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Functional Analysis of the Molluscum Contagiosum Virus MC160 Death Effector Domain-Containing Protein RxDL Motif

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Functional Analysis of the *Molluscum Contagiosum* Virus MC160 Death Effector Domain-Containing Protein RxDL Motif

By

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Abstract

The *Molluscum contagiosum* virus (MCV) is a member of the *Poxviridae* family that causes benign skin lesions. MCV lesions persist on average for 8-12 months in otherwise healthy individuals. MCV lesions are characterized by reduced inflammation. The persistence and reduction of inflammation at the site of MCV lesions have been attributed to MCV immune evasion genes. MCV encodes two death effector domain (DED) containing proteins, MC159 and MC160. DEDs are found in cellular proteins such as FADD and procaspase-8. These cellular proteins are involved in several innate immune responses such as apoptosis and activation of interferon (IFN). Presumably, MC159 and MC160 bind to host DED-containing proteins as a means to prevent the formation of innate immune signaling complexes. The RxDL motif is conserved among several host and viral DED-containing proteins and has previously been shown to be required for protein function. The hypothesis of this study was the MC160 protein requires the RxDL motif to inhibit the activation of host inflammatory pathways. MCV mutants with mutated RxDL motifs were assessed for the ability to inhibit TBK1- and MAVS- induced activation of interferon-β. Surprisingly, the RxDL mutants retained the ability to inhibit IFN-β activation as assessed by the activity of a firefly luciferase gene under the control of the IFN-β enhancer. Therefore, the RxDL motif of the MC160 protein is not required for the inhibition of IFN activation.

Introduction

The *Molluscum contagiosum* virus (MCV) is a member of the *Poxviridae* family. Since the eradication of the variola virus, the causative agent of smallpox, MCV is the only poxvirus that strictly infects humans. MCV causes benign neoplasms that persist from months to years and are characterized by reduced inflammation (Shisler & Moss, 2001). In immunocompromised patients, MCV infections tend to be more severe and result in the formation of larger and more numerous than normal neoplasms (Theiler et al., 2011; Chularojanamontri et al., 2010; Drain et al. 2014). The virus spreads primarily through skin to skin contact, including sexual activity. MCV can also spread by autoinoculation. Poxviruses typically have a broad tissue or host tropism (Randall & Shisler, 2013). In contrast, MCV only infects epidermal keratinocytes with 122 million cases reported worldwide as of 2012 (Hay et al., 2014).

Presently, MCV has no FDA approved cure, no vaccine, and no rapid diagnostic tool. The most common treatment for MCV includes administration of cantharidin or imiquimod, cryotherapy, curettage, and cidofovir (Coloe & Morrell, 2009). The problems associated with these physical treatments are blistering or post-treatment pigment changes, anxiety and pain. Cidofovir can cause kidney damage or failure as well as a number of other undesirable side effects for the patient. However, none of these treatment methods are 100% effective for curing MCV lesions and there can be reoccurrence of the virus (Shisler, 2015).

Despite its prevalence, MCV remains under researched. MCV cannot be propagated in tissue culture. Despite this limitation, the genome of MCV has been sequenced. MCV is predicated to encode 77 potential host interacting proteins (Senkevich et al., 1996). Six of these proteins have been characterized in various host signaling pathways. The immune evasion proteins of MCV have been characterized as MC159, MC160, MC54, MC148 and MC007 (Randall & Shisler, 2013). MC54 inhibits interleukin-18 by binding to interleukin-18 (Xiang & Moss, 2003). MC148 binds to CCR8 and CxC12α and inhibits chemotaxis (Jin et al., 2011; Luttichau et al., 2001). MC007 inhibits a tumor suppressor protein, Retinoblastoma protein (pRb) (Mohr et al., 2008). MC66 inhibits apoptosis by inhibition of hydrogen peroxide and UV light (Shisler et al., 1998). The immune evasion protein MC159 inhibits apoptosis and Nuclear Factor-kappa B (NF-κB). MC160 has shown no anti-apoptotic function but does inhibit NF-κB and will be discussed in detail later (Shisler, 2015).

Immune evasion molecules antagonize immune responses to neutralize the host antiviral state to allow for viral proliferation. MCV upregulates the expression of Toll-like receptor (TLR) 3 and 9 which detect viral RNA and DNA within the cell and tumor necrosis factor (TNF) and IFN-beta (IFN-β) are highly expressed in the infected neoplasm and surrounding tissue (Ku et al., 2008). MC159/MC160 has already been shown to dampen the activation of type I interferon (IFN) transcription factors. The upregulation of these proinflammatory genes suggests MCV lesions would be highly inflamed. However, the lack of

inflammation at MCV neoplasms indicates the initial infection is not sufficient to induce the innate immune system. Immune cells involved in the inflammatory response such as T cells, natural killer cells, and Langerhans cells are not recruited to the site of MCV infections (Viac & Chardonnet, 1990). Within the cytoplasm, poxvirus transcription produces dsRNA as a transcriptional byproduct (Willis et al., 2011). dsRNA induces several pattern recognition receptors (PRRs) which in turn induce the expression of interferon beta (IFN-β) through activation of interferon response factor 3 (IRF 3). However, like other members of the *Poxviridae* family, MCV viral proteins antagonize the host's innate immune response.

