesenchymal transition in Hepatocellular Carcinoma. (Xiao S et al, 2016).

These findings confirm the role of ACTL6A not only in cells that are in an undifferentiated, stem cell like state, but also in restoring a stem cell like phenotype in terminally differentiated cells, as well as in promoting some of the hallmarks of cancer such as metastasis and EMT. As mentioned earlier in this section, we also know that ACTL6A interacts – as part of the p400 protein complex – with the ST region of MCV. This indicates a possible pathway involving ACTL6A in switching cells from a terminally differentiated state to a cancerous phenotype in MCPyV+ MCC.
We also know that various proteins in the BAF complex, including ACTL6A – are mutated in different cancers (Cigall Kadoch and Gerald R. Crabtree, 2015). The types and frequencies of mutations in ACTL6A and ACTL6B are shown in Figure 5 and Figure 6.

Our primary research will therefore focus on comparing the levels of ACTL6A and ACTL6B between Polyomavirus positive and both, Polyomavirus negative MCC cells as well as non MCC cells. To identify the transforming role of ACTL6A, we will also compare cell proliferation and cell apoptosis levels between ACTL6A+ and ACTL6A knockdown cell lines.

My hypothesis is that MCPyV+ MCC cells should have a higher expression of ACTL6A compared to MVPyV- MCC cells as well as non-MCC cells. I expect to find an upregulation of ACTL6A and a downregulation of ACTL6B in MCPyV+ MCC cells. If my research results are as expected, I will find higher levels of both, the ACTL6A protein and ACTL6A mRNA in MCPyV+ MCC cells as compared to non-MCC cells and MCPyV- MCC cells. I also expect to see enhanced cell proliferation and decreased apoptosis in MCPyV+ MCC cells with the ACTL6A gene intact as compared to cells in which the ACTL6A gene has been knocked out.

This research will, therefore, provide us with a better understanding of the role of ACTL6A and ACTL6B in MCC. It will also give us insights on additional pathways that might lead to this disease, as well as potential therapies.
Definition of Terms

“Merkel Cells”: Oval receptor cells found in the skin of vertebrates that have synaptic contacts with somatosensory afferents.

“Merkel Cell Carcinoma (MCC)”: A rare type of skin cancer that usually appears as a flesh-colored or bluish-red nodule, often on the face, head or neck. Merkel cell carcinoma is also called neuroendocrine carcinoma of the skin.

“Polyomavirus”: Unenveloped, double stranded DNA viruses consisting of a circular genome of around 5000 base pairs enclosed in an icosahedral shaped viral capsid. The genome typically consists of early and late transcribed genes.

“MCPyV+”: Polyomavirus positive Merkel Cell Carcinoma cells.

“MCPyV-“: Polyomavirus negative Merkel Cell Carcinoma cells.

“LT”: Large T Antigen of the Merkel Cell Polyomavirus.

“ST”: Small T Antigen of the Merkel Cell Polyomavirus.

“Nucleosome”: A basic unit of DNA packaging in eukaryotes. It consists of DNA wrapped around eight histone protein cores.

“Epigenetics”: Study of genetic effects in an organism not coded within the genes themselves but which occur as a result of modifications that do not result in a change in the nucleotide sequence.

“Histone”: Highly alkaline proteins which are the primary components of chromatin. They act as spools around which DNA winds. Histones and their acetylation/methylation states play a role in gene activation and inactivation.

“ESC”: Embryonic Stem Cells.
“EMT”: Epithelial to Mesenchymal Transition, a process in which epithelial cells lose their polarity and adhesion to become migratory and become mesenchymal stem cells. One of the most important hallmarks of cancer that can lead to metastasis.

“SWI/SNF”: Switch/Sucrose Non Fermentable. It is a nucleosome remodeling complex found in both eukaryotes and prokaryotes.

“BAF”: A protein complex whose name stands for Brahma (BRM or SMARCA2) or Brahma Related Gene 1 (BRG1) Associated Factors. A chromatin remodeling complex that is the human analog of SWI/SNF (SWI/SNF-A).

“PBAF”: A protein complex whose name stands for Polybromo Associated BAF. A chromatin remodeling complex that is the human analog of SWI/SNF (SWI/SNF-B).

“nBAF”: Neuronal analog of BAF.

“npBAF”: Neuronal analog of PBAF.


“BAF53A”: A sub-component of the BAF protein complex. Widely expressed. Also known as ACTL6A.

“BAF53B”: A sub-component of the BAF protein complex. Also known as ACTL6B. Understood to be exclusive to neurons (Kuroda et al, 2002).

“Euchromatin”: Non-condensed and actively transcribed portion of the genome, appears as light-colored bands when stained and observed under a microscope.

“Heterochromatin”: Highly condensed and non-transcribed portion of the genome, appears as dark-colored bands when stained and observed under a microscope.

“Histone Acetylation”: Adding of an acetyl group to histone proteins. Normally results in gene activation, and is associated with euchromatin.
“Histone Acetyltransferase”: Enzymes that acetylate conserved lysine amino acids on histone proteins by transferring an acetyl group to them.

“Histone Methylation”: Adding of methyl groups to histone proteins. Can result in gene activation or inactivation depending upon which histone residues are methylated and how many methyl groups are added.

“Histone Methyltransferase”: Enzymes that transfer methyl groups from S-Adenosyl Methionine to Lysine or Arginine residues of H3 or H4 histones.

“EP400”: E1A binding Protein p400. A component of the NuA4 Histone Acetyltransferase complex also known as the p400 or Tip60 complex with roles in acetylation of histones H1, H2, H2A and H4.

“CRISPR-Cas9”: Acronym for Clustered Regularly Interspaced Short Palindromic Repeats-Cas9. A gene editing technique used to knockout, knockdown, or edit genes of interest.

MCPyV Replication and Tumorigenesis

MCPyV is a small, double-stranded, DNA virus that does not encode its own polymerases and requires the host cell machinery for transcription and replication. The T antigens are essential to this process, and they are transcribed immediately upon virus entry into the host cell nucleus. These antigens make the cellular environment hospitable to viral replication by inducing the host cell to enter S-phase. The viral miRNA regulates the level of gene transcription – once there is enough T antigen available, the early stage gene transcription is turned off and focus is turned towards viral replication as well as late stage gene transcription (Whitehouse A. et al, 2014).
The LT antigen plays an important role in the initiation of viral replication. LT oligomerises into hexameric molecules. Two hexamers of LT bind to the origin of replication region in the NCCR region through their OBD domain. The LT helicase region then unwinds the DNA strands and replication proceeds. The bromodomain containing protein Brd4, a member of the BET family, also interacts with LT and recruits various factors that facilitate the tethering of DNA polymerase δ. Brd4 is an epigenetic reader protein and has a role in reading the acetylation state of histones H3 and H4. It is predicted that Brd8, a component of the p400 complex, binds to acetylated Lysine residues on histones (Human p400 Complex and Cancer, Hiroshi Y. Yamada), plays a similar role as Brd4 (DeCaprio et al., manuscript in production).

The Small T antigen ST is needed for enhancing viral replication. While the exact mode is still not completely clear, one of the ways in which ST accomplishes this is by hyperphosphorylating certain regulating proteins which in turn lead to an increase in production of host proteins needed for viral replication. ST also inhibits the degradation of LT by binding to the SCF<sub>Fbw7</sub> ligase through its LT Stabilizing Domain (LSD). ST significantly increases the half-life of LT - from 3-4 hours to 24 hours. While ST is not sufficient for replication, its knockdown leads to inhibition of replication.

