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Mutational Analysis of the Molluscum Contagiosum Virus MC160 protein

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Mutational Analysis of the Molluscum Contagiosum Virus MC160 protein

By

Henri Estanbouli

Submitted in partial fulfillment of the requirements for the degree

Master of Sciences

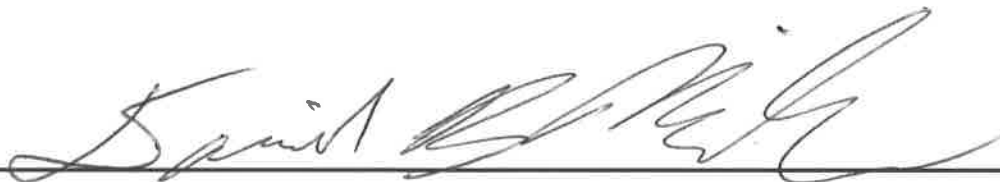
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December 2017

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My Mother, Father, and Brother

Lab Mates

Friends

Abstract

The Molluscum Contagiosum Virus (MCV), belongs to a family of large DNA viruses called *Poxviridae*. MCV causes a common skin infection resulting in benign neoplasms. 122 million cases of MC were reported in 2010. The virus is not primarily lethal, though fatalities have occurred due to secondary bacterial infections. Even with the high volume of reported cases, MCV is very much understudied, and its pathogenicity is not well understood. Thus, with that in mind, a more in-depth understanding of the MCV-host interactions is a priority. Presumably during infection, MCV utilizes immune evasion molecules to inhibit activation of the host pro-inflammatory signal pathways allowing the infection to persist. However, only nine of a predicted 77 immune evasion molecules, encoded by the MCV genome, have been characterized. This study focuses on one in particular, the MC160 protein. MC160 contains a pair of death effector domains (DEDs). MC160 expression inhibits various innate immune responses (IIRs), such as TNF α -induced NF- κ B activation and MAVS-mediated activation of type I interferons (IFN). The first death effector domain (DED1) inhibits the activation of IRF3, a vital transcription factor in the activation of type I IFNs. To further discover the region(s) of interest within the first DED1 of MC160 that are required for MAVS-induced IRF3 inhibition, five MC160 truncation mutants were generated, by deleting predicted α -helixes from DED1. Constructs expressing truncated MC160 mutants were evaluated for the ability to inhibit MAVS-mediated IFN β activation using the dual luciferase activity. Preliminary data, from transfection of HEK 293T cells with MC160 truncation mutants identified the second α -helix as being critical for the MC160 protein's ability to inhibit

activation of IFN β . By identifying the region of MC160 required to antagonize host immune signaling, we hope to gain a better understanding of how MC160 expression antagonizes this important IIR.

Introduction

Molluscum Contagiosum and Poxvirus Background

The Molluscum Contagiosum Virus (MCV) is a dermatotropic poxvirus, which exclusively infects human keratinocytes (Randall et al., 2013). An MCV infection results in benign skin neoplasms which can persist for months in even healthy patients (Chen et al., 2013). There are two types of MCV lesions have been recorded. The first, inflammatory MC (I-MC), presents clear signs of inflammation, whereas the second type, non-inflammatory MC (NI-MC), perseveres with little evidence of host inflammatory responses (Vermi et al., 2011). The MCV genome is predicted to encode numerous host immune evasion molecules, which likely contribute to the development of NI-MC lesions, allowing MCV to persist within the host by evading local immune responses (Senkevich et al., 1997). On the other hand, out of a predicted 77 immune evasion molecules in the MCV genome, only nine MCV proteins with immune evasion properties have been described.

Poxviruses contain a linear, double stranded DNA (dsDNA), ranging between 130-300 kbp. These large dsDNA viruses replicate exclusively within the cytoplasm of host cells. Once in the target cell, the virus is then transcribes its genome via a carefully regulated cascade of early, intermediate, and late genes. The first step essentially requires that early gene transcription occur in the nucleocapsid with early gene transcription factors already bound to early promoters. This early formation allows for the flow of early messengers through pores into the cytoplasm of the target cell. Many viral early

genes code for immune evasion molecules in order defend against host immune response. Furthermore, late transcription factors are produced into viral particles, called lateral bodies, that thought to be delivered to the host cell, to allow the virus to hijack the host's innate immune response. Many poxvirus immune evasion molecules are needed for its pathogenicity.

Host innate immune responses

Several cytokines are produced in to antagonize poxvirus infection. For example, TNF is extremely detrimental to poxvirus infection as mutant poxviruses lacking genes to antagonize TNF result in attenuated infection in animal models. TNF can induce multiple outcomes in the cell including NF- κ B activation and apoptosis. In the case of NF- κ B activation, Once bound to the receptor, TNF-R1, adaptor proteins (TNF-R)-associated death domain protein (TRADD), TNF-R2-associated factor 2 (TRAF2), Fas-associated death domain (FADD), and receptor-interacting protein 1 (RIP1) are recruited to the cytoplasmic domain of the receptor. This signaling complex recruits additional factors leading to the phosphorylation and activation of the IKK signaling complex, which consists of IKK β , IKK α , and the regulatory component IKK γ . Once activated, the IKK complex phosphorylates the inhibitory molecule I κ B α leading to its ubiquitination and subsequent degradation. Upon degradation of I κ B α , the nuclear localization sequence on the NF- κ B p65/p50 dimer is unmasked allowing NF- κ B to translocate to the nucleus. Once at the nucleus, NF- κ B binds to enhancer elements upregulating the transcription of pro-inflammatory molecules such as TNF and IL-6.

Alternatively, the TRADD, FADD, and procaspase-8 complex can release from the receptor and form the death-inducing signaling complex (DISC), which results in the oligomerization and auto cleavage pro-caspase-8 to active caspase-8. Caspase-8 is an initiator caspase capable of cleaving and activating downstream effector caspases such as caspase-3. In addition, caspase-8 can cleave Bid into tBid which promotes the release of cytochrome *c* from the mitochondria thereby triggering the intrinsic apoptotic response as well. In addition to TNF, the FasL can also trigger apoptosis by binding to the Fas receptor leading to FADD and procaspase8 forming the DISC which triggers caspase-8 mediated apoptosis. To ensure successful virus replication, poxviruses have evolved mechanisms to prevent both the extrinsic and intrinsic pathways which will be discussed below.

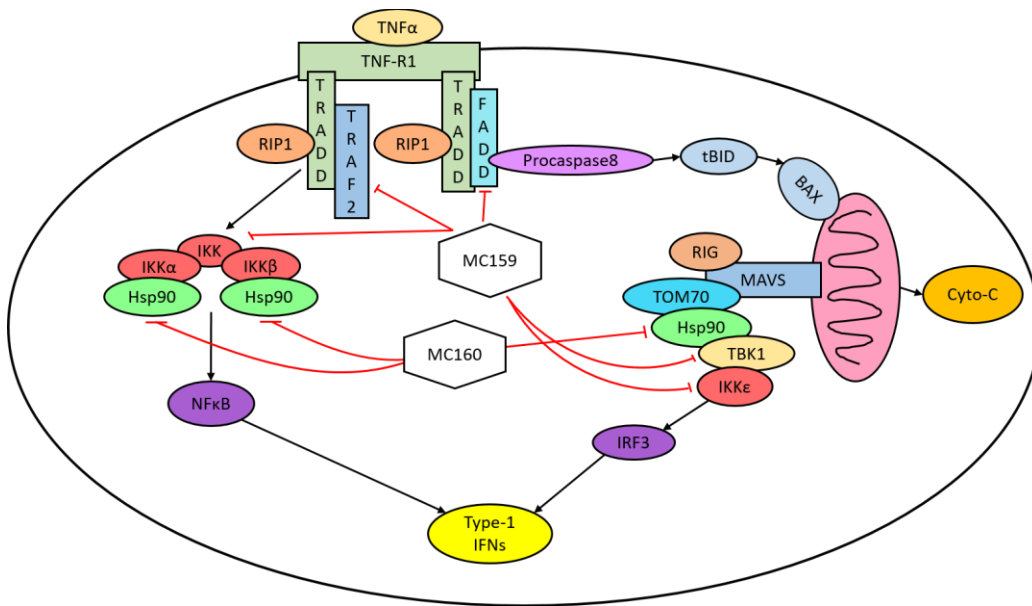


Figure 1: TNF- α Signaling Pathway

*All figures are made by the author, unless otherwise noted.

Host cells possess an array of pattern recognition receptors (PRRs) to detect and respond to virus infection inside the cell. For example, dsRNA is an important pathogen associated molecular pattern that is produced as a by-product of poxvirus replication. RIG1/MDA5/MAVS-induction of type 1 interferons. Poxviruses are involved in both extrinsic and intrinsic pathways.

One of the key intrinsic pathways of the cell that poxvirus must inhibit is the RIG1/MAVS-induction of Type 1 interferons (IFNs). In this pathway, retinoic acid-inducible gene 1 (RIG-1) activates mitochondrial antiviral signaling protein (MAVS) via caspase-recruitment domain (CARD). MAVS plays a key role in activation of the interferon regulatory factor (IRF) signaling pathway with stimulates the production of Type I interferons (IFNs) in the nucleus. For this, MAVS interacts with stimulator of interferon genes (STING). This molecule associates with both the TRAP complex and SEC16 translocon to mediate the pathway. MAVS then interacts with translocase of the outer membrane 70 (TOM70), which in turn also interacts with heat shock protein 90 (HSP90), which couples TANK-binding kinase 1 (TBK1) and interferon regulating factor 3 (IRF3) within proximity of the MAVS complex. From there, TBK1 associates with I κ B kinase ϵ (IKK ϵ) which then phosphorylates IRF3 and IRF7, stimulating Type I IFN production (West et al., 2011).