The immune evasion proteins of MCV, MC159 and MC160, are homologs of the cellular Fas-associated death domain-like interleukin-1-beta converting enzyme (FLICE)-like inhibitory protein (FLIP) family of proteins (Fig. 1) (Randall & Shisler, 2013). Included in this family of proteins are viral FLIPs (vFLIP) such as the Kaposi's sarcoma-associated herpesvirus (KSHV) K13 protein and the cellular FLIP (cFLIP) proteins of which there are three variants: cFLIP long ($cFLIP_L$), cFLIP short ($cFLIP_S$), and $cFLIP_R$. Both vFLIPs and cFLIPs are procaspase-8 and-10 homologues that lack an active site and are therefore catalytically inactive. The hallmark of the FLIP family of proteins is the presence of tandem death effector domains (DEDs) (Fig. 1) (Li et al., 2006). DED-containing proteins interact with various signaling pathways to mediate biological

processes such as apoptosis and activation of proinflammatory transcription factors IRF-3 and NF-κB (Garvey et al., 2002A; Shisler, 2014).

The death-inducing signaling complex (DISC) is formed when a death receptor receives a signal mediated by highly specific protein-protein interactions that generate oligomeric signaling. DISC is comprised of Fas, the Fas Receptor, Fas-associated death domain (FADD), and caspase-8 or -10 are assembled via homotypic associates between the death domains of the Fas Receptor, FADD and the DEDs of FADD and the pro domain of caspase-8 and -10 (Randall et al., 2012; Murao & Shisler, 2005). The interaction between FADD and the DED-containing pro-domain of caspase-8 initiates proteolytic auto-processing of procaspase -8 and -10 resulting in the activation of effector caspase -3 and -7 will result in the commitment of the cell to apoptosis.

Both vFLIPs and cFLIPs inhibit cell death by preventing proteolytic cleavage and subsequent activation of procaspase-8 after it associates with FADD, thereby inhibiting the formation of a functional DISC (Chaudhary et al., 2000; Shisler & Moss, 2001; Garvey et al., 2002A). MCV MC159 and MC160 vFLIPs bind FADD and procaspase-8 presumably through interactions with DEDs (Shi et al., 2006; Thome et al., 1997; Shisler & Moss, 2001; Shisler & Nichols, 2009). However, while expression of the MC159 protein protects cells from Fas-mediated apoptosis, MC160 expression does not (Randall et al., 2014).

Most single and tandem DEDs contain a highly conserved charged triad (Yang et al., 2005). The charged triad is a unique feature of DEDs and is a

network of hydrogen bonds between side chains of charged residues composed of E/D-RxDL and is highly conserved amongst DED containing proteins (Twomey et al., 2013). This triad is homologous amongst DED containing proteins such as MC160, MC159, procaspase-8 and procaspase-10 (Fig. 1A). The crystal structure of MC159 revealed this hydrogen bonded charged triad on the surface of DED 1 and DED 2 where the triad is referred to as E/D-RxDL where x represents a variable amino acid (Yang et al., 2005). The hydrogen bonds formed between the three amino acids are believed to be involved in maintaining a precise organization of the side chains and a local role in maintaining the conformation of this region of the DED. The bonds form between the Asp and Arg residues on helix H6 and negatively charged residues in helix H2 of the RxDL motif (Yang et al., 2005). In caspase-8, DED2 the E/D-RxDL motif has a Glu Lys and Ser at the three charged residue positions (Fig. 1B). This could be due to the change of Arg to Lys lowers the hydrogen bonding potential so that it now only interacts with one negatively charged residue in the triad. MC159 RxDL mutants lost the ability to antagonize apoptosis induced by Fas (Garvey et al., 2002B; Yang et al., 2005). These DEDs bind to different proteins and kinases in the signaling pathway to initiate either the expression of NF-κB for a proinflammatory response or interferon-β for initiating an antiviral response (Valmiki & Ramos, 2008).

Figure 1: The alignment of different DED containing proteins to show the homology between the MCV immune evasion proteins MC159, MC160 and cellular proteins.