It is important to note that the mere presence – or replication – of MCPyV in host cells does not lead to tumorigenesis. In fact, MCPyV infection is quite common in the population and is normally quite harmless.

The following two events – not necessarily in this order - must occur before MCPyV becomes carcinogenic (Figure 7):

1. The viral genome must integrate into the host genome.

2. The LT part of the viral genome must get truncated.
These two changes in the host/viral genome lead to expression of viral proteins that cause cell proliferation as well as cellular transformation. It is interesting to note that this truncation of LT occurs at the carboxy terminus and leads to a loss of domains that are required for viral replication. The truncation site is random and is different in different tumors, but the pRb binding domain of LT is always preserved.

When the non-mutated virus is integrated into the host genome, it has the ability to initiate viral DNA replication of the integrated viral origin of replication. This can result in a collision with a host cell DNA replication fork which results in DNA damage and ultimately in cell death. In addition, the C-terminus of the LT antigen has growth suppression properties (Cheng et al, 2013). Therefore LT truncation – and consequent loss of replicative and growth inhibitory capability – is essential for carcinogenesis.

Since the LT of most polyomaviruses binds to pRb and p53 the Rb and p53 binding domains of LT were considered to be the major contributor of polyomavirus carcinogenesis. MCPyV LT, including both the full-length wild type form and the truncated form mutated in MCC, binds to and inactivates the pRb tumor suppressor protein. However, unlike other polyomaviruses including SV40, MCPyV LT does not interact with p53. Therefore its cell transformation capacity is diminished. Also, neither full length nor truncated LT is sufficient to promote cellular transformation. Therefore, the role of MCPyV ST seems to be equally – if not more – important in carcinogenesis of MCC (Whitehouse A. et al).

In fact, we see that 92% of MCC tumors are positive for ST whereas only 75% are positive for LT. Also, knocking down ST in MCPyV+ cells severely limits their growth and proliferation. Furthermore, expression of ST is sufficient to induce rodent fibroblast transformation, loss of contact inhibition, anchorage-independent and serum independent growth, while expression of LT does not lead to these changes (Shuda et al, 2011).
It therefore seems appropriate to further evaluate the role of ST and its interacting proteins on carcinogenesis.
Recent studies have focused on the role of chromatin remodeling complexes and their interaction with the ST region of the MCC polyomavirus. Specifically, it has been shown (Cheng J. and DeCaprio J., manuscript in production) that ST coimmunoprecipitates with the p400 protein complex, which contains the ACTL6A and ACTL6B proteins. These two proteins are also part of the BAF complexes (Figures 8, 9). Both these complexes have roles in chromatin remodeling.

Figure 8 shows various BAF complexes and their subunits within their specific tissue type. On the surface of various subunits are domains that interact with DNA. The question marks (in the cardiac progenitors) indicate that the subunits and their functionality is unknown.

It should also be noted that various protein complexes – including various subunits of the BAF complex – are involved in terminal differentiation of Embryonic Stem Cells into various functional cell types. Recent evidence also points to emerging consensus that BAF subunits may also be responsible for reversing this terminal differentiation process, i.e., in reverting fully differentiated cells back into their progenitor pluripotent forms (Bao X et al, 2013).

The p400 complex – also known as the NuA4 or the TIP60 complex - is composed of 16 subunits, and its primary role is in controlling chromatin remodeling and transcription through its Histone Acetyltransferase (HAT) activity, as well protein/enzyme activity through acetylation.

Nearly 100 exome sequencing studies recently have shown a very high frequency of mutations in the genes that encode the subunits of ATP dependent chromatin remodeling complexes (Kadoch et al, 2015). Most of these mutations are in the genes that encode various subunits that form the BAF chromatin modeling complex, which is the SWI/SNF analog in
humans. An understanding of the function of these subunits and their possible role in cancer, is therefore critical.

ACTL6A and ACTL6B are two proteins that, as part of the p400 complex, co-precipitate with MCPyV ST in MCC cells. The ACTL6A and ACTL6B genes code for the ACTL6A and ACTL6B proteins which are the 53kDa subunits – BAF53A and BAF53B - of the BAF and PBAF chromatin remodeling complexes, which are the human analogs of SWI/SNF complex. Both ACTL6A and ACTL6B are Actin Related Proteins and have a role in many cellular processes including not only vesicular transport, spindle orientation and nuclear migration, but also chromatin remodeling.

The exact role of ACTL6A/B protein in MCC is unknown. We know from previous studies that mutations in various sub-components of both BAF and PBAF play a role in many cancers (Figure 9).

ACTL6B is a protein that is found exclusively in cells with a neuronal crest origin. The transition from a proliferating neuronal stem cell/progenitor state to a terminally differentiated neuron requires an exchange of the ACTL6A component of the npBAF neuronal progenitor protein complex with the ACTL6B component of the nBAF neuronal protein complex. While this switch does not cause the transition from a neuronal stem cell to a post-mitotic neuron, it is needed for dendritic growth. Since ACTL6A/B are also part of the p400 complex, I propose that their interaction with ST as part of this complex may also affect cell differentiation.

If ACTL6B is indeed expressed exclusively in cells of neural lineage, and an ACTL6A to ACTL6B swap occurs during neuronal development, then the fact that ACTL6A/B coprecipitate with ST in MCPyV+ MCC points to the possibility of a neural origin for MCC cells. More importantly, from the point of view of this research, it points to a possible role for ACTL6A in switching cells from a terminally differentiated state to a carcinogenic stem-cell like state in
MCPyV+ cells. A role for ACTL6A has been found, for example, in switching to a progenitor state in epidermal cells (Bao X et. al, 2013). ACTL6A has also been associated with metastasis and increased epithelial to mesenchymal transition, and leads to poor prognosis in Hepatocellular carcinoma (Xiao S et al, 2016).

We also know that trimethylation of H3K4 is an epigenetic change that results in gene activation. While Cheng J. and DeCaprio J. have shown that ST co-precipitates with proteins shown in Figure 1, and that ST, EP400 and MAX ChIP-seq peaks overlap with H3K4me3 ChIP-seq peaks, we do not yet know the exact nature of the interaction between these proteins and H3K4me3.

Furthermore, Cheng J. and DeCaprio J. have given us significant insight into gene expression profiles of EP400+ and EP400 KO MCPyV+ cells, but similar studies do not exist for gene expression profiles of ACTL6A KO MCPyV+ cells.

Finally, there are no current studies that have looked at MCPyV+ cell proliferation and survival both in the presence and absence of ACTL6A.
Research Goals and Hypothesis

There are two important factors that led me to explore possible roles for ACTL6A and ACTL6B in MCC.

First, we know that these two proteins are involved in the process of neuronal cell differentiation. As multipotent neuronal stem cells differentiate into specialized neurons, the ACTL6A protein in the npBAF complex gets switched with ACTL6B in the nBAF complex. Since a neuronal origin is believed to be one possible scenario for MCC, it is reasonable to hypothesize that the process that leads to the conversion of normal cells into a stem cell like, cancerous phenotype in MCC, might have pathways that are common with neuronal cell differentiation, and therefore might involve ACTL6A and ACTL6B.

Second, it is clear from previous research that ACTL6A and ACTL6B are not only mutated at a high rate of frequency in many different cancers, but that they are also involved in two major hallmarks of cancer - metastasis and epithelial to mesenchymal transition. We also know that ACTL6A and ACTL6B are components of the p400 complex, and MCPyV ST binds to p400 - therefore, it is reasonable to hypothesize that they play an important role in virus-positive MCC as well.

Based on these observations, the primary goal of this research is to evaluate the role of ACTL6A and ACTL6B in the pathogenesis of Merkel Cell Carcinoma.