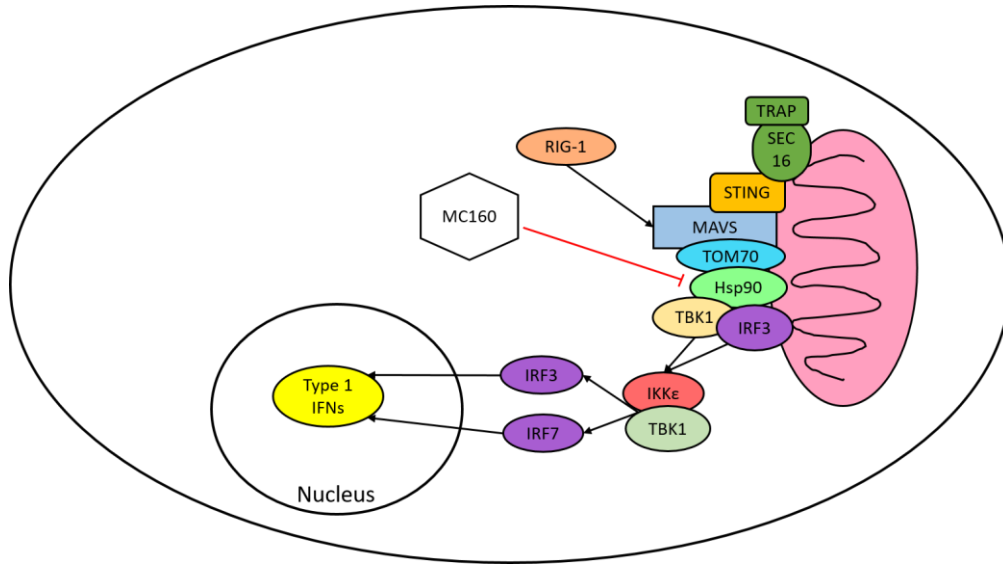


Figure. 2: Intrinsic RIG-1/MAVS-induction of Type I interferons.

Molluscum Contagiosum Virus Immune Evasion Strategies

To ensure successful replication, MCV encodes an array of immune evasion molecules to target a variety of host cell immune responses. The MC054 protein plays a key role in binding Interleukin-18 (IL-18) and inhibiting the pro-inflammatory cytokine's role in both the innate and adaptive immune response (Xiang et al. 2003). MC066 is a glutathione peroxidase that inhibits the apoptotic response initiated by ultraviolet light and hydrogen peroxide (Shisler et al. 1998). The MC132 protein blocks NF- κ B activity via direct activity with the p65 subunit, leading to its degradation (Brady et al. 2015). MC148 acts as a chemokine-like protein, binding to both CxCL12 and CCR8, inhibiting chemotaxis (Jin et al. 2011; Luttichau et al. 2001). The MC007 is a mitochondrial localizing protein that isolates the retinoblastoma protein (pRb) (Mohr et al. 2008). MC005 inhibits NF κ B activation by targeting IKK γ , part of the IKK complex (Brady et al., 2015). The MC163 protein aids in virulence, by inhibition of TNF α -induced apoptosis, via prevention of mitochondrial membrane permeabilization (MMP) (Coutu et al. 2016). MC159 and MC160 target a variety of IIRs including apoptosis, NF- κ B activation, and inhibition of MAVS signaling. The main focus of this paper is aimed at understanding the molecular mechanisms through which the MCV protein MC160 dampens host immune pathways.

<u>MCV Protein</u>	<u>Function</u>	<u>Mechanism</u>
MC005	Inhibits NF- κ B Activation	Targets IKK γ in the IKK complex
MC007	Inhibits pRb	Binds pRb
MC54	Inhibits IL-18	Binds IL-18
MC66	Inhibits apoptosis	Inhibits hydrogen peroxide
MC148	Inhibits chemotaxis	Binds to CCR8 and CXCL12 α
MC159	Inhibits apoptosis	Binds FADD
MC160	Inhibits NF κ B	Binds caspase-8, Hsp90
MC163	Inhibits TNF- α	Inhibits MMP

Table 1: Characterized Molluscum Contagiosum Virus Immune Evasion Molecules

MCV MC159 and MC160 Proteins

Both MC159 and MC160, belong to a family of proteins called FADD-like Interleukin 1 β converting enzyme inhibitory proteins (FLIPs). This family of proteins is unique in the fact that they contain specialized death effector domains (DEDs). MC159 and MC160 contain two tandem DEDs (Figure 1). The MC159 protein inhibits Tumor Necrosis factor-alpha (TNF- α), and Fas mediated apoptosis through interactions with procaspase-8 and FADD. By binding FADD and procaspase-8, MC159 prevents the formation of DED-filaments thus preventing the oligomerization and subsequent auto cleavage of procaspase-8 into the active caspase-8 form (Nichols et al. 2017). MC159 expression also prevents TNF- α -induced NF- κ B activation through association with the regulatory subunit IKK γ (Randall et al. 2012). Despite binding to both FADD and procaspase-8, the MC160 protein does not inhibit apoptosis. However, MC160 expression does dampen TNF- α -induced NF- κ B activation. This is accomplished through the binding of Heat Shock protein 90 (Hsp90) and the subsequent degradation of the I kappa kinase (IKK) complex (Nichols & Shisler 2006; Nichols & Shisler 2009). In addition, both MC159 and MC160 inhibit MAVS-mediated activation of type I interferons (IFNs). The MC159 protein binds both TBK1 and IKK ϵ and prevents their phosphorylation and subsequent activation. While MC160 expression blocks TBK1 and IKK ϵ phosphorylation, the molecular mechanism and cellular target of MC160 in the MAVS pathway has yet to be identified. Using a panel of MCV truncation mutants, Randall et al. demonstrated that the DED1 and C terminal of MC160 independently inhibit IRF3 activation, whereas the second death effector domain was dispensable

(Randall et al., 2012). A summary of the function of the MC160 domains is listed in

Table 2.

MC160 DED	Inhibits TNF-induced NFκB	Inhibits TBK1-induced IFNbeta	Inhibits IKK-induced NF-κB	Inhibits MAVS-induced IFNβ activation
D1	No	Yes	No	Yes
D2	Yes	No	Yes	No
MC160 C terminal	Yes	Yes	Yes	Yes

Table 2: Summary of MC160 domains functions

The purpose of this thesis was to identify the critical region in MC160 DED1 required to inhibit MAVS-mediated activation of IRF3 and subsequent IFN β activation. To this end, several MC160 mutants were generated including a panel of MC160 truncation mutants lacking predicted alpha-helices in DED1. These mutant proteins were expressed in HEK293T cells and detected via immunoblot. The MC160 mutants were further characterized for their ability to inhibit MAVS- and TBK-1 induced IFN β activity via the luciferase assay. MC160 mutants lacking the predicted second alpha-helix lost the ability to inhibit IFN β activation suggesting this region is required for key interactions between MC160 and target proteins.

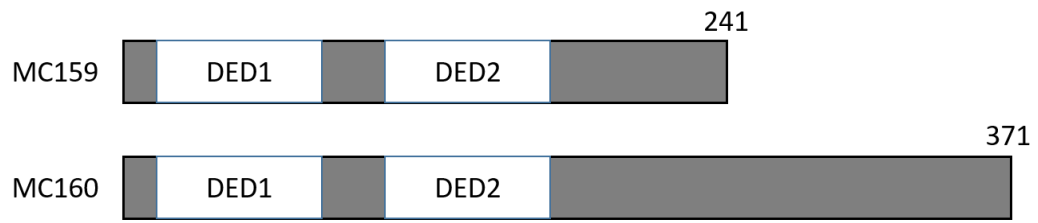


Figure. 3: MCV MC159 and MC160 proteins. Both proteins contain in tandem dual death effector domains (DEDs). MC159, a.a. 241; MC160, a.a. 371.

Materials and Methods

Mutagenesis

Construction of the truncated MC160N mutants began by performing a sequence alignment on the MC160 and the homologous protein MC159. The crystal structure of the MC159 protein was previously solved (Yang 2005, Shi 2005) and MC160 is predicted to fold similar to that of MC160 (Beaury 2017). Custom primers were then developed to specifically remove each α -helix of MC160 DED1. Due to the rich GC content of the region, the primers were well above the melting temperature (T_m) parameters, and thus several silent mutations had to be introduced to lower the T_m to the desired range while preventing any changes in the amino acid sequence. The template that was used was a N-terminus truncated MC160 protein, MC160N lacking the C-terminus. Target sequences were amplified by a two-step PCR process. In the first reaction, each individual α -helix region using the respective forward primers (table 3) and adding the first half of the HA tag, and the reverse primer, pCI_{rev}HT (5'-CTTATCATGTCTGCTCGAAGCGGCCGC -3', Table 4). The second step was to attach the second half HA-sequence, using the forward primer Universal Secondary Primer_2 (USP_2, Table 4). After each step round of PCR, products were then run on a 0.7% agarose gel for verification. PCR products were purified using the PCR purification kit (Promega). The amplified products were then digested via a double-restriction enzyme digest, using EcoRI/XbaI and ligated into a pCI mammalian expression vector linearized using the aforementioned restriction enzymes (Promega). All constructs were engineered to contain the HA-tag at the N-terminus. Vectors were transformed into DH5 α competent

cells and incubated overnight. Individual colonies were picked and grown overnight in LB broth containing 100 $\mu\text{g}/\text{mL}$ ampicillin. Following 16-22 hours incubation at 37°C, plasmid DNA was isolated and restriction digested using EcoRI and XbaI to verify the presence of the insert. A PCR reaction using primers pCIseqFOR and pCIseqREV was used to further verify the presence of the insert. The PCR products were purified using the PCR purification kit and amplicons were sent out for DNA sequencing (Genscript) to verify that the correct sequence was present (Figure. 11).