DED-containing proteins, such as FADD, also bind to various proteins and kinases the signaling pathways to initiate the interferon (IFN) antiviral response (Valmiki & Ramos, 2008). Type I IFNs, such as IFN-α and IFN-β, are responsible for activating the antiviral state within infected host cells. The innate immune response utilizes type I IFNs to stimulate the activity of natural killer cells, antibody-dependent cytotoxicity, and T suppressor cells (Müller et al., 1994). The expression of IFN-β can be achieved by many different signaling pathways such as retinoic inducible gene I (RIG-1) and melanoma differentiated associated gene 5 (MDA5) (Kalai et al., 2008). The upstream cellular sensors for cytosolic dsRNA trigger different signaling pathways, such as sensors that activate IFN-β and NF-κB. RIG-1 will interact with procaspase-8 to trigger mitochondria associated viral signaling (MAVS) adaptor protein activation by binding at CARDs (Takeuchi & Akira, 2008; Kawai et al., 2005; Johnson & Gale, 2006; Roth & Ruland, 2011). MAVS will form a signaling complex with FADD, TNF receptor associated factor 3 (TRAF3) adaptor protein, TRAF family member associated NF-κB activator (TANK), and tumor necrosis factor receptor type I associated death domain (TRADD) to activate Interferon Regulatory Factor (IRF)-3 and NF-κβ. Tank will bind TRAF3 and TRAF2 recruit TANK binding kinase 1 (TBK1) and I kappa Beta kinase ε (IKKε) phosphorylation of IRF3. IRF3, NF-κB, and activating protein 1 (AP-1) induce the expression of type I IFNs (Randall & Shisler, 2014). Expression of both MC159 and MC160 block MAVS- and TBK-1 induced activation of IFNβ. MC159 associates with TBK1 and IKKε. However, MC160

did not associate with either kinase (Randall et al., 2014). Given the known association with MC160 and FADD, one possibility may be that MC160 interacts with FADD to inhibit the formation of MAVS-signaling complexes. However, the functionality of the MC160-FADD interaction has not been evaluated. MC159 inhibits IRF3 by binding to the TBK1:IKKε complex (Fig. 2).

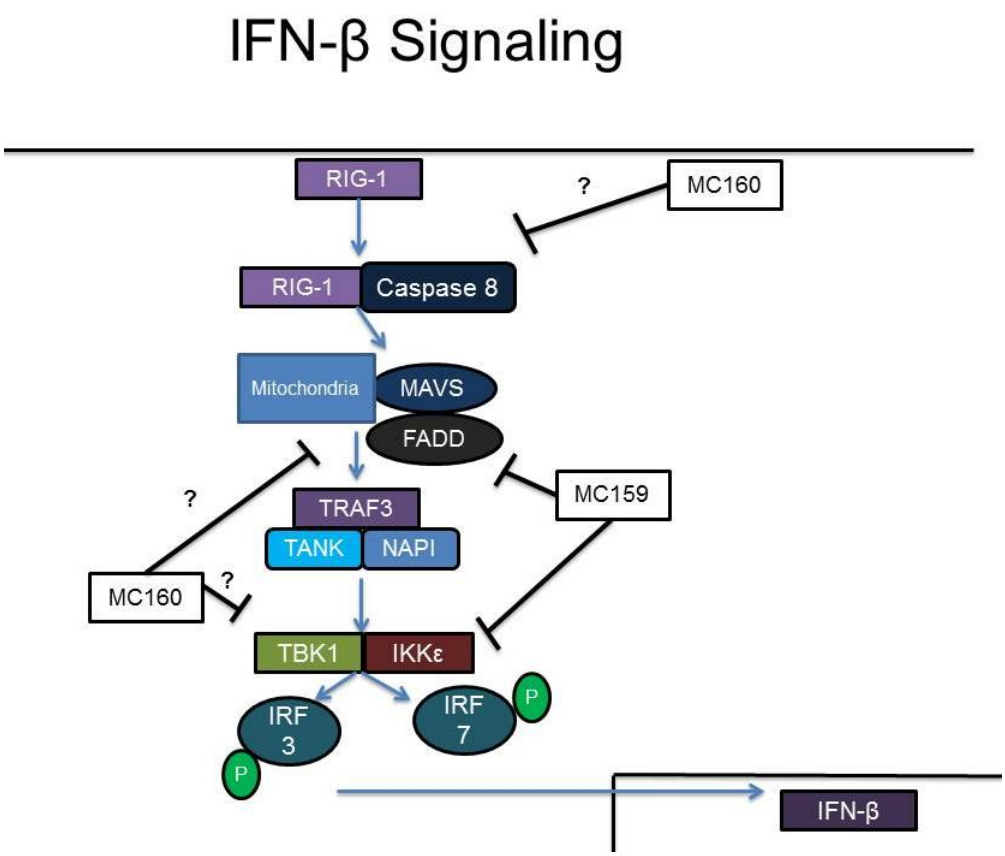

Figure 2: The signaling of IFN-β by viral sensors in the cytoplasm. The viral dsDNA is converted by RNA polymerase III to 5'-triphosphate RNA which will induce IFN-β production through RIG-1 and (MDA5). RIG-1 and MDA5 will activate MAVS through CARD-CARD interactions. MAVS will then recruit TRADD and TANK to bind TRAF3 and TRAF2 which promotes TBK1 and IKKε phosphorylation of IRF3 that induces the expression of type I interferon genes (IFN-β).