I hypothesize that:

i) ACTL6A is upregulated in virus-positive MCC cells compared to both virus-negative MCC cells and non-MCC cells, and that the ratio of ACTL6A to ACTL6B in virus-positive MCC cells is higher than that in non-MCC cells.
ii) *ACTL6A* knockout, virus-positive MCC cells have a lower proliferation rate than virus-positive, *ACTL6A*+ MCC cells.

iii) *ACTL6A* knockout, virus-positive MCC cells have a higher rate of apoptosis than virus-positive, *ACTL6A*+ MCC cells.

Primary Objective: Evaluate the role of *ACTL6A* and *ACTL6B* in MCC.

Specific Aim 1: Show upregulation of *ACTL6A* in MCPyV+ MCC cell lines and compare the difference in *ACTL6A*/*ACTL6B* expression between MCPyV+ MCC and MCPyV- MCC as well as non-MCC cell lines.

Methods: Extract total protein and total mRNA from MCPyV+ and MCPyV- MCC cells and compare *ACTL6A* and *ACTL6B* cDNA levels as well as *ACTL6A* and *ACTL6B* protein levels in these cell lines against that in Human Foreskin Fibroblasts, a non-MCC cell line.

Specific Aim 2: Compare apoptosis and cell proliferation levels between *ACTL6A*+ and *ACTL6A* KO MCPyV+ MCC cell lines.

Methods: Knockdown *ACTL6A* in virus-positive MCC cell lines using CRISPR-Cas9, measure apoptosis and cell proliferation levels and compare them with *ACTL6A*+ MCC cell lines.

Our research will therefore specifically evaluate the carcinogenic role of *ACTL6A* in MCPyV+ cells by:

i) Comparing the *ACTL6A*/*ACTL6B* ratio in MCPyV+ and MVPyV- MCC cells.

ii) Comparing the total gene expression between *ACTL6A*+ and *ACTL6A* KO MCPyV+ and MCPyV- MCC cells.
iii) Comparing cell proliferation levels – through cell viability studies - between $ACTL6A^+$ and $ACTL6A$ KO MCPyV+ MCC cells.

iv) Comparing apoptosis levels between $ACTL6A^+$ and $ACTL6A$ KO MCPyV+ MCC cells.
Chapter II
Materials and Methods

Acknowledgments

All experiments listed under this section were performed by me under the guidance of Dr. Jingwei Chen. In addition, for some of my experiments, I obtained starting material from my colleagues in the DeCaprio lab, as listed below:

RT-qPCR (Table 3 and 4): cDNA for HFFs, WaGa, MS-1 and UISO cell lines were obtained from Esther Donglim Park.

Western Blot (Figures 11 and 12): Protein extracts for HFFs, WaGa, MS-1 and UISO cell lines were obtained from Esther Donglim Park.

Western Blot (Figures 13 and 14): Protein extracts for HFFs, HFFs with an empty vector knocked in, HFFs with early regions of LT and ST knocked in, as well as for MKL-1 cells with either both LT and ST regions inhibited by shRNA or with just the ST region inhibited by shRNA were obtained from Reety Arora.

CRISPR-Cas9 Knockout: Pre-digested BsmBl1 Lentivirus plasmid was obtained from Roxana Tarnita.
Cell Lines

A total of 8 human cell lines were used in this study – wild type Human Foreskin Fibroblasts (HFF), polyomavirus positive MCC cell lines MKL-1, PeTa, MS-1, and WaGa and polyomavirus negative MCC cell lines MCC-13, MCC-26 and UISO. Each cell line was split into two parts, one used for total protein extraction and the other for RNA extraction. All samples were frozen at -80°C.

Protein Extraction

Frozen cell lines were thawed. A lysis master buffer was created containing 500μL RIPA buffer, 5μL (100x) Protease inhibitor and 5μL (100x) Phosphatase inhibitor. To each sample, we added 50μL of this lysis buffer. The samples were kept on ice for 15 minutes to start the lysis process. Each sample was centrifuged at 21,000 rpm for 5 minutes. The pellet was discarded and the supernatant containing total protein for each sample was extracted and frozen at -20°C.

Protein Concentration

Protein concentration was measured for each sample on using the Bradford assay. The following measurements were made:

PeTa: 0.8081 mg/ml
MCC-13: 1.6302mg/ml
MCC-26: 0.7321 mg/ml

RNA Extraction

A lysis buffer consisting of 350μL RLT and 3.5μL β-Me was added to each thawed cell sample. The samples were vortexed for 30 seconds. The homogenized lysate was transferred to a
gDNA eliminator column placed in a 2ml collection tube. This was centrifuged for 30 seconds at 10000g. The column (with the DNA) was discarded and 350μL of 70% ethanol was added to the flow through. The sample was then transferred to an RNeasy spin column placed in a 2ml collection tube. To each RNeasy mini-spin column, 700μL of RW1 buffer was added and centrifuged for 15 seconds at 10000g. The flow through was discarded – the RNA sticks to the tube. 500μL of RPE buffer was then added to the RNeasy spin column and centrifuged for 2 minutes at 10000g. This was further dried by placing the RNeasy column in a new 2ml collection tube and centrifuging for 1 minute. The RNeasy column was now placed in a 1.5ml collection tube, 30μL-50μL RNase free water was added and the tubes were centrifuged for 1 minute at 10000g.

The concentration of the RNA samples for each cell line were measured and noted as follows:

- MKL-1: 25.7 ng/μL
- PeTa: 20.2 ng/μL
- MCC-13: 209.8 ng/μL
- MCC-26: 49.9 ng/μL

The samples were frozen at -80°C.

Western Blotting

Detection of ACTL6A and ACTL6B was performed using Western Blotting with a Criterion TGX 4-20% Precast Gel. SeeBlue Plus2 Pre-stained protein standard was used as the ladder. A voltage of 150V for 1 hour was used for protein migration. Protein transfer was performed to a nitrocellulose membrane using a voltage of 100V for 30 minutes.
After protein transfer, the nitrocellulose membrane was blocked for 1 hour using a 3% TBST buffer consisting of 1X TBST and Milk powder. Rabbit monoclonal primary antibodies in a 1:5000 dilution ratio with the TBST buffer were used for detection of ACTL6A and ACTL6B. Mouse monoclonal primary antibody in a 1:30,000 dilution ratio with the TBST buffer was used to detect the Vinculin control protein. All secondary antibodies were used in a 1/5,000 dilution ratio. The incubation period for all primary and secondary antibodies was 1 hour. There were three washes performed after incubations with primary and secondary washes, each for 10 minutes with 1X TBST.

After applying antibodies, the nitrocellulose membrane was placed for 5 minutes in a transparent folder, in 3ml Millipore Immobilon Western Chemiluminescent HRP Substrate for chemiluminescent detection. The membrane was then developed using GeneSnap from SynGene.

The protein loading volumes for each blot were based on protein concentrations measured, and are listed below.

i) Western Blot, Figure 10: Protein concentrations and Loading volumes

PeTa: .8081 mg/ml
MCC-13: 1.6302 mg/ml
MCC-26: 0.7321 mg/ml

Total loaded protein was normalized based on the lowest (MCC-26) concentration. We need 20μl of MCC-26 which gave us a total of 0.7321x20=14.64μg protein. We added 5X SDS buffer=14.64/5=2.92μl SDS buffer.

To get 14.64μg total MCC-13 protein, we needed 14.64/1.6302 = 8.98μl protein and 8.98/5=1.8 μl of SDS buffer.