Primer Name	Primer Sequence
MC160_α-helix_1	5'- CCATATGACGTGCCAGACTATGCAGCAGAGCTGGAC -3'
MC160_α-helix_2	5'- CCATATGACGTGCCAGACTATGCACTGTGCCGAGATGTG -3'
MC160_α-helix_3	5'- CCATATGACGTGCCAGACTATGCAAGACGGCGTCTG -3'
MC160_α-helix_4	5'- CCATATGACGTGCCAGACTATGCAGCGCTACGACGCTTT -3'
MC160_α-helix_5	5'- CCATATGACGTGCCAGACTATGCATTTGGTATGACGCGC -3'
Universal Secondary Primer_2	5'- TCGAATTCGCCACCATGGCTTACCCATATGACGTG -3'
pCI _{rev} HT	5'- CTTATCATGTCTGCTCGAAGCGGCCGC -3'

Table 3: List of primers used for MC160 truncations

Primer Name	Primer Sequence
MC160_α-helix_1	5'- CCATATGACGTGCCAGACTATGCAGCAGAGCTGGAC -3'
MC160_α-helix_2	5'- CCATATGACGTGCCAGACTATGCACTGTGCCGAGATGTG -3'
MC160_α-helix_3	5'- CCATATGACGTGCCAGACTATGCAAGACGGCGTCTG -3'
MC160_α-helix_4	5'- CCATATGACGTGCCAGACTATGCAGCGCTACGACGCTTT -3'
MC160_α-helix_5	5'- CCATATGACGTGCCAGACTATGCATTTGGTATGACGCGC -3'
Universal Secondary Primer_2	5'- TCGAATTCGCCACCATGGCTTACCCATATGACGTG -3'
pCI _{rev} HT	5'- CTTATCATGTCTGCTCGAAGCGGCCGC -3'

Table 4: MC160 truncation mutant primers

Plasmid DNA preparations

Plasmid DNA was isolated from the bacterial cultures using the PureYield Plasmid Miniprep system (Promega) according to the protocol presented by the manufacturer. The *E.coli* containing experimental constructs were incubated for 16-22 hours in Luria broth (LB) supplemented with ampicillin at 100 µg/mL (Life Technologies). Following incubation, the bacteria were harvested via centrifugation at 14,000 rpm for 1 minute, then re-suspended in 600 µL of 1X Tris-EDTA buffer (TE) and then the Promega protocol was followed. Once the Miniprep protocol was completed, the plasmid DNA was eluted in 30 µL of 1X TE buffer, assed for purity and measured concentration of samples using a BioDrop spectrophotometer, and stored at 4°C. During the assessment process, plasmid DNA was deemed acceptable and utilized in subsequent experiments with O.D. 260/280 values within the range of 1.8-2.0.

Cell culture and Transfections

Human embryonic kidney 293T (HEK 293T) cells were maintained in Dulbecco's modified eagle medium (DMEM, Sigma or HiMedia) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (sigma) at 37C, with 5% CO₂, in a humidified incubator. The transfection of HEK 293T cells was performed using Mirus Trans-IT 2020 reagent in Opti-MEM (Gibco) at 3 µL/µg of total DNA.

Immunoblotting

HEK 293T cells were plated at 2.0×10^5 cells/well in a 12 well plate (Cellbind, Corning). Following overnight incubation, cells were transfected with one μg of either pCI, MC160N/pCI, or the indicated MC160N truncation mutant using the Mirus TransIT 2020 transfection reagent. 24 hours post transfection, the cells were then collected and lysed in 100 μL RIPA lysis buffer (150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.5% Sodium Dexycolate, 50 mM Tris, 0.1% SDS) supplemented with protease inhibitor cocktail added according to the manufacturers recommended concentration (Amresco, M250: AEBSF, Aprotinin, E-64, Bestatin, Leupeptin, Pepstatin). Cells were lysed for 30 minutes, the centrifuged at 14,000 rpm to remove cellular debris. The supernatants were collected and subsequently boiled for 5 minutes with 5% 2-mecaptoethanol in 1X sodium dodecyl sulfate (SDS) sample buffer. Once boiling was completed, samples were either placed on ice till use or stored at -80°C . Once ready for use, prepared lysates were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVFD) membranes (Millipore), blocked in 5% milk (carnation non-fat dry milk) in 1X Tris-buffered saline with Tween®20 (1X TBST), for 30 minutes or if needed by overnight. Following blocking, the PVFD membranes were then probed for 1 hour in primary antibody (anti-HA, 1:1000, Sigma) diluted in 0.5% milk TBST. The membranes were then washed three times in 0.5% milk TBST, then probed again, this time using a secondary antibody (goat-a-mouse-HRP, 1:1000) for 1 hour, and washed again, three times, using 0.5% milk TBST. Lastly, the membranes were then visualized using SuperSignal™ West Femto (Thermo Scientific) chemiluminescent reagent, according to

the manufactures instructions. Blots were visualized using a FluorChem E imaging system (Protein Simple).

Luciferase Assay

HEK 293T cells were plated at 2.5×10^5 cells per well into 12-well plates, and allowed to grow for 24 hours at 37C in a 5% CO₂ humidified incubator. Following the 24 hours, the cells were then transfected with 300 ng of plasmid DNA (pCI, pHA-MC160N, pΔ α -1, pΔ α -2, pΔ α -3, pΔ α -4, pΔ α -5, pD1 and pD2), 300 ng of mitochondrial antiviral-signaling protein (MAVS) or TANK-binding kinase 1 (TBK1), 200 ng reporter plasmids (100 ng pIFN- β -luciferase, 100ng pRenilla), 200 μ L of Opti-MEM, and Mirus Trans-IT 2020 reagent (Mirus) at a ratio of 3 μ L/ μ g of total DNA. Upon completion of transfection, the cells were then incubated for another 24 hours at 37C in a 5% CO₂ incubator. Post-24 hour incubation, the cells were then lysed for 10 minutes with 1X passive lysis buffer (Promega). The lysates were then assayed for firefly and sea pansy luciferase activity using a Dual-reporter assay according to the manufacturer's specifications (Promega). The ratio of Firefly to *Renilla* luciferase activity was used to determine relative activation of the IFN β enhancer element. The data was then measured for statistical significance using the student's t-test.

Results

Cloning the MC159 & MC160 Death Effector Domain Swap Chimeras:

The initial goal of our research was to see if the individual death effector domains (DEDs) of both the MC159 and MC160 proteins were interchangeable, and if so, would there be a gain or loss of function for the respective proteins. Two MCV proteins, MC159 and MC160, contain two tandem death effector domains (DEDs). Both MC159 and MC160 belong to a group of proteins collectively known as viral FLICE-like Inhibiting Proteins (vFLIPs) (Shisler et al. 2015; Senkevich et al. 1997; Senkevich et al. 1996). These MCV vFLIPs provoke a diverse array of host-mediated immune responses including apoptosis and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation (Nichols & Shisler 2009; Nichols & Shisler 2006; Murao & Shisler 2005; Randall et al., 2012). Previous research done by Yang et al, identified that the sequences of both viral proteins were similar. A total of six mutants were designed, and a cloning protocol was made to create them (Figure 6). First specific primers, with overhang, were made for each DED and a custom general forward and reverse primer as well (Table 2). Once the primers were made, a two-step overlap extension PCR was done to create the domain swapped chimeras. Both the MC159 wt and MC160 truncation mutant, MC160N, were used as templates for the initial step of the PCR process was to amplify and clone only the desired regions of each template. Once completed, the PCR products were then used as the templates for the second round of PCR using both the pCI_{for} and pCI_{rev} primers (Table 3) to make the final constructs. The final constructs were then run on a 0.7% agarose gel for verification (Figure 7). All constructs were engineered

to contain an N-terminal HA epitope tag to detect expression. PCR amplicons were then cloned into the pCI mammalian expression vector and sequenced. Expression of all constructs in HEK293T cells were then verified by immunoblotting and detection with anti-HA antibody.

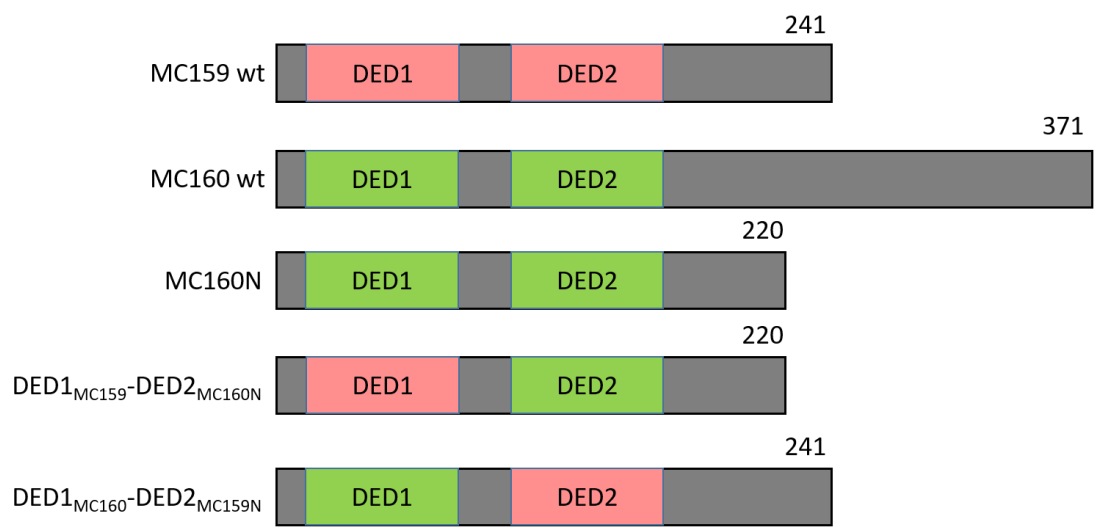


Figure 4: MC159 and MC160 chimeric domain swapped mutants characterized in this study. The MC159 wild-type (wt), a.a. 1-241; MC160 wt, a.a. 1-371; MC160N, a.a. 1-220, DED1_{MC159}-DED2_{MC160} (abbreviated as DED1_{MC159} in data sets), a.a. 1-220; DED1_{MC160}-DED2_{MC159} (abbr. as DED1_{MC160} in data sets), a.a. 1-241.