Both MC159 and MC160 expression dampen tumor necrosis factor (TNF)-induced NF-kB activation. TNF-α is a proinflammatory cytokine involved in apoptotic signaling, activation of Mitogen-activated protein kinase and NF-κβ (Murao & Shisler, 2005). Many poxviruses have developed mechanisms for inhibiting TNF-α mediated cytotoxicity to prevent cytokine regulatory cascades. Once the tumor necrosis factor receptor (TNFR) is bound to TNFα, a signaling complex composed of tumor necrosis factor receptor type I associated death domain (TRADD), TRAF2, and receptor interacting protein (RIP) is assembled (Hayden & Ghosh, 2008; Li et al., 2013). The signalsome subsequently recruits the IKK complex consisting of kinases IKKα and IKKβ and the regulatory IKKγ. The IKK complex induces phosphorylation of the IκB inhibitory protein. IκB sequesters the NF-κB transcription factor in the cytoplasm. Upon phosphorylation, IκB is tagged for ubiquitination and degradation which frees NF-κB allowing it to translocate to the nucleus and induce expression of proinflammatory cytokines and apoptosis (Hu et al., 2000; Bhoj & Chen, 2009). A second signaling complex consisting of ubiquinated RIP-1 binding to a signaling complex consisting of TRADD, FADD, procaspase-8 the NF-κβ-IKKβ and IKKα complex (Grunert et al. 2012). This signaling complex can signal the activation of either apoptosis or NF-κB depending on whether procaspase-8 undergoes proteolytic cleavage or is inhibited by binding to cFLIP. MC159 inhibits NF-κB activation by binding to TRAF2 and IKKγ (Shisler & Murao, 2005; Randall et al., 2014). MC160 also inhibits TNF-induced NF-kB activation. MC160 associates

with procaspase-8 and may also inhibit the formation of the IKK complex, presumably by disrupting IKKα and heat shock protein (Hsp) 90 interactions. The MC160 DED 2 is sufficient for this inhibition (Nichols & Shisler, 2009).