To get 14.64μg total PeTa protein, we needed 14.64/0.8081=18.10μl protein and 18.10/5=3.62μl of SDS buffer.
ii) Western Blot, Figures 11 and 12: Protein concentrations and Loading volumes

Protein extracts for HFF, MS-1, WaGa and UIISO were obtained from Esther Donglim Park. These proteins were pre-normalized to 3.67 mg/ml. Fresh proteins were then extracted from MKL-1 cell lysates. Their concentration was measured at 5.9240 mg/ml.

We took 20μL each of HFF, MS-1, WaGa and UIISO proteins. Therefore, the amount of loaded MKL-1 protein was 20x3.67/5.9240=12.40μL.

iii) Western Blot, Figure 13: Protein concentrations and Loading volumes

a) MKL-1 and ACTL6A KO MKL-1: Protein concentration for total protein extract from unmodified MKL-1 cells was measured at 8.9392 mg/ml. Protein concentration for ACTL6A KO MKL-1 cells through CRISPR-Cas9 using the 3B single guide RNA was measured at 2.0042 mg/ml.

We took 20μL of total protein extract for ACTL6A KO MKL-1 cells, which amounted to 20 x 2.0042≈40μg of total protein. We added 4μl of loading buffer to this.

To get 40μg total protein, we needed 40/8.9392≈4.5μl of WT MKL-1 cells. We added 0.9μl of loading buffer to this.

b) WT HFFs and HFFs with early regions of LT and ST knocked in: Protein extracts with loading buffer added were obtained from Reety Arora. The following volumes were used to load on to the gel, each with 50μg of total protein:

WT HFF: 5.39μl
HFF Empty Vector: 4.92μl
HFF Early Region: 7.49μl

c) MKL-1 cells and MKL-1 cells with either both the LT and ST regions inhibited, or just the ST region inhibited: Protein extracts with loading buffer added were obtained
from Reety Arora. The following volumes were loaded on to the gel, each with 50μg of total protein:

sh.Scrambled: 8.33 μl
sh.panT: 7.29 μl
sh.ST: 3.44 μl

cDNA Synthesis

RNA samples were normalized for total RNA content based on the lowest concentration RNA sample (PeTa). We took 10μL of PeTa which had 20.2x10=202ng of RNA. To get the same amount of RNA we took 200/25.8≈8μL of MKL-1 RNA, 200/209.8≈1μL of MCC-13 RNA and 200/49.9≈4μL of MCC-26 RNA and added RNase free water to bring the volume of each sample to 10μL.

A 50μL 2X RT master cDNA synthesis mix was prepared for the four samples (MKL-1, PeTa, MCC-13, MCC-26). Ratios determined by Table 1 were used.

10μL of the master mix was pipetted into 5 tubes each. Then, 10μL of RNA from one of the four cell lines was added to each tube. The 5<sup>th</sup> tube was our control and we added RNase free water instead. The tubes were briefly centrifuged to eliminate any air bubbles and then loaded into the thermal cycler. A reverse transcription run was performed using settings listed in Table 2. The synthesized cDNA was stored at -20°C.

In addition, pre-synthesized cDNA for HFF, WaGa, MS-1 and UISO cell lines were obtained from Esther Donglim Park. Each cDNA was synthesized from 2000ng of total RNA.
RT-qPCR Primer selection

A ThermoFisher primer match was performed for \textit{ACTL6A} and \textit{ACTL6B}. A total of 16 primers – eight forward and eight reverse for each \textit{ACTL6A} and \textit{ACTL6B} - were ordered.

After running a qPCR efficiency measurement test, the following primers were chosen for cDNA amplification (all sequences are 5’ to 3’):

\textit{ACTL6A}:

P2: CGGTCCCACCTACTACATAGA
P2R: CTATCCAGTCTTAACCATCC

\textit{ACTL6B}:

P4: TCCCAAGTGCTGGGATTATAG
P4R: CACAAACTCACACACACACACATAC

RT-qPCR cDNA Amplification

\textit{ACTL6A} and \textit{ACTL6B} cDNA was amplified and quantified through RT-qPCR, using the selected primers and three reference gene primers (ACTB, B2M, TBP).

Amplifications were performed using three biological cDNA replicates and either two or three technical replicates for each set of cell line and primer. The exact plate configurations are shown in Table 1 and Table 2.

CRISPR-Cas9 – Gene Knockdown

Oligonucleotide selection, Annealing, and Ligation

The following 8 forward and reverse oligonucleotides were ordered from Invitrogen, 4 each for \textit{ACTL6A} (1 through 4) and \textit{ACTL6B} (5 through 8):
Oligo 1: CACCGGGCGATAAAGGCAAACAAGG
Oligo 1R: AAACCCTTGTTCCTTTATCGCCC
Oligo 2: CACCGTGTTCCGAGGGAGAATATGG
Oligo 2R: AAACCCATATTCTCCCTCGGAACAC
Oligo 3: CACCGGATGGCGATAAAGGCAAACA
Oligo 3R: AAACCTTTTGCCCTTTATCGCCATCC
Oligo 4: CACCGGCGTGTTCCGAGGAGAATA
Oligo 4R: AAACCTATTCTCCCTCGGAACACGCC
Oligo 5: CACCGGGGAGTGTCATTGTCACCG
Oligo 5R: AAACCCGGTGACAATGACACTCCCC
Oligo 6: CACCGCGTCAAGTCCCCTCTGGCAG
Oligo 6R: AAACCTGCCAGAGGGGACTTGACGC
Oligo 7: CACCGGAGCGACTCCGCATCCCTGA
Oligo 7R: AAACCTCAGGGATGCGGAGTCGCTCC
Oligo 8: CACCGGGCTCCTTCTCAGTCCGCG
Oligo 8R: AAACGCGCGGACTGAGAAGGAGCCC

Forward and Reverse oligo pairs were annealed using 1μL of forward oligo, 1μL of reverse olio, 1μL of 10X T4 ligation buffer, and 7μL of nuclease free H₂O. The solution was then annealed in a thermocycler using the following settings - 37°C for 30 minutes, 95°C for 5 minutes, and 5°C/minute ramp down to 25°C.

The oligos were then diluted 1:200 with nuclease free water. 2μL of each annealed oligo pair and 1μL of 10X T4 Ligase buffer were added to a 50ng/μL pre-BsmBl digested LentiCRISPR plasmid obtained from Roxana Tarnita. The mix was topped off to a total of 10μL with nuclease free H₂O. The, 1μL of T4 Ligase was added to get a total of 11μL solution. The
solution was incubated at room temperature for 1 hour and then frozen at -20°C for future transformation into competent cells.
Transformation

Ten samples of 12.5μL each of Stbl3 competent cells were used for initial transformation. To each tube, 2μL of one ligated oligo pair was added and the tubes were labelled. A positive control reference annealed oligo was added to a ninth tube and negative control of nuclease free water was added instead of annealed oligos to the tenth tube. The contents were mixed by light tapping and the mix was kept on ice for 30 minutes.

Heat shock was then applied to the ten tubes by immersing them in 42°C water bath for 30 seconds. The heat shock creates pores in the cell membrane of the competent cells and allows the ligated plasmid to enter. After 30 seconds, the tubes were put back on ice for 2 minutes. 300μL of LB media was then added to each tube. The tubes were then put in a shaker at 37°C for 1 hour. After an hour, contents of each tube was poured onto an LB Agar plate with Ampicillin. Each plate was clearly labelled and put in a 30°C incubator for 48 hours.

Multiple bacterial colonies for annealed oligos #5, 6 and 7 were seen established on each plate after 48 hours. The transformation was then repeated with DH5α competent cells and well established colonies were detected for oligos #1, 2, 3, 4 and 8.