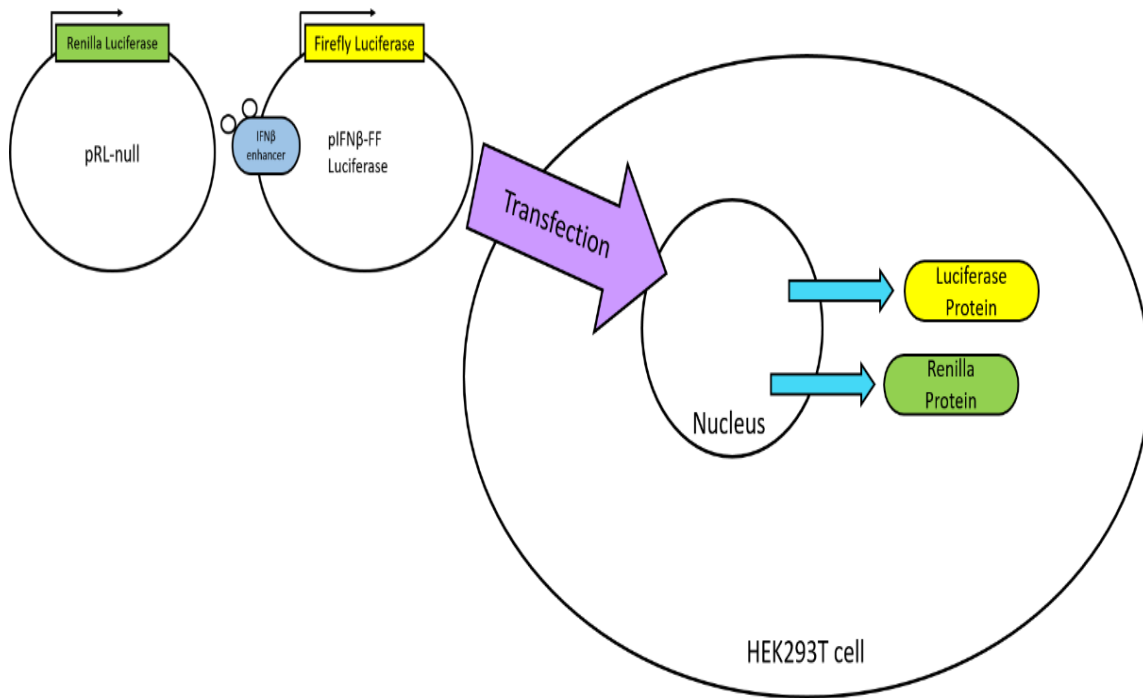


Figure 5: Dual Luciferase Assay Mechanism

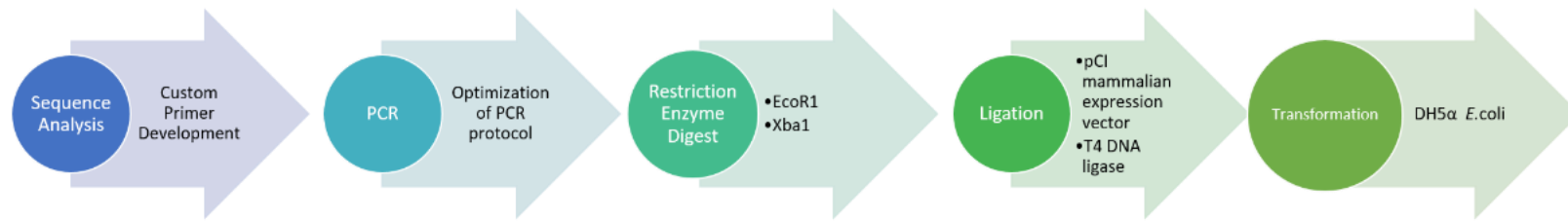


Figure 6: Cloning Strategy for the MC159 and MC160 domain swapped chimeric mutants

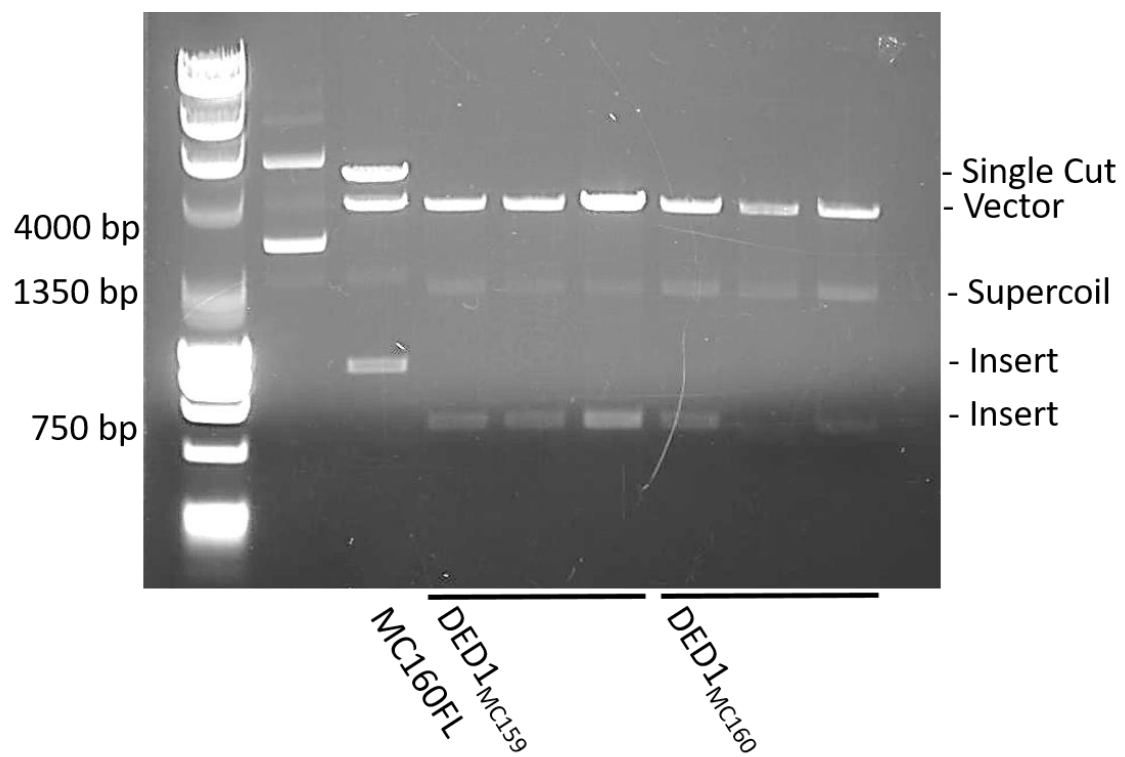


Figure 7: MC159 and MC160 Domain Swap Chimeras Duel-Restriction Enzyme Digest

Domain-swapped mutants inhibit MAVS-mediated activation of IFN β :

Both MC159 and MC160 proteins play a key role in inhibition of the MAVS pathway, especially the DED1 of each protein, though both DEDs of MC159 inhibit this pathway (Shisler et al. 2015; Randall et al. 2013). Both chimera were utilized to see if swapping the DED of each would maintain their ability to inhibit the MAVS-mediated activation of IFN β . HEK293T cells were transfected with either empty vector, pHA-MC159, pHA-MC160, pDED1_{MC159}, pDED1_{MC160}, or were left untransfected. All cells were also transfected with pIFN β fire fly luciferase and pRL-null *Renilla* luciferase. In cells transfected with empty vector, treatment with MAVS resulted in significant increase in the levels of IFN β activation as detected by an increase in firefly luciferase values (Figure 9). Interestingly, pDED1_{MC159} and pDED1_{MC160} expressing cells showed inhibition of MAVS-induced activation of IFN β with luciferase values similar to cells transfected with the wild-type pHA-MC159 and pHA-160 (Figure 9). Thus, the data indicates that the DED domains of MC159 and MC160 are interchangeable.

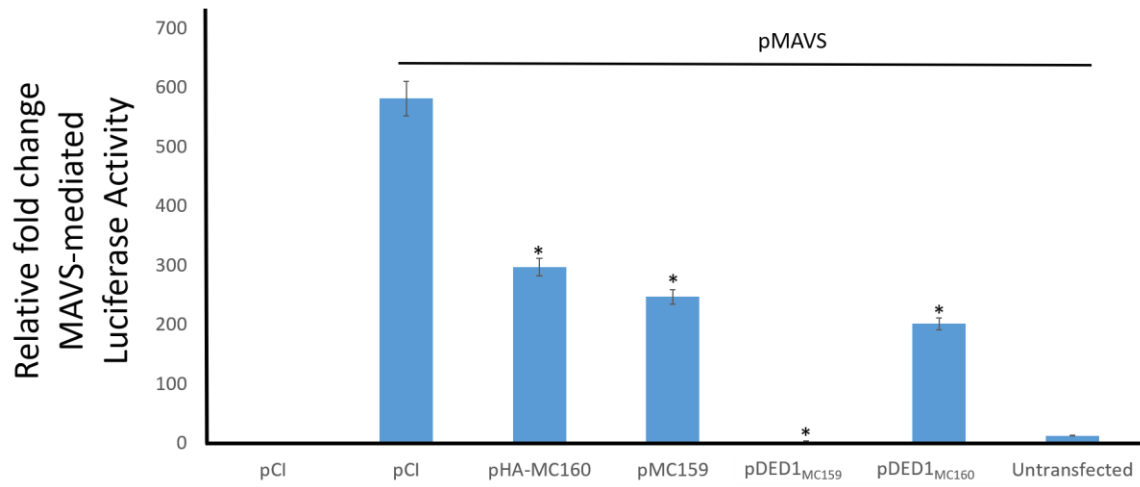


Figure 8: Dual Luciferase Activity Analysis of MC159 and MC160 domain swapped chimeric mutants effect on MAVS-mediated $\text{INF}\beta$ Activation; experiments were performed in triplicate, n=3.