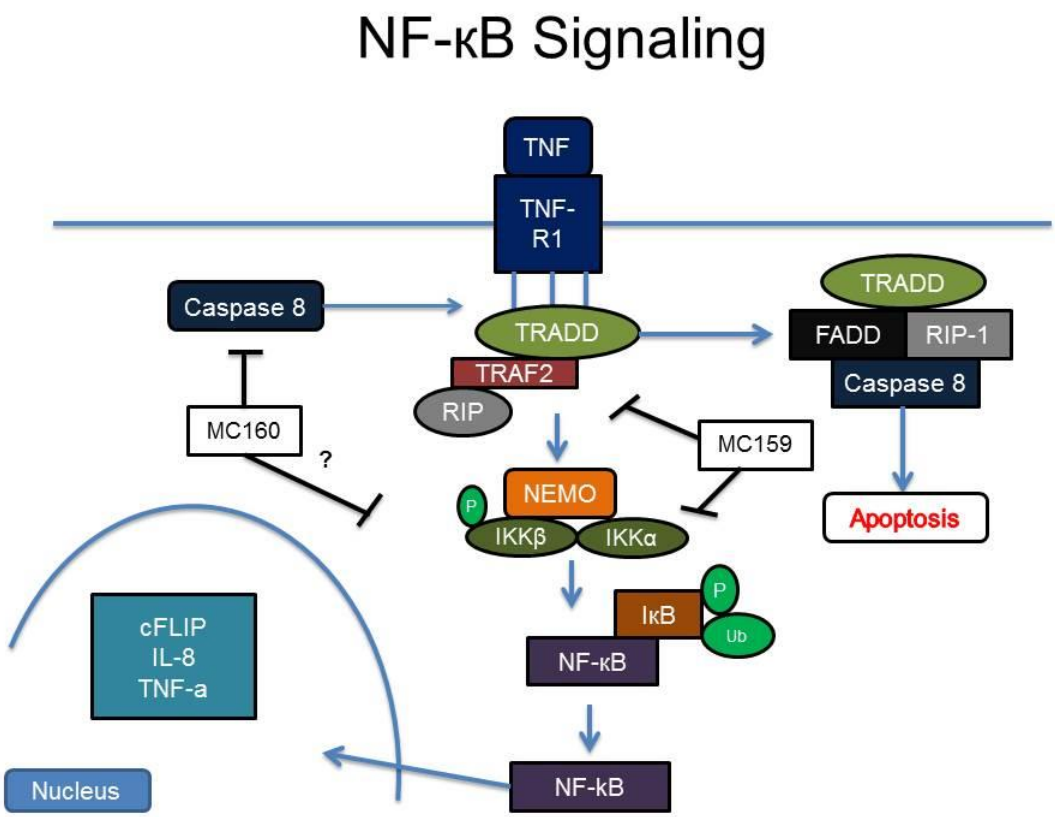

Figure 3: NF-κB signaling when stimulated by cytokines through binding of the TNF-α receptor. Upon ligand binding, Tumor necrosis factor receptor 1 (TNF-R1) binds the homologous domain of TRADD, a death domain containing cytoplasmic adaptor protein. TRADD activates a kinase cascade by recruitment of RIP, a death domain containing protein, and TRAF2. The DED of FADD binds to the N-terminal prodomain of caspase-8 which will then induce apoptosis. If FADD is not recruited to the complex then the signaling complex of RIP, TRAF2, and TRADD will activate NEMO which will then cause phosphorylation dependent ubiquitination of IkB freeing NF-κB to translocate to the nucleus and induce the expression of proinflammatory cytokines.

The purpose of this thesis was to analyze the RxDL motif of MC160 in various signaling pathways that activate IFN-β and NF-κB.The MCV protein MC159 when mutated at the RxDL motif resulted in a loss of function in apoptotic pathways. Due to the homology between MC159 and MC160 and the conserved nature of the RxDL motif amongst DED-containing proteins the working hypothesis is that the RxDL motif of MC160 is required for the function of MC160 as a means of host inflammatory pathways (Garvey et al., 2002B). A loss of function MC160 mutant could yield valuable clues on the molecular mechanism utilized by the MC160 protein to inhibit host innate immune responses. Additionally, the role of the RxDL motif will be characterized in TBK1- and MAVS-induced IFN-β signaling. The RxDL motif was mutated within each of the DEDs and then tested in TBK1- and MAVS-induced IFN-β and RIP-1- and procaspase-8-induced NF-κB signaling pathways. The mutations were generated in the first DED RxDL motif at R67AD69A and the second DED RxDL motif at R160AD162A (Fig. 4). An RxDL double mutant was generated in order to test both RxDL motif changes.

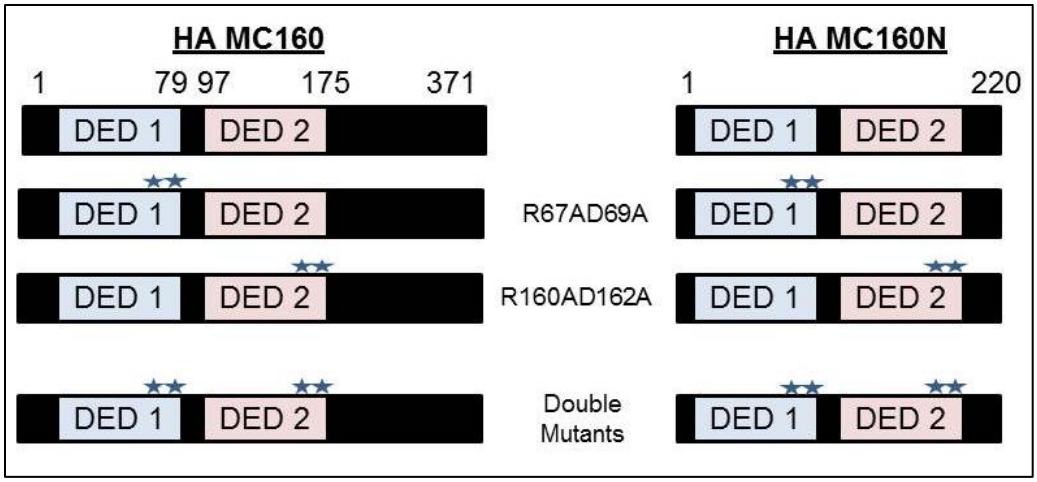

Figure 4: The mutations generated on the RxDL motif of the tandem death effector domains present on HA MC160 and the N-terminus of HA MC160 missing the C-terminus. The mutations were generated on the charged triad at R67AD69A and R160AD162A for a single mutation test and a double knockout generated with both R67AD69A and R160AD162A with the positively charged arginine replaced with alanine which has a neutral charge. The relative area of the RxDL motif for each DED changed is indicated with (★).