DNA extraction from Transformed cells

Total DNA was extracted from 2-3 randomly selected samples on each plate using the QIAprep Spin Miniprep Kit. Each bacterial sample was pelleted by centrifuging at 21000 rpm for 3 minutes at room temperature. The bacterial cells were resuspended in 250μL Buffer P1 and transferred to a microcentrifuge tube. To initiate lysis, 250μL of Buffer P2 (with LyseBlue reagent at a 1 to 1000 ratio) was added and mixed by inverting 4-6 times. The lysis reaction was allowed to proceed for 5 minutes and then 350μL of Buffer N3 was added to stop the lysis. The tubes were then centrifuged at 13000 rpm for 10 minutes in a microcentrifuge.
800μL of the supernatant was then added to the QIAprep 2.0 spin column by pipetting, and centrifuged at 21000 rpm for 60 seconds. The flow-through was discarded. The spin column was then washed by adding 0.5ml Buffer PB and centrifuging at 21000 rpm for 60 seconds. The flow-through was discarded. The spin column was washed again by adding 0.75ml Buffer PE and centrifuging at 21000 rpm for 60 seconds. The flow through was discarded. The column was centrifuged for an additional 60 seconds at 21000 rpm to remove any residual buffer.

The QIAprep spin column was then placed in a 1.5 ml microcentrifuge tube. DNA was eluted by adding 50μL Buffer EB to the center of the spin column, letting stand for 1 minute and then centrifuging for 1 minute.

DNA samples were then sent for sequencing to confirm transformation.

Transfection, Transduction and Selection

The following AddGene protocol was followed for creating lentivirus particles, except that PEI was used for transfection instead of Fugene or Lipofectamine:

**Day 1:** For each set of *ACTL6A* KO sgRNA plasmid to be transfected, one plate of HEK-293T cells were incubated at 37°C, 5% CO₂ overnight. In addition, one plate was incubated for positive control. We had a total of 5 plates – 4 plasmids and 1 control.

**Day 2:**

a. Performed the transfection in the late afternoon so that the transfection mix was only incubated with the cells for 12-15 hours.

b. In polypropylene microfuge tubes, I made the following cocktail for each transfection:

- 5 μg DNA CRISPR plasmid
- 3.75μg of psPAX2 packaging plasmid
• 1.25μg pVSV.G envelope plasmid

• 30μl serum-free OPTI-MEM

c. Created a master mix of 120μl PEI transfection reagent (30μl per sgRNA) and 1480μl of serum-free OPTI-MEM (370μl per sgRNA). In a polypropylene tube, I added OPTI-MEM first. Pipetted PEI directly into the OPTI-MEM – did not allow PEI to come in contact with the walls of the tube before it was diluted. Mixed by swirling and gently flicking the tube. Incubated for 5 minutes at room temperature.

e. Added 400μL of PEI master mix to each tube from Step b. Pipetted master mix directly into the liquid and not onto the walls of the tube. Mixed by swirling and gently flicking the tube.

f. Incubated for 20-30 minutes at room temperature.

g. Retrieved HEK-293T cells from incubator. The cells were 50-80% confluent and in DMEM that did not contain antibiotics.

h. Without touching the sides of the dish, I gently added DNA:PEI mix dropwise to cells. Swirled to disperse mixture evenly.

i. Incubated cells at 37°C, 5% CO₂ for 12-15 hours.

Day 3:

In the morning, changed the media to remove the transfection reagent. Replaced with 5 mL fresh DMEM + 10% FBS + penicillin/streptomycin. Pipetted the media onto the side of the plate so as not to disturb the transfected cells.

I then incubated the cells at 37°C, 5% CO₂ for 48 hours.

Day 5:

The medium/supernatant was collected from the cell plates into a syringe. This supernatant has the lentivirus particles. This supernatant was then filtered through a 0.45 micron syringe to flush out any debris/dead cells.
MKL-1 cells were then added by inoculation. This mix of MKL-1 cells and lentivirus was then centrifuged at 800rpm for 30 minutes. This results in better transduction efficiency. This is an optional step.

**Day 6:**

For each plate, the media with the lentivirus was removed. Fresh media (10ml) added to each tube and the cells were plated. Puromycin was added in a 1μg/ml ratio to each plate for selection. The cells were then incubated at 37°C, 5% CO₂ for 3 days.

**Day 9:**

By day 9, the selection process is complete. The cell palette was then obtained by centrifuging and protein extraction was performed for Western Blots.

Two separate biological replicates were created using the above protocol.

**Cell Proliferation**

Three samples were chosen for comparing cell proliferation – wild type MKL-1 cells and MKL-1 cells with *ACTL6A* knocked out through transduction with the CRISPR lentivirus using either sgRNA 1A or 3B. These two sgRNAs were confirmed to have successfully knocked out *ACTL6A* in MKL-1 cells (Figures 13, 14).

On Day 0, an equal number of cells (2.1 x 10⁷) were taken for each sample. They were then divided into three biological samples each, and incubated at 37°C, 5% CO₂ for 6 days.

On the 6th day, two separate cell counts were performed for each biological sample using the Invitrogen Countess Automated Cell Counter with Trypan Blue Dye. The counts at Day 6 are listed
Chapter III

Results

ACTL6A and ACTL6B Protein expression

I wanted to check ACTL6A and ACTL6B protein expression in MCPyV+ and MCPyV- cells. To do this, we extracted total protein from whole cell lysates of four MCV+ cell lines (MKL-1, MS-1, WaGa and PeTa) and three MCV- cell lines (MCC-13, MCC-26 and UISO) as described in the “Protein Extraction” section in Materials and Methods. Western Blots were then performed as described in the “Western Blot” section in Materials and Methods. Human Foreskin Fibroblasts (HFFs) were used as an independent primary control, and Vinculin and Actin were used as loading controls for the Western Blot.

As seen in Figure 10, ACTL6A was expressed in both polyomavirus positive (PeTa) and polyomavirus negative MCC cells. An interesting observation was that PeTa cells did not express Vinculin even though they did express Actin. The ACTL6A protein level was also higher in MCPyV+ cells compared to HFF (Figure 11).

ACTL6B protein levels were also higher in MCPyV+ cells as compared to Human Foreskin Fibroblasts whereas we did not detect any ACTL6B protein expression in MCPyV- UISO MCC cell line (Figure 12).

We then checked if higher ACTL6A levels correlated with either the ST or the LT antigen of the MCC polyomavirus. Cell lysates were obtained from Reety Arora for HFFs with the early region of tumor derived MCV knocked in, as well as for MKL-1 cells with either the ST region inhibited or both the LT and ST regions inhibited using pre-designed shRNA. HFF cells with an empty vector knocked in, and MKL-1 cells with a scrambled knockout version of shRNA were used as negative controls.
As seen in Figure 13, there was low expression of ACTL6A in HFFs with early region MCV knock in. This was similar to that in wild type HFFs as well as control. MKL-1 cells with inhibited PanT (both LT and ST) region had diminished expression of ACTL6A as compared to MKL-1 cells with treated with scrambled shRNA, and the expression of ACTL6A further decreased when only ST was inhibited.

A Western blot was then repeated for ACTL6A in biological duplicates of MKL-1 cells with shRNA inhibited PanT or ST regions (Figure 14). As seen in the first blot, this second experiment confirmed that compared to wild type MKL-1 cells, there was a decrease in ACTL6A in MKL-1 cells with both LT and ST regions inhibited and a further decrease in MKL-1 cells with only the ST region inhibited. There was an inexplicable absence of ACTL6B in the control (sh.Scr).