Cloning the MC160 $\Delta\alpha$ -mutants:

The goal of the project was to identify the region within the death effector domain 1 (DED1) of MC160 required to inhibit MAVS- induced IFN β activation. Through previous research, five α -helix regions were identified within the DED1 of the MC159, and hypothesized in the DED1 of the MC160 (Yang et al. 2005). Furthermore, it has been reported that the MC160 protein is predicted to fold similar to MC159, with high confidence (Beaury et al. 2017). A sequence alignment of MC159 and MC160 DEDs is illustrated in Figure 9. Five MC160 truncation mutants, designated; $\Delta\alpha$ -1-5, were generated, by deleting the predicted α -helices (α -1-5) from DED1 (Figure 10). A cloning protocol was made to create the truncation mutants (Figure 11). First, specific primers were designed to create the truncation mutants, using the MC160N truncated mutant of the full length MC160 protein. Both the HA-tag sequence and the DED1 of MC160 are both rich in GC content, making the cloning particularly difficult. To circumvent this problem, silent mutations were made to codon optimize the nucleotide sequence of the primers, to both decrease the GC content and the melting temperature (T_m), to fit into the constructed cloning parameters. These mutations maintain the integrity of the wild-type amino acid sequence. Because the C-terminus of MC160 also inhibits MAVS/TBK1- induced IFN β activation, the MC160N (a.a. 1-220) construct which lacks the MC160 C-terminus was used as the template for cloning using PCR. Thus, any loss of function due to deletion of amino acid in the DED1 of MC160 will not be masked by the presence of the MC160C-terminus. Once the specific primers were made, samples were run through a two-step PCR process. The first round of PCR amplifies the 5' region of the MC160

deletion and adds part of the HA tail. This first PCR reaction required the use of the MC160_α-helix_1-5 as forward primers and pCI_{rev}HT as the reverse primer (Table 4). The second round required the use of the same reverse primer; however, a Universal Secondary Primer_2 (USP_2, Table 4) was used to add the remaining HA-tag. Once the each round of PCR was completed, the PCR products were run on a 0.7% agarose gel for verification (Figure 12 & 13, respectively). Each gel showed the distinct bands decreasing in size which occurs as each α-helical region is stripped from the DED1 of the MC160 protein. All constructs were engineered to contain a N-terminal HA epitope tag to detect expression. Once the both PCR processes were completed, the PCR amplicons were the cloned into the pCI mammalian expression vector and sequenced (Figure 15). For further verification of a successful ligation and transformation, a dual restriction enzyme digest, using EcoR1 and Xba1, was conducted. The restriction enzyme digest products were then run on a 0.7% agarose gel and analyzed, showing the same distinct bands decreasing in size, with the vector consistently shown at around 4000 base pairs (bp) (Figure 14). Expression of the constructs in HEK293T cells were verified by immunoblotting and detection with anti-HA antibodies (Figure 16). All MC160N mutants were detected, however, Δα-1, Δα-2, and Δα-4 mutants were detected at a much higher level at 22 kD, 19 kD and 17 kD, respectively, than the Δα-3 and Δα-5, which were seen at a much lower level (Figure 16). Since Δα-3 was detected at low levels, I performed initial analysis with truncation mutants one and two.

Sequence View: Similarity Format, Color areas of high matches at same base position

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MC159      1  MSDSKEVPSLPFLRHLEELDSHEDSLLLFLCHDAAPGCTTVTQALCSLSQQRKLTLAALVEMLYVLQRM
MC160      1  --MAHEPIFFSFLRNLLAELDASEHEVLRFLCRDVAPASKTAEDALRALQRRLLTLSSMAELLCALRRF

MC159     71  DLLKSRFGLSKEGAEQLLGTSTFLTRYRKL M VCVGEELDSSELRALRLFACNLNPSLSTALSESSRFVELV
MC160     69  DVLKVRFGMTRE CAGRL LGHGFLSQYRLQVAAINNMVGS E DLRVMCLCAGKLLPPSCTPRC----LVDLV