Materials and Methods

DNA Preparations

Bacteria with plasmid inserts were cultured in a BD Falcon tube with 3 milliliters of Luria broth (Sigma Aldrich) with 100µg/mL of ampicillin (Amp) at 37°C rocked at 225 rpm in the Incu-Shaker Mini. The cultures were allowed to grow between 16-19 hours prior to extracting the DNA. The cultured bacteria were concentrated by centrifugation at 14,000 rpm for 1 minute in an eppendorf tube and then re-suspended in nuclease free water and combined in one eppendorf tube. The DNA was then harvested following the manufacturer's protocol for the PureYield Plasmid Miniprep System (Promega). All plasmids were generously donated by Dr. Joanna Shisler (University of Illinois). The plasmids used were MAVS, TBK1, IFN-β-luciferase, NF-κB-luciferase, pHA-MC160, pHA-MC160N, pCI, pRenilla TK, C360S, RIP-1, pHA-MC160C, and pHA-MC160 DED 2.

Mutagenesis

The pHA-MC160 or pHA-MC160N, a truncated version of pHA-MC160 composed of the DED containing N-terminus; DNA was diluted to 10 ng from a DNA stock and used in PCR. Sequence changes were made using primers created based on the known sequence of pHA-MC160 or pHA-MC160N to change the amino acid sequence from arginine/aspartate to alanine. The cycling conditions for each mutant are listed in Table 1 and the primers used to generate each mutant are listed in Table 2. All of the PCRs used to generate the mutants

were run with equal parts of forward and reverse primers at 10 µM. Reactions were run following the manufactures' protocol for the Phusion High Fidelity kit (New England BioLabs). The fragment sizes were verified by gel electrophoresis on a 0.6% agarose gel run with 1X Tris-acetate buffer (TAE) made from a 50X stock of TAE (2.0 M Tris-Acetate, 0.5 M Ethylenediaminetetra acetic acid (EDTA), pH 8.3 and nuclease free water up to a liter). After the fragment sizes were verified the DNA was either PCR purified or gel extracted following the manufacturers protocol (Promega). Then the forward and reverse fragments were used as the DNA template in the PCR and fused together using the NFor and CRev (pHA-MC160) or NFor and FRev (pHA-MC160N) as shown in Figure 5.

Table 1: The optimized cycling conditions for the PCR reactions for R67AD69A and R160AD162A mutations to pHA-MC160 or pHA-MC160N DNA. These conditions were used to generate the forward and reverse mutated fragments and then to anneal the mutated fragments together and generate the entire mutated sequence.

Cycle Steps	Cycles	Temperature	Time
Initial Denaturation	**1**	**98°C**	**30 seconds**
Denaturation	**30**	**98°C**	**10 seconds**
Annealing	**30**	**60°C**	**15 seconds**
Extension	**30**	**72°C**	**10 seconds**
Final Extension	**1**	**72°C**	**10 min**
Hold	**1**	**4°C**	**∞**

Table 2: The created primer sequences based on pHA-MC160 or pHA-MC160N to change the amino acid sequence in the regions of R67AD69A and R160AD162A to an alanine and control primers for pHA-MC160 or pHA-MC160N. The underlined nucleotides represent the changed nucleotides. Sequencing primers were used to sequence the HA-MC160, HA-MC160N, abd RxDL motif mutants DNA. The primers, pCIseq, HA-MC160 509-528 Reverse (Rev), and HA-MC160 509-528 Forward (For) were created to sequence the mutated regions, R67AD69A, R160AD162A, and double mutants, to confirm the changes were present in comparison to the wild-type sequence of HA-MC160 or HA-MC160N.

Primer Name	**Primer Sequence**	**DNA Sample Used**
HA-MC160 NFor	5’ CGA GAA TTC GCC ACC ATG TAT CCA 3’	HA-MC160 AND HA-MC160N
HA-MC160 DED 2 Rev	5’ GGA AAG AGC ATA AGC GCA AAC GGC 3’	HA-MC160 AND HA-MC160N
HA-MC160 CRev	5’ CGT CTA GAC GCT CGC TAG TAG G 3’	pHA-MC160
HA-MC160 DED 2 For	5’ GCC GTT TGC GCT TAT GCT CTT TCC 3’	pHA-MC160 AND pHA-MC160N
HA-MC160 DED 1 Rev	5’ GAG AAC AGC AAA AGC CCG GAG C 3’	pHA-MC160 AND pHA-MC160N
HA-MC160 DED 1 For	5’ CGC TCC GGG CTT TTG CTG TTC TCA AG 3’	pHA-MC160 AND pHA-MC160N
HA-MC160 FRev	5’ GGT CGA CTC TAG ATT ACC CCG C 3’	pHA-MC160N
pCIseq	5’ GTC CAC TCC CAG TTC AAT TAC AG 3’	-
HA-MC160 509-528 Rev	5’ CAC GGA AAG ATC GTA TCT GC 3’	-
HA-MC160 509-528 For	5’ GCA GAT ACG ATC TTT CCG TG 3’	-