This blot also confirmed successful ACTL6A knockout in the second biological replicate of lentivirus transduced MKL-1 cells by sgRNAs labelled 1A and 3B.

ACTL6A and ACTL6B mRNA amplification

After looking at ACTL6A and ACTL6B protein levels through Western blots, I wanted to confirm my findings by quantifying the ACTL6A and ACTL6B mRNA expression in MCPyV+ and MCPyV- MCC cells. To do this, I extracted total RNA from two MCPyV+ cell lines (MS-1 and WaGa) as well as one MCPyV- cell line (UISO), as described in the “RNA Extraction” section under Materials and Methods. I then synthesized cDNA from the extracted mRNA using RT-qPCR, as described in the RT-qPCR cDNA Amplification section in Materials and Methods. Human Foreskin Fibroblasts were used as the independent primary control and cDNA for these cells was obtained from Esther Donglim Park at the lab. The RT-qPCR amplification settings are listed in Table 2 and the well configuration is shown in Table 3.
To confirm my RT-qPCR results, I then ran RT-qPCR for biological duplicates and triplicates (Table 4) using cDNA for WaGa, MS-1, UISO and HFF cell lines provided by Esther Donglim Park. HFFs were used as the independent primary control. Primers for ACTB and B2M genes were used as reference.

The RT-qPCR analysis showed that there was significant upregulation (p-values << 0.05 as seen in Table 10) of ACTL6A in MCPyV+ MCC cells but not in MCPyV- MCC cells (Tables 5, 6, 7, 10, and Figure 16).

Similarly, we saw between significant increase in levels of ACTL6B mRNA in MCPyV+ MCC cells (p-values << 0.05 as seen in Table 11) as compared to HFFs (Tables 5, 8 and 9, 11, and Figure 17).

The upregulation of both ACTL6A and ACTL6B in MCPyV- MCC cells was insignificant (p >> 0.05, Tables 10, 11, Figures 16, 17).

Apoptosis in ACTL6A KO cells

Having confirmed that both ACTL6A and ACTL6B are amplified in MCPyV+ MKL-1 cells, I then wanted to check if knocking out ACTL6A would result in increased apoptosis in MCPyV+ MCC cells.

To do this, I first knocked out the ACTL6A gene from MKL-1 cells using a CRISPR-Cas9 Lentivirus plasmid, as described in the Materials and Methods chapter. I then performed a Western blot for two proteins that are typically cleaved during apoptosis – PARP and Caspase 3. The size of full length PARP protein is 116kDa. During apoptosis, PARP gets cleaved into a large segment of 89kDa and a smaller segment of approximately 24kDa. Similarly while full length Caspase 3 is 35kDa, during apoptosis it gets cleaved into two segments of 17kDa and 12kDa respectively.
We looked for differences in expression of these two apoptotic proteins in CRISPR

\textit{ACTL6A} KO MKL-1 cells, in HFF cells that had the early regions of ST and LT antigens of MCPyV knocked in, and in MCPyV+ MKL-1 cells with either the ST region or the PanT region inhibited by short hairpin RNA.

As seen in Figure 15, cleaved PARP protein – the full length and the large fragment - was found in both MKL-1 WT cells as well as in MKL-1 \textit{ACTL6A} KO cells, although there seemed to be reduced cleavage in the knockouts. We did not find any cleaved Caspase 3 in either the wild type MKL-1 cells or the \textit{ACTL6A} KO cells. Similarly, no Caspase 3 cleavage was found in any of the HFF cell lines. Surprisingly the sh.PanT and the sh.ST MKL-1 cell lines did not have any cleaved PARP but the sh.Scr MKL-1 cell line did.

I then wanted to see if there was any difference in cell proliferation between \textit{ACTL6A} KO MKL-1 cell and wild type MKL-1 cells. To measure this, we started with an equal number (2.1 x 10^6) cells for wild type MKL-1 cells, MKL-1 cells where \textit{ACTL6A} had been knocked out using sgRNA 1A, and MKL-1 cells where \textit{ACTL6A} had been knocked out using sgRNA 3B. Each sample was then divided into three equal biological samples and incubated at 37°C, 5% CO₂ for 6 days. Cell number measurements were then taken for each sample on the 6th day.

As can be seen from Table 10, there was a significantly large (p << 0.05) reduction in cell proliferation in \textit{ACTL6A} KO MKL-1 cells as compared to wild type MKL-1 cells.
Significance of Results

The primary hypothesis of my research was that ACTL6A is upregulated in MCPyV+ MCC compared to both MCPyV- MCC and non-MCC cells.

By looking at the results from the gene amplification, protein expression, and cell proliferation experiments that I performed, it is clear that my hypothesis was correct and that both ACTL6A and ACTL6B play a role in virus-positive MCC.

As expected, I saw increased ACTL6A protein levels as well as multi-fold increased levels of ACTL6A mRNA in MCPyV+ cells as compared to HFF cells, whereas the fold increase in ACTL6A mRNA for MCPyV- cells was not significant.

My initial hypothesis assumed an increase in ACTL6A expression in MCC cells and a corresponding decrease in ACTL6B. This was based on the assumption that pathogenesis of MCC might follow pathways analogous to neural cell differentiation from neural stem cells. The surprising result was the between 530 to over 97,700 fold increase in ACTL6B mRNA levels in MCPyV+ cell lines as compared to non-cancerous HFFs. This result shows us that both ACTL6A and ACTL6B play an important role in MCPyV+ MCC but that they might function through independent pathways. The increase in ACTL6B mRNA in MCPyV- cells was not significant.

We also saw a significant decrease in cell proliferation (Table 12, Figure 18) in MCPyV+ MCC cells when ACTL6A was knocked out as compared to wild type MCPyV+ cells. Once again, this shows that ACTL6A plays an important role in MCV positive MCC.

There was no effect observed on cell apoptosis - as measured by PARP and Caspase 3 cleavage - by knocking out ACTL6A.
Study Limitations and Future Research Directions

The main hypothesis of my research, that ACTL6A is upregulated in virus-positive MCC was clearly proven through my experiments, by an increase in ACTL6A protein levels, a significant increase in ACTL6A mRNA, and a decrease in cell proliferation upon knocking out ACTL6A.

While this research proves my hypothesis and shows a significant amplification of both ACTL6A and ACTL6B in virus positive MCC, it does not closely examine if this gene amplification is part of a larger amplicon, or look at oncogenes in the neighborhood of ACTL6A and ACTL6B. Future studies should examine genes in the neighborhood of ACTL6A and ACTL6B, and explore if a larger amplicon is involved in MCC.

Also, while the focus of our research was on ACTL6A and ACTL6B, the CRISPR knockout experiments were designed and performed for only ACTL6A, since our original hypothesis assumed an increase in ACTL6A expression in MCC cells coupled with a decrease in ACTL6B expression. This was because we hypothesized that the mechanism of MCC pathogenesis would be analogous to the differentiation of neurons from neuronal stem cell.

The hundreds to thousands fold overexpression of ACTL6B in MCPyV+ MCC cells was an unexpected finding, and it therefore makes sense to conduct further cell apoptosis studies, cell proliferation and viability studies, and cell morphology studies on ACTL6B KO MCPyV+ MCC cell lines.

The study did not see any increase in apoptosis – as measured by cleavage of PARP and Caspase 3 - in ACTL6A knockout MKL-1 cells. However, it might be worthwhile to check for increased apoptosis using other apoptotic markers, and any such future studies should be performed in both ACTL6A and ACTL6B knockout cells.
Prior studies have shown that micro RNAs have a significant effect on regulation of
ACTL6A and the role of micro RNAs should be explored further. In my research, I have
consolidated some preliminary information from previously published papers, and extending
these studies to MCC should be further explored.