MC159    141  LALENVGLVSPSSVSVLADMLRTLRRDL C QQLVEYEQQ--
MC160    135  SALEDAGAI SPQDVSVLV TLLHAVCRYDLSVALSAVAHG M

```

Annotations above the sequences show the start and end of each α -helix region within the DED1:

- α -helix_1: |<--a-helix_1-->| (residues 19-26)
- α -helix_2: |<---a-helix_2--->| (residues 32-40)
- α -helix_3: |<----a-helix_3---->| (residues 51-60)
- α -helix_4: |<-----a-helix_4----->| (residues 66-75)
- α -helix_5: |<----a-helix_5---->| (residues 79-87)

Figure 9: Sequence alignment of the MC159 and MC160 DED1 and DED2 regions. The red brackets indicate the start and end of the death effector domain 1 (DED1) for both proteins. The blue bracket represents the predicted start of the second DED. The annotations above the sequences show the start and end of each α -helix region within the DED1. For the purpose of this experiment, the MC160 α -helices locations are of importance: α -helix_1, a.a. 19-26; α -helix_2, a.a. 32-40; α -helix_3, a.a. 51-60; α -helix_4, a.a. 66-75; α -helix_5, a.a. 79-87.

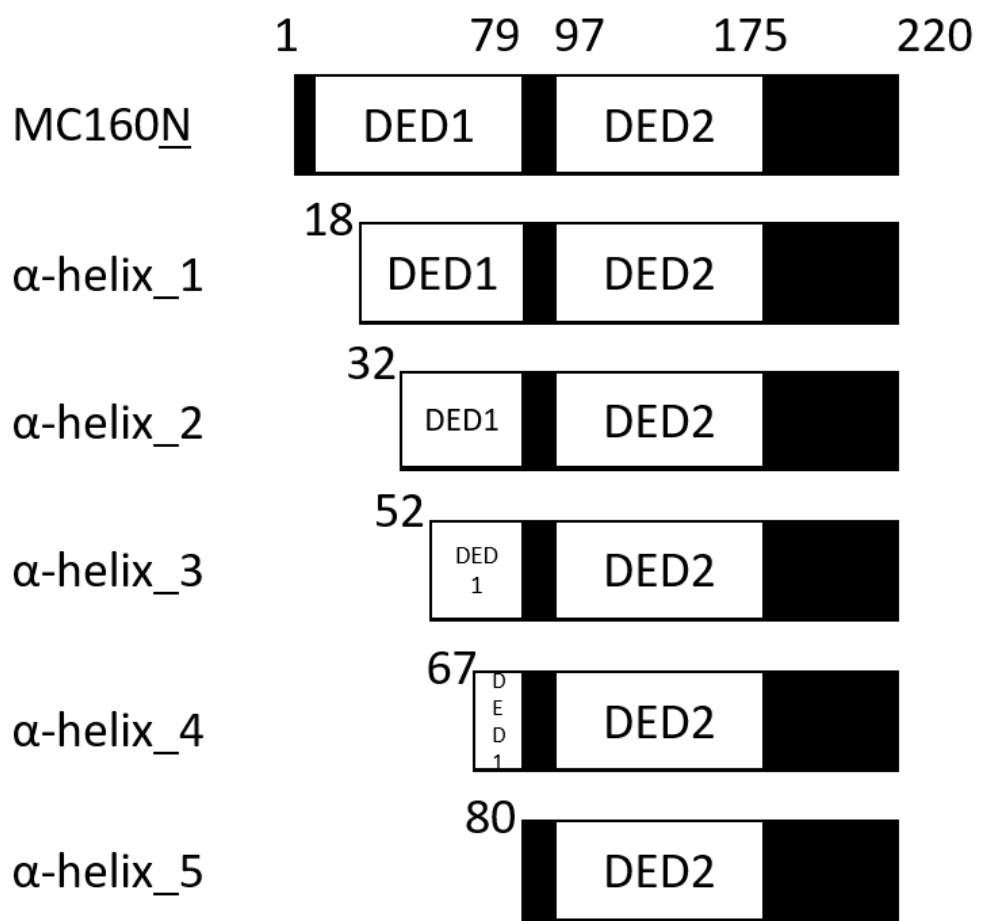


Figure 10: MC160N $\Delta\alpha$ mutants characterized in this study. MC160N, a.a. 1-220; $\Delta\alpha$ -1 a.a. 18-220; $\Delta\alpha$ -2 a.a. 32-220; $\Delta\alpha$ -3 a.a. 52-220; $\Delta\alpha$ -4 a.a. 67-220; $\Delta\alpha$ -5 a.a. 80-220

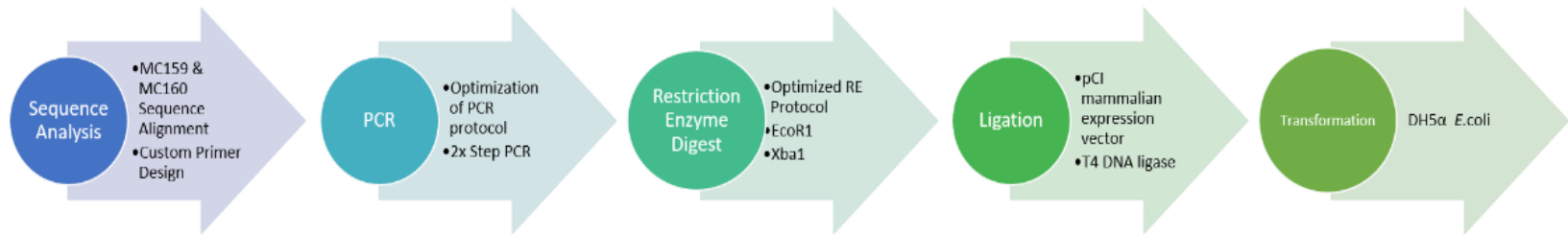


Figure 11: Cloning Strategy for MC160 truncation mutants

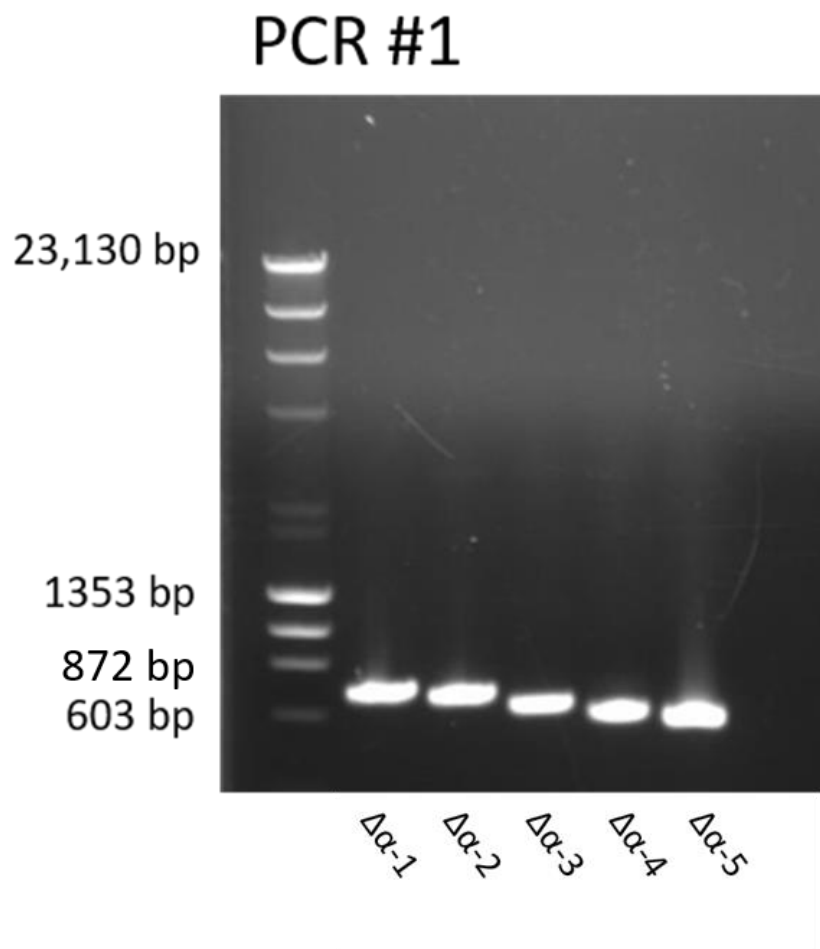


Figure 12: Gel Electrophoresis result for PCR #1 amplicons. The distinct decreasing ladder

PCR #2

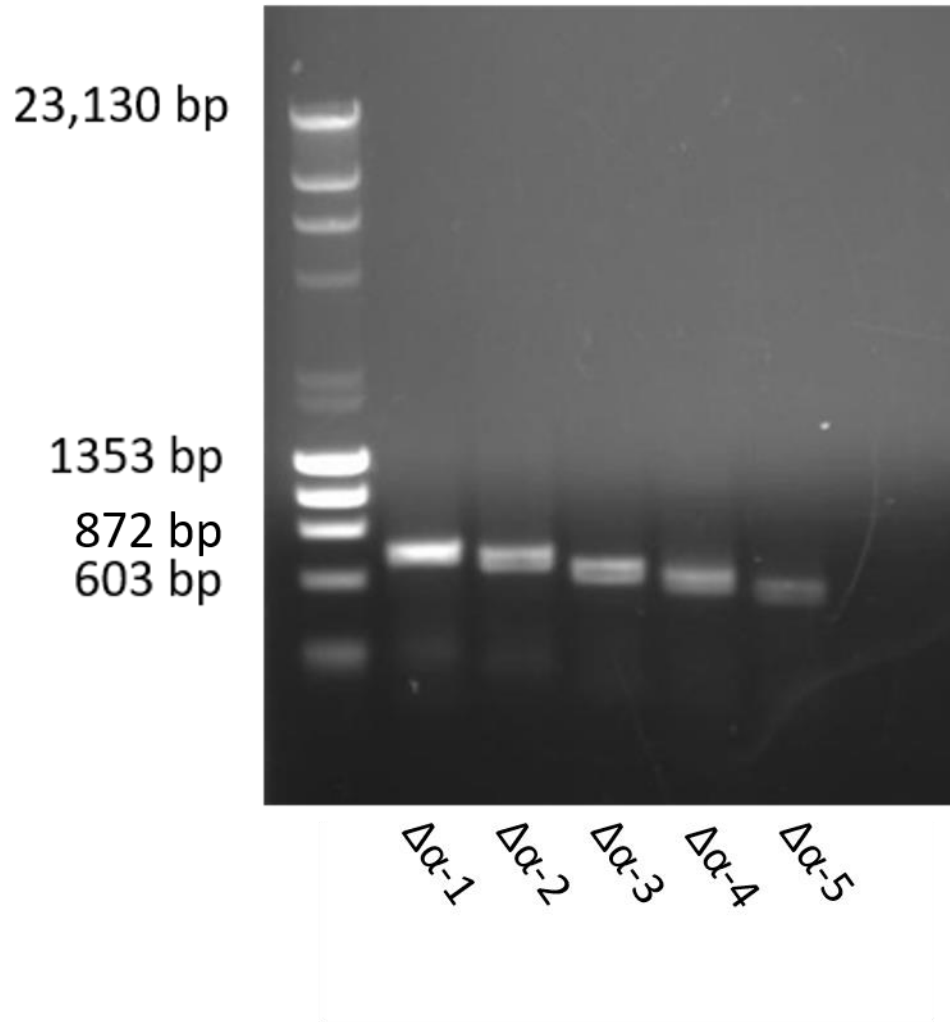


Figure 13: Gel Electrophoresis result for PCR #2 amplicons. The distinct decreasing ladder

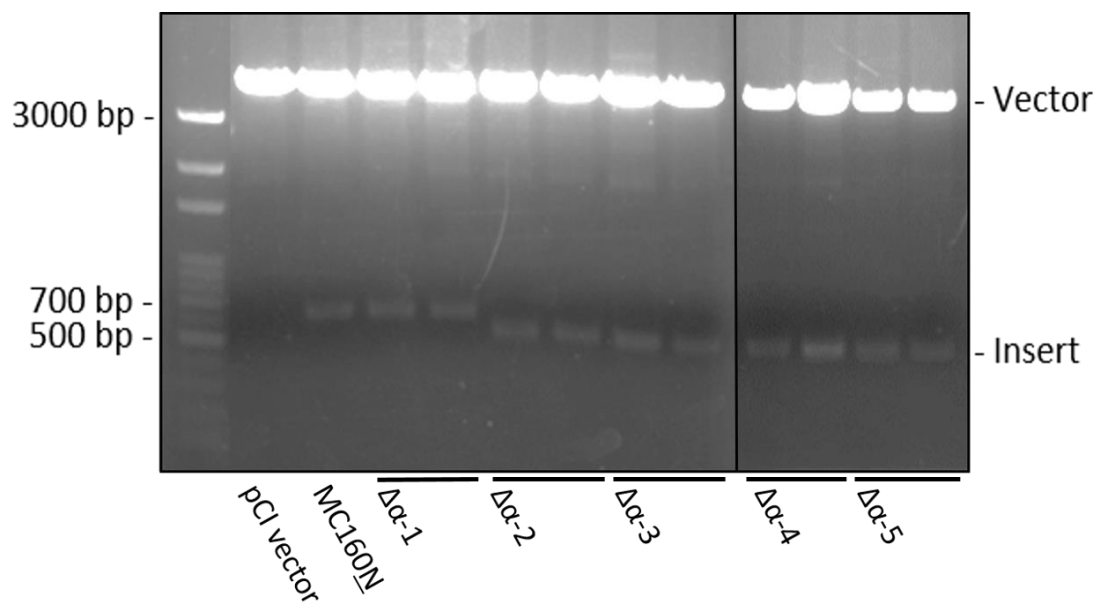


Figure 14: Restriction Enzyme Digest result. pCI mammalian vector is 4006 bp long, and can be seen across all

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MC160N      1 ATM-YPYDVPDYAAHEPIPFSLRNLLAELDASEHEVLRFLCRDVAPASKTAEDALRALQRRRLTLSSMAELLCALRRFDVLKVRFGMTRECAGRLLGHGFLSQYRLQVAAINNMVGSDEL RVMCLCAG
a-1         1 ATMAYPYDVPDYA-----AELDASEHEVLRFLCRDVAPASKTAEDALRALQRRRLTLSSMAELLCALRRFDVLKVRFGMTRECAGRLLGHGFLSQYRLQVAAINNMVGSDEL RVMCLCAG
a-2         1 ATMAYPYDVPDYA-----LCRDVAPASKTAEDALRALQRRRLTLSSMAELLCALRRFDVLKVRFGMTRECAGRLLGHGFLSQYRLQVAAINNMVGSDEL RVMCLCAG
a-3         1 ATMAYPYDVPDYA-----RRRLTLSSMAELLCALRRFDVLKVRFGMTRECAGRLLGHGFLSQYRLQVAAINNMVGSDEL RVMCLCAG
a4          1 ATMAYPYDVPDYA-----ALRRFDVLKVRFGMTRECAGRLLGHGFLSQYRLQVAAINNMVGSDEL RVMCLCAG
a5          1 ATMAYPYDVPDYA-----FGMTRECAGRLLGHGFLSQYRLQVAAINNMVGSDEL RVMCLCAG

MC160N     130 KLLPPSCTPRCLVDLVSALEDVGAISPQDVSVLVTL LHAVCRYDLSVALSAVAHGHTVGVGTPVQDEPMDVLEVDDAEPMEATPACDEIGVVKLAGAASAG
a-1        117 KLLPPSCTPRCLVDLVSALEDVGAISPQDVSVLVTL LHAVCRYDLSVALSAVAHGHTVGVGTPVQDEPMDVLEVDDAEPMEATPACDEIGVVKLAGAASAG
a-2        104 KLLPPSCTPRCLVDLVSALEDVGAISPQDVSVLVTL LHAVCRYDLSVALSAVAHGHTVGVGTPVQDEPMDVLEVDDAEPMEATPACDEIGVVKLAGAASAG
a-3         84 KLLPPSCTPRCLVDLVSALEDVGAISPQDVSVLVTL LHAVCRYDLSVALSAVAHGHTVGVGTPVQDEPMDVLEVDDAEPMEATPACDEIGVVKLAGAASAG
a4          69 KLLPPSCTPRCLVDLVSALEDVGAISPQDVSVLVTL LHAVCRYDLSVALSAVAHGHTVGVGTPVQDEPMDVLEVDDAEPMEATPACDEIGVVKLAGAASAG
a5          58 KLLPPSCTPRCLVDLVSALEDVGAISPQDVSVLVTL LHAVCRYDLSVALSAVAHGHTVGVGTPVQDEPMDVLEVDDAEPMEATPACDEIGVVKLAGAASAG

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Figure 15: Sequence alignment of MC160 truncation mutants based on Sanger Sequencing.

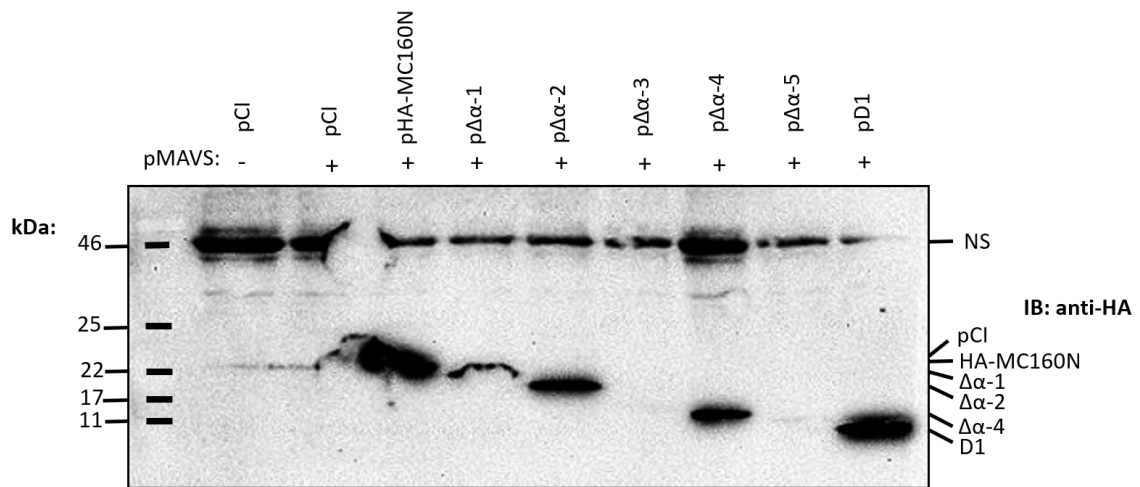


Figure 16: Expression of MC160 truncation mutants

MC160 inhibits MAVS-mediated activation of IFN β :

MAVS plays a key role in activation of the interferon regulatory factor (IRF) signaling pathway with stimulates the production of Type I interferons (IFNs) in the nucleus. Viral regulation of IFN β is a key component to immune evasion. The DED1 of MC160 inhibits TBK1-induced IRF-3 activation by preventing phosphorylation of TBK1. Therefore, the panel of MC160 truncation mutants were utilized to determine the region of the MC160 DED1 responsible for the inhibition the MAVS-mediated activation of IFN β . HEK293T cells were transfected with either empty vector, pHA-MC160N, p $\Delta\alpha$ -1, p $\Delta\alpha$ -2, p $\Delta\alpha$ -3, p $\Delta\alpha$ -4, or p $\Delta\alpha$ -5, pD1 as well as pIFN β fire fly luciferase and pRL-null *Renilla* luciferase. In cells transfected with empty vector, treatment with MAVS resulted in significant increase in the levels of INF β activation as detected by an increase in firefly luciferase values (Figure 17). Expression of HA-MC160N inhibited MAVS-mediated IFN β regulated activity constant with previous research as did HA-MC160 $\Delta\alpha$ -1 (Nichols et al., 2009). Interestingly, $\Delta\alpha$ -2 expressing cells failed to inhibit MAVS-induced activation of IFN β with luciferase values similar to cells transfected with the empty vector and then treated with MAVS (Figure 17). Furthermore, the results from D1 expressing cells did not fully inhibit MAVS-induced IFN β activation. This result was unexpected as Randall et al. reported D1 inhibits TBK1-induced signaling to levels similar to that of MC160N (Randall et al., 2013).