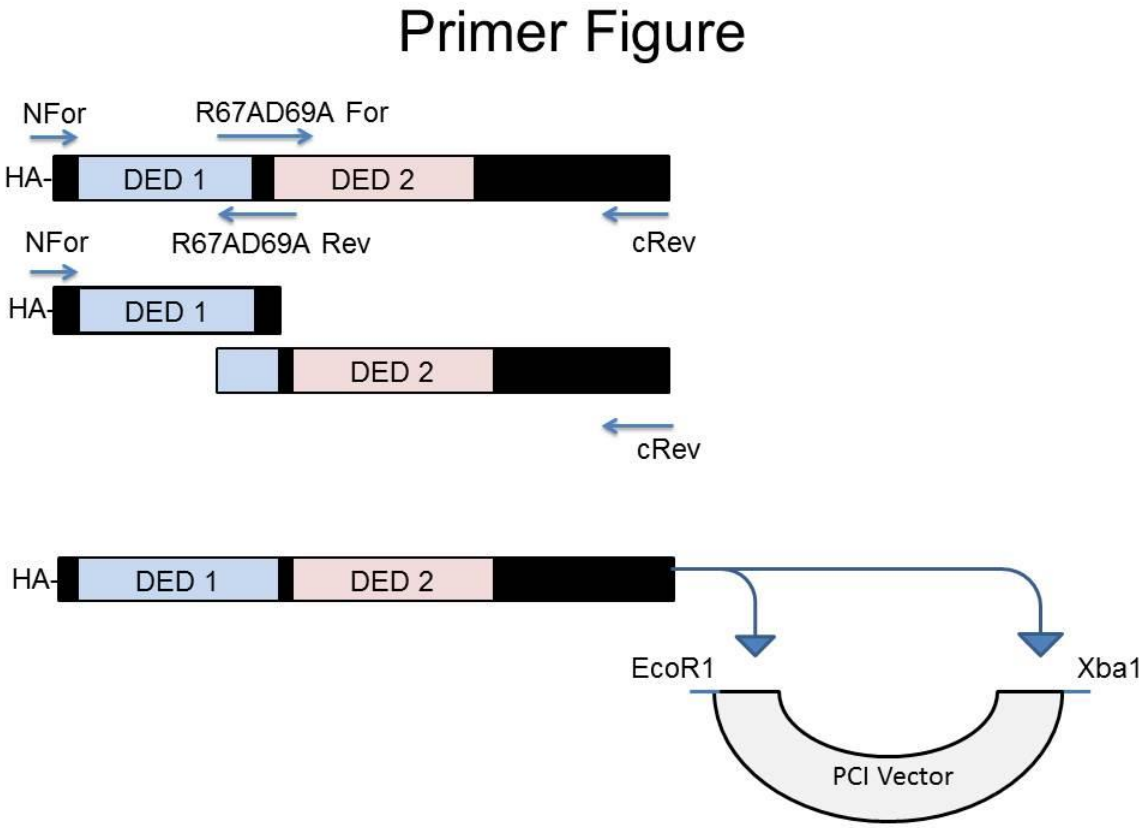

Figure 5: An example of the process of mutagenesis in order to change the RxDL motif of pHA-MC160 using the primers for R67AD69A. The HA tag and restriction enzymes sites were on the primers. Once the entire strand is formed with the mutation, the DNA was enzyme digested with Xba1 and EcoR1 to linearize the vector, pCI, to allow for the insert to be ligated into the vector.

Then 1 µg of mutated pHA-MC160 or pHA-MC160N DNA samples were restriction enzyme digested with Xba1 and EcoR1 to linearize the DNA. A pCI mammalian expression vector (Promega) was used as the vector. pCI contains the human cytomegalovirus immediate-early gene enhancer/promoter region and promotes constitutive expression of the cloned DNA inserts in mammalian cells. The vector pCI and all mutant DNA samples were restriction enzyme digested to linearize the DNA. In an eppendorf tube 5 µL of 2.1 buffer (New England BioLabs), 1 µL of Xba1 enzyme (New England Bio Labs), and nuclease free water (Promega) were combined for a total volume of 50 µL. The tubes were placed in a tube holder and incubated at 37°C water bath for 90 minutes to digest.