The process of creation of miR-9 and miR-124 from their primary micro RNAs in human
cells, and their base pair sequence is depicted in Figure 19.

A previous study (Yoo et al, 2011) has shown that micro RNAs miR-9 and miR-124 are
selectively expressed in post-mitotic neurons, and that they repress ACTL6A by binding to 3’
UTR region on the mRNA. This study also identified the 3’ UTR sites on ACTL6A that bind
miR-9 and miR-124 (Figures 20 and 21). In addition, they found that mutation of these 3’ UTR
sites led to persistent expression of ACTL6A and defective dendritic activity in post-mitotic
neurons, and that overexpression of miR-9 and miR-124 led to reduced proliferation.

Another study (Veija et al) has shown that miR-34a, miR-30a, miR-142-3p and miR-
1539 are underexpressed in MCPyV-MCC. Similarly, studies have shown that miR-9 is
downregulated in lung fibroblasts in Idiopathic Pulmonary Fibrosis (Li et al, 2016) and also that
expression of miR-9 and miR-124 induces the conversion of human fibroblasts to neurons (Yoo
et al, 2011).

Other studies (Conaco C. et al) have also shown that REST – Repressor Element I
Silencing Transcription Factor - represses miR-9 and miR-124 and that expression of REST in
post-mitotic neurons led to de-repression of ACTL6A. The interaction of REST, miR-9, miR-124
and ACTL6A is shown in Figure 22.

Now that we have shown that ACTL6A is upregulated in MCPyV+ MCC cells, we should
explore the following questions about the role of REST and micro-RNAs in MCC:
i) Are miR-9 and miR-124 downregulated in MCPyV+ MCC cells compared to MCPyV- cells and non-MCC cells?

ii) Is REST upregulated in MCPyV+ MCC cells as compared to both MCPyV- MCC cells and non-MCC cells?

iii) Are miR-9 and/or miR-124 binding sites on ACTL6A in MCPyV+ MCC cells mutated?

Finally, our study compared MCC cells with Human Foreskin Fibroblasts, and not with cells that have a neuronal heritage. Since there is still some debate over the exact cell of origin of MCC, it would be worthwhile to compare ACTL6A and ACTL6B expression in MCC cells with that in cells with a neuronal origin, for example, neuronal stem cells.
Conclusion

Based on prior research in the DeCaprio lab, and on subsequent analysis of the role of
ACTL6A and ACTL6B in neuronal development, I hypothesized that a role might exist for
ACTL6A and ACTL6B in the development of Merkel Cell Carcinoma. Specifically, I
hypothesized that ACTL6A is overexpressed in MCPyV+ MCC. Based on my experiments with
both virus-positive and virus negative MCC cells, and with non-MCC cells, this hypothesis was
proven to be correct. The significant increase in expression of ACTL6B was an unexpected result,
and was confirmed in tests on multiple biological samples.

Upon knocking out ACTL6A, I saw a significant decrease in cell proliferation for
MCPyV+ MCC cells, also as predicted by my hypothesis.

From these results, we can conclude that ACTL6A and ACTL6B have a significant role to
play in virus positive Merkel Cell Carcinoma. We also conclude that this role is different from
the roles that these genes play in neural development - different pathways seem to be involved
rather than a simple switch between ACTL6A and ACTL6B that is observed in neuronal
differentiation. Further studies must be undertaken to identify the exact biological pathways
through which they act.

We can also conclude from our research that there does not seem to be as large a role for
these two genes in virus negative MCC cells. The gene expression for both ACTL6A and
ACTL6B in MCPyV- MCC cells was very similar to wild type HFFs.

Furthermore, as seen by the levels of cleaved apoptotic proteins in ACTL6A knockouts,
apoptosis in of MCPyV+ cells does not seem to be directly affected by ACTL6A.
Appendix I

Tables

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for 10μl (μl)</th>
<th>Volume for 45μl Master mix</th>
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<tbody>
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<td>10X RT Buffer</td>
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<td>10.0</td>
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<tr>
<td>25X dNTP Mix (100mM)</td>
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<td>10X RT Random Primers</td>
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<td>10.0</td>
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<td>MultiScribe Reverse Transcriptase</td>
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</tr>
<tr>
<td>RNase Inhibitor</td>
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<td>TOTAL</td>
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Table 1: cDNA Master Mix

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<tr>
<th></th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
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<td>4</td>
</tr>
<tr>
<td>Time (min)</td>
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Table 2: RT-qPCR settings
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<th>WaGa P2</th>
<th>WaGa P4</th>
<th>WaGa 36B4</th>
<th>MS-1 P2</th>
<th>MS-1 P4</th>
<th>MS-1 36B4</th>
<th>UISO P2</th>
<th>UISO P4</th>
<th>UISO 36B4</th>
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<td>HFF 36B4</td>
<td>WaGa P2</td>
<td>WaGa P4</td>
<td>WaGa 36B4</td>
<td>MS-1 P2</td>
<td>MS-1 P4</td>
<td>MS-1 36B4</td>
<td>UISO P2</td>
<td>UISO P4</td>
<td>UISO 36B4</td>
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<td>WaGa P2</td>
<td>WaGa P4</td>
<td>WaGa 36B4</td>
<td>MS-1 P2</td>
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<td>UISO P4</td>
<td>UISO 36B4</td>
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</table>

**Table 3: ACTL6A and ACTL6B Amplification through RT-qPCR**

P2: Primer for ACTL6A amplification; P4: Primer for ACTL6B amplification;

36B4: Reference Gene
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<thead>
<tr>
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<th>HFF-1</th>
<th>HFF-1</th>
<th>HFF-1</th>
<th>WaGa-1</th>
<th>WaGa-1</th>
<th>WaGa-1</th>
<th>MS1-1</th>
<th>MS11-1</th>
<th>MS1-1</th>
<th>UISO-1</th>
<th>UISO-1</th>
<th>UISO-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technical Replica 1</td>
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<td>HFF-1</td>
<td>HFF-1</td>
<td>WaGa-1</td>
<td>WaGa-1</td>
<td>WaGa-1</td>
<td>MS1-1</td>
<td>MS11-1</td>
<td>MS1-1</td>
<td>UISO-1</td>
<td>UISO-1</td>
<td>UISO-1</td>
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<td>HFF-1</td>
<td>HFF-1</td>
<td>WaGa-1</td>
<td>WaGa-1</td>
<td>WaGa-1</td>
<td>MS1-1</td>
<td>MS11-1</td>
<td>MS1-1</td>
<td>UISO-1</td>
<td>UISO-1</td>
<td>UISO-1</td>
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<tr>
<td>Biological Replica 2</td>
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<td>HFF-2</td>
<td>HFF-2</td>
<td>WaGa-2</td>
<td>WaGa-2</td>
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Table 4: *ACTL6A* Amplification through RT-qPCR: Biological Replicas

Two Biological Replicas and Three Technical Replicas

P2: Primer for ACTL6A amplification; P4: Primer for ACTL6B amplification; ACTB, B2M: Reference Genes

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<thead>
<tr>
<th>Ct Values</th>
<th>ΔCt Values</th>
<th>ΔΔCt Values</th>
<th>Fold Change</th>
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Table 5: Biological sample I - *ACTL6A, ACTL6B* mRNA fold change: RT-qPCR cDNA amplification results for MCPyV+ (WaGa, MS-1) and MCPyV- (UISO) cell lines as compared to HFF; Ct values are average of technical replicates; Fold Change=2^ΔΔCt
Table 6: Biological sample II - *ACTL6A* cDNA fold change. RT-qPCR cDNA amplification results for MCPyV+ (WaGa, MS-1) and MCPyV- (UISO) cell lines as compared to HFF; Ct values are average of technical replicates; Fold Change=$2^{\Delta\Delta Ct}$