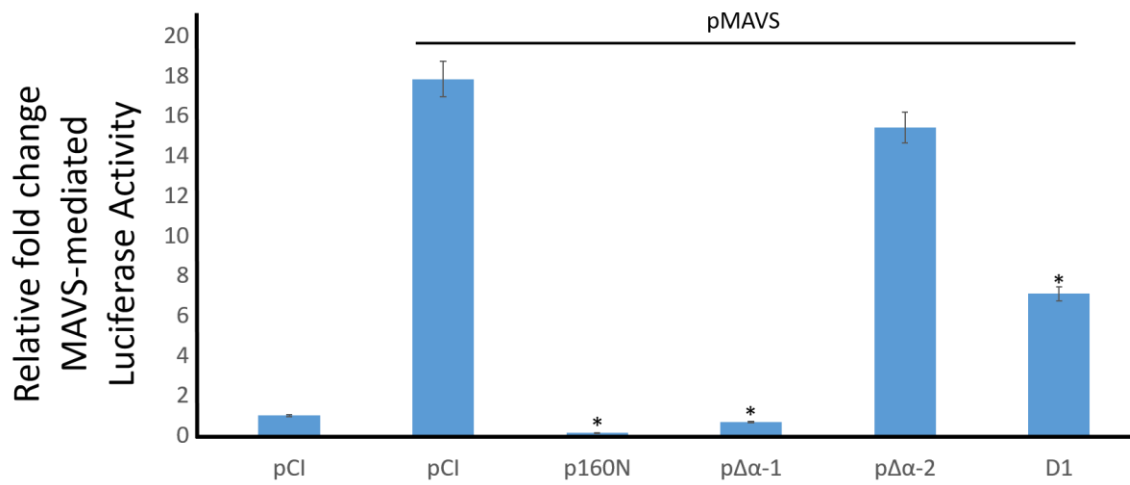


Figure 17: Dual Luciferase Activity Analysis of MAVS-mediated $\text{INF}\beta$ Activation; experiments were performed in triplicate, $n=3$. An asterisk indicates statistically significant inhibition ($P < 0.05$).

MC160 inhibits TBK1-mediated activation of IFN β :

Another key downstream component of the RIG1/MDA5/MAVS pathway is TANK-binding kinase 1 (TBK1). TBK1 is coupled with interferon regulating factor 3 (IRF3) via heat shock protein 90 (Hsp90). From there, TBK1 associates with I κ B kinase ϵ (IKK ϵ) which then phosphorylates IRF3 and IRF7, stimulating Type I IFN production (West et al., 2011). Therefore, we tested to see which regions of the DED1 on the MC160 protein was the key in performing this function, in which it would inhibit the TBK1-mediated activation of IFN β . HEK293T cells were transfected with either empty vector, pHA-MC160N, p $\Delta\alpha$ -1, or p $\Delta\alpha$ -2, as well as pIFN β fire fly luciferase and pRL-null *Renilla* luciferase. In cells transfected with empty vector, treatment with TBK1 resulted in significant increase in the levels of IFN β activation as detected by an increase in firefly luciferase values (Figure 18). Expression of HA-MC160N inhibited TBK1-mediated IFN β regulated activity consistent with data obtained from Randall et al. as did HA-MC160 Δ -1. Interestingly, $\Delta\alpha$ -2 expressing cells failed to block TBK-1-induced IFN β , suggesting that the second alpha-helix of MC160 is required for its inhibitory activity. (Figure 18). On the other hand, D1 expressing cells showed complete inhibition of TBK1-induced IFN β activity, which is significant, since as stated in the previous section, that D1 expressing cells did not fully inhibit MAVS-mediated IFN β (Figure 17).

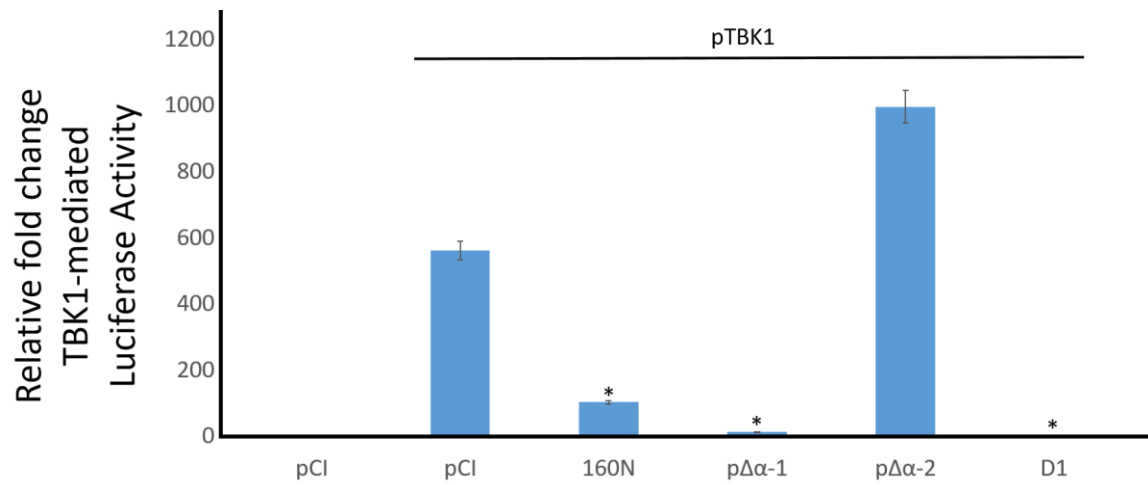


Figure 18: Dual Luciferase Activity Analysis of TBK1-mediated $\text{INF}\beta$ Activation; experiments were performed in triplicate, $n=3$. An asterisk indicates statistically significant inhibition ($P < 0.05$).

Discussion

The Molluscum Contagiosum Virus (MCV) codes two molecules, MC159 and MC160, which inhibit the MAVS-induced IFN activation.. Swapping death effector domains (DEDs) between the MC159 and MC160 proteins results in function proteins maintaining the ability to inhibit activation of type I IFNs. Further, we present evidence to suggest that the predicted second α -helix (α -2) within the DED1 of the MC160 protein plays a key role in its ability to inhibit activation of Type I interferons, via blocking the MAVS pathway.

The initial design of this thesis was to investigate whether or not the DEDs of the MC159 and MC160 proteins were interchangeable, due to similarity of their sequences and structures, and if so, would they maintain their original function or even gain functionality from the other. Thus to initiate that, using the predicted sequences aligned (Yang et al., 2005) we generated the domain swapped chimeras. The data that was collected and presented above is still preliminary. However, it does coincide with previous data and research. One chimera mutant, DED1_{MC159}, can be seen to actually function better than the wild-type (Figure 8). Nevertheless, both mutants inhibit the MAVS-mediated activation of INF β . This data suggests that the DEDs of MC159 and MC160 are interchangeable. The DED1 of MC160 is required to inhibit TBK1-induced activation of IFN β , whereas both MC159 DEDs independently inhibit IRF3 activity (Randall et al 2014). Future steps that will be undertaken, will include verification of expression levels, as well as cloning an HA-epitope tag to the DED1_{MC159} mutant. In future studies, the two chimeric mutants will be evaluated for additional immune evasion

functions attributed to either MC159 or MC160 including effects on apoptotic and NF- κ B signaling.

The second goal of this thesis was to identify the critical region of the DED1 of the MC160 protein. The MC160 DED1 is predicted to possess five α -helices. Each individual α -helix was removed, creating five total truncation mutants, $\Delta\alpha$ -1-5 (Figure 10). The analysis presented interesting preliminary data. The predicted $\Delta\alpha$ -2 resulted in a loss of function in both MAVS-mediated and TBK-induced activations of IFN β (Figure 17 & 18). This result would indicate that the α -2 that is required to for the MC160 protein to inhibit the MAVS-mediated IFN β pathway. Previous studies show there are key charge residues found within the α -2 region on the MC159 protein are required for its function in inhibiting activation of both apoptosis and NF- κ B signaling (Garvey et al., 2002; Yang et al., 2005; Fu et al., 2016; Randall et al., 2012). Furthermore, within this region lies a predicted glutamate (E24) that comprises part of the charged triads typical in many DED containing proteins. This charged triad is predicted to maintain local structural integrity of DED proteins such as MC159 and orient adjacent amino acids to interact with target proteins. However, a mutant MC160 proteins with alanine substitutions in the charged triad mutant maintained the ability to inhibit MAVS-mediated IFN activation as well as NF- κ B activation suggesting that the charged triad of MC160 may not be required as it is in MC159 (Beaury et al. 2017). However, given, that the MC160 delta 2 mutant lost the ability to inhibit MAVS-mediated activation of IFN β , it seems reasonable to speculate that one or more of these amino acid(s) may be required for MC160 to bind to target proteins.

With this preliminary data, future steps include, verification that the MC160 protein is in fact folding correctly, with the absence of the α -2 region. For this, we would test the constructs against TNF- α induced NF- κ B activation. We predict that it should still inhibit TNF- α induced NF κ B, due to the fact that the DED2 of the MC160 protein is required for NF- κ B inhibition, whereas DED1 is dispensable. The data presented above, leads to the observation, that DED1 of the MC160 protein does not fully inhibit the MAVS-mediated IFN β activation. Nevertheless, it still does inhibit at the level of TBK1-induced IFN β . The current hypothesis is that each of the two MC160 DEDs are key in inhibiting MAVS-mediated response pathways. MAVS signaling can trigger activation of both NF- κ B and IRF3. Therefore, the current data suggests that DED1 is required for the TBK1-mediated processes, whereas DED2 is needed for inhibition of NF κ B-mediated processes. Since the IFN β enhancer has both NF κ B and TBK1, for the MAVS pathway, it is required to have both, DED1 and DED2, to fully inhibit the MAVS signaling (Figure 19A). To support this, the data presented above, shows that TBK1 is fully inhibited by D1 (Figure 18). The significance of this is, that TBK1 induces only IRF3, however, MAVS can induce both NF κ B and IRF3 activities, and thus is only partially inhibited by D1 (Figure 17), whose main target is IRF3. In addition the C-terminus of MC160 also independently inhibits MAVS-mediated processes. In light of current data recent literature review, the working hypothesis is that the MC160 C-terminus binds to Hsp90 and thereby prevents the association of MAVS to downstream signaling kinases (Nichols et al., 2009). This model is currently being experimentally validated.

In conclusion, the above data implicates the second α -helix as a key functional region within the DED1 of the MC160 protein's function to inhibit the activation of Type I interferons. In addition, current data suggests that both DEDs of MC160 are necessary for complete inhibition of the MAVS signaling pathway. The next step will be to further verify this finding by creating an MC160 mutant that contains all predicted α -helices, except α -2 (Figure 19B). Future studies will concentrate on the identification and validation of suspected cellular targets of the MC160 DEDs. Based on data from Randall et al (Randall et al., 2013), we believe that target to be TBK1, at least for the MC160 DED1. The loss of function mutant identified in the current study can be utilized to determine if binding to TBK1 is indeed required for MC160's ability to inhibit IRF3 phosphorylation. Thus, this mutant can be used to ultimately verify one molecular mechanism by which MC160 targets the cell's innate immune signaling network to inhibit activation of type I IFNs.

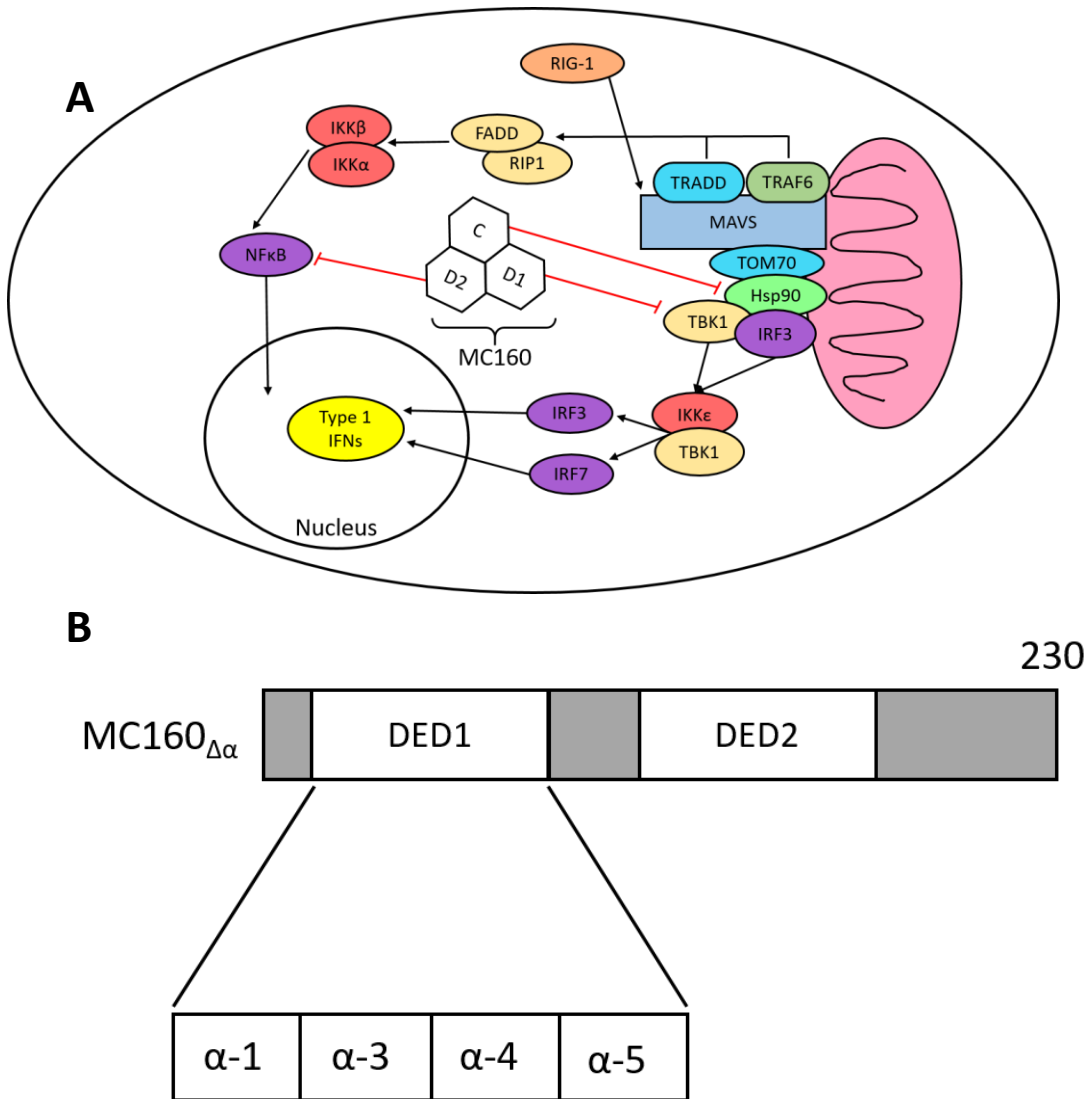


Figure 19: MCV MC160 protein interaction of the MAVS signaling pathway and Future Mutants. A) The DED1 of MC160 is predicted to bind, and inhibit the TBK1-induced activation of Type 1 interferons, especially IFN β . Furthermore, the DED2 of MC160 is required to inhibit NF κ B. Lastly, the C-terminus of the MC160 protein is predicted to bind to heat shock protein 90 (Hsp90), aiding in inhibition of the downstream targets of the intrinsic MAVS-mediated signal pathway. B) Proposed model of a future MC160 truncation mutant, denoted temporarily as MC160 $\Delta\alpha$, a.a. 1-230. This proposed model would be lacking the second α -helix, α -2.

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