<table>
<thead>
<tr>
<th></th>
<th>Ct</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>Fold Change</th>
</tr>
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<tr>
<td></td>
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<td>REF</td>
<td>6A</td>
<td>REF</td>
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<tr>
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Table 7: Biological sample III - *ACTL6A* cDNA fold change. RT-qPCR cDNA amplification results for MCPyV+ (WaGa, MS-1) and MCPyV- (UISO) cell lines as compared to HFF; Ct values are average of technical replicates; Fold Change=$2^{\Delta\Delta Ct}$

<table>
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<tr>
<th></th>
<th>Ct</th>
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<th>ΔΔCt</th>
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Table 8: Biological sample II - *ACTL6B* cDNA fold change. RT-qPCR cDNA amplification results for MCPyV+ (WaGa, MS-1) and MCPyV- (UISO) cell lines as compared to HFF; Ct values are average of technical replicates; Fold Change=$2^{-\Delta \Delta Ct}$

<table>
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Table 9: Biological sample III - *ACTL6B* cDNA fold change. RT-qPCR cDNA amplification results for MCPyV+ (WaGa, MS-1) and MCPyV- (UISO) cell lines as compared to HFF; Ct values are average of technical replicates; Fold Change=$2^{-\Delta \Delta Ct}$

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<tr>
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<td>UISO</td>
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| p-values | 0.0060 | 0.002 | 0.412 |

Table 10: ACTL6A RT-qPCR Ct and p-values

<table>
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<tr>
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<th>MS-1</th>
<th>UISO</th>
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| p-values: | 0.00017 | 5.08 x 10⁻⁵ | 0.52 |

Table 11: ACTL6B RT-qPCR Ct and p-values
<table>
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<tr>
<th>Biological Replicate Number</th>
<th>$ACTL6A$ KO – 1A Cell count/ml</th>
<th>$ACTL6A$ KO – 3B Cell count/ml</th>
<th>MKL-1 WT Cell count/ml</th>
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</thead>
<tbody>
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<td>$9.0 \times 10^4$</td>
<td>$1.5 \times 10^6$</td>
</tr>
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<td>$8.0 \times 10^4$</td>
<td>$4.0 \times 10^4$</td>
<td>$1.0 \times 10^6$</td>
</tr>
<tr>
<td>4</td>
<td>$2.0 \times 10^4$</td>
<td>$3.0 \times 10^4$</td>
<td>$1.1 \times 10^6$</td>
</tr>
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<td>$4.0 \times 10^4$</td>
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<td>6</td>
<td>$8.0 \times 10^4$</td>
<td>$3.0 \times 10^4$</td>
<td>$2.4 \times 10^6$</td>
</tr>
</tbody>
</table>

**Table 12: Cell Proliferation and p-values:** Cell count at Day 6 for wild type MKL-1 and $ACTL6A$ KO MKL-1 cells; p-value (1A versus WT) = $6.6 \times 10^{-5}$; p-value (3B versus WT) = $4.87 \times 10^{-5}$
Appendix II

Figures

Figure 1: MCC Polyomavirus structure

Figure 2: MCPyV Antigen mapping
Figure 3: Protein co-precipitation with ST

Figure 4: Role of p400 in protein complexes that co-precipitate with ST

Source: Cheng J and DeCaprio J, manuscript in production
Figure 5: Cross-cancer alteration summary for ACTL6A (147 studies / 1 gene)

Source: http://www.cbioportal.org/

Figure 6: Cross-cancer alteration summary for ACTL6B (147 studies / 1 gene)

Source: http://www.cbioportal.org/
Figure 7: MCPyV integration and cell transformation


Figure 8: BAF subcomplexes in various tissues

Figure 9: BAF and PBAF subunit mutations in cancers


Figure 10: ACTL6A expression in MCC cell lines
Figure 11: ACTL6A expression in MCPyV+ cells

Figure 12: ACTL6B expression in MCPyV+ cells
Figure 13: ACTL6A expression with ST, LT: Change in ACTL6A in HFF cells with early regions of MCV LT and ST knocked in, in MKL-1 cells with the ST region inactivated by short hairpin RNA

Figure 14: ACTL6A Expression with T Antigen inhibition: MKL-1 Biological Replica 2: Wild Type MKL-1; MKL-1 sh.Scr: MKL-1 cells with scrambled shRNA; MKL-1 sh.PanT: MKL-1 cells with both ST and LT regions inhibited by shRNA; MKL-1 sh.ST: MKL-1 cells with only ST region inhibited by shRNA
Figure 15: Apoptotic Protein expression

A: Full length PARP (116 kDa); B: Cleaved PARP (89kDa); C: Full length Caspase 3 (35kDa)

Figure 16: *ACTL6A* mRNA Fold Change in MCC cells compared to HFF

p-value (WaGa versus HFF) = 0.006; p-value (MS-1 versus HFF) = 0.002; p-value (UISO versus HFF) = 0.412
Figure 17: *ACTL6B* mRNA Fold Change in MCC cells compared to HFF

p-value (WaGa versus HFF) = 0.00017; p-value (MS-1 versus HFF) = $5.08 \times 10^{-5}$; p-value

(UISO versus HFF) = 0.52

Figure 18: Cell Proliferation and p-values: Cell count/ml of MKL-WT and *ACTL6A* KO MKL

cells measured on Day 6; p-value (1A versus WT) = $6.60 \times 10^{-6}$; p-value (3B versus WT) = $4.87 \times 10^{-6}$
Figure 19: miR-9 and miR-124 synthesis and structure

![Diagram of miR-9 and miR-124 synthesis and structure]

Figure 20: miR-9 and miR-124 binding sites

AGCTACAATCAGGGAATTGCACTCCAGGCTGGACAGAGGACATTTCTCTCCAAAAAATT
TTTAAGGCAATTTCTAGTAAAGAGAAGGCTAATAAATATTAAAATTTTTCCCTTATACGGGTACCTTTCT
AACAGATGGATTCCAAAGCAAGAATATGAAGAAGGAGAGGAAAGAGTGTTGAAAGAGAAATGCCCTTG
AGAAGAGAGTCCCCAGCCTTCTACCTICCTTTGTCACCTTACGTTTCTAGCTTTTAGTCTACTCAAGAAA
AGAATGACCATCTTTGTAGAATGTTTATACATTTTTGATATTTCAATTTCCACTAAATTATTAAAG
CTTTAAGGGCTCTATAATATAGTTTGTGTTTCTCTTTCGCATATATTCATTTCTATTTACTCAAGCATTCTA
TTTTTTGATAAATGCTATTTTCTCTAAATATTATTGTCTTTCAAGTAAAGATGTTCTTCCAACCTCTGTGTTAG
TGTATTAATATTACCTAGGGATTGGTAGAAGACTGCTTTTTATTGACTAGTAAAGTTACTGCTCTAGCTTTT
ACCTAGGCTTACGAATTTAAATAAAAATAGCCATTCCAGAAAATA

**Yellow**: miR-9* binding site

**Green**: miR-9 binding site

**Blue**: miR-124 binding site

Figure 21: miR-9 and miR-124 binding sites on *ACTL6A*

![Diagram of miR-9 and miR-124 binding sites on ACTL6A](image)

**Figure 22**: REST, miR-9, miR-124 and *ACTL6A* interaction
References