The 2.1 buffer was converted to EcoR1 buffer by adding the following to each of the sample tubes: 3 µL of 1.5 M Tris pH 7.9, 1.25 µL of 1% Triton, and 1 µL EcoR1 enzyme (New England BioLabs). The tubes were placed in a tube holder and incubated at 37°C water bath for 90 minutes to digest. All of the samples were then loaded into a 0.6% agarose gel and ran at 100V for 30-45 minutes with 1 kb ladder (New England BioLabs). The fragments were gel extracted per manufacturers protocol (Promega) using a UV illuminator.

The linearized RxDL mutant pHA-MC160 and pHA-MC160NDNA was ligated into the pCI vector using T4 ligase (New England Biolabs) with a 3:1 insert to vector molar ratio of restriction digested DNA. Combined in each eppendorf tube was 2 µL of T4 ligase buffer, 1 µL T4 ligase, 1X restricted digested pCI DNA, 3X

restriction digested mutated pHA-MC160 DNA, and nuclease free water to 20 µL. The reaction was allowed to run between 10-15 minutes at room temperature.

After the ligation reaction, 40 µL of DH5α competent cells (Life Technologies) and 5 µL of the ligation reaction were combined and placed on ice for 30 minutes. Then heat shocked for 45 seconds in a 42°C water bath; and placed back on ice for 2 minutes. 900 µL of S.O.C media (Life Technologies) was added to each BD Falcon tube and then placed in a 37°C Incu-Shaker Mini for an hour. From each tube 50 µL was plated on a Luria broth agar plates containing 100 µg/mL ampicillin and grown between 16-21 hours in a 37°C incubator. Isolated colonies were extracted and grown (Page15 DNA Preparations). The samples were restriction enzyme digested with Xba1 and EcoR1 (Page 20) to verify the correctly sized insert was present.

Samples with the correct size insert were sent for sequencing to Genewiz Incorporated. In a PCR strip each tube contained 500 ng/ µL of sample DNA, 5 µM of primer (Table 2; pCIseq, HA-MC160 509-528 Rev, HA-MC160 509-528 For), and nuclease free water to 15 µL. After the samples were processed, the sequences were compared to the control of pHA-MC160 or pHA-MC160N sequences and analyzed using Clone Manager. The chromatograms for the generated sequences were analyzed with Chromas Lite.

Cell Culture

The human embryonic kidney 293T (HEK 239T) cells were obtained from ATCC. The murine embryonic fibroblast wild-type (MEF) cells were obtained from

Laurent Poliquin (University of Quebec, Montreal). Cells were cultured in Dulbecco's Modified Eagle's Media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained in T75 flasks in a humidified incubator at 37°C with an air saturation of 6.0% CO_2. Once cells reached at least 80% confluence as observed under the microscope, cells were passaged at either 1:5 or 1:10.

Luciferase Assays

In 12 well plates, HEK 293T or MEF wt cells were plated at 2.0 x 10^5 cells per well. The wells were then transfected with 500 ng of plasmid DNA, 500 ng MAVS, TBK1, RIP-1 wt or 250 ng C360S, 225 ng reporter mix of IFN-β-luciferase or NF-κB-luciferase and 25 ng of Renilla TK, and 3.75 µL of Mirus Trans-IT 2020 reagent (Mirus) or 3 µL of P3000 and 4.5 µL of Lipofectamine 3000 (Invitrogen). At 100% confluence the cells were harvested between 24-52 hours post-transfection using 100 µL of 1X passive lysis buffer. The plates were rocked on a BioRocker for 20-30 minutes and then 10 µL of each of the lysates were assayed for sea pansy and firefly luciferase activity by using the Dual-reporter assay (Promega).

After rocking, 10 µL of lysate from each well were placed in a 96 well opaque plate (Costar) and firefly luciferase substrate was added to each well. The luciferase activity was measured as relative light units using the SpectraMax M5 reader and analyzed with SoftwareMax Pro. After the firefly readings were saved, sea pansy luciferase substrate was added to each well and read. All

assays were performed in triplicates. For each experimental point, firefly luciferase activity was divided by sea pansy luciferase activity to correct for differences in transfection efficiencies. The resultant ratios were normalized to those of the appropriate control cells, consisting of cells co-transfected with pCI, IFN-β-luciferase or NF-κB-luciferase, and pRenilla TK null, whose value was taken as 1. Results were displayed as relative change in luciferase activity compared to pCI-transfected cells. Statistical significance was determined by using two tailed t test with significance set at $p< 0.05$.

Immunoblotting

HEK 239T cells were plated at 3.0×10^5 cells per well in 6 well plates. The wells were transfected with 1 µg of plasmid DNA, 100 µL OptiMEM (Life Technologies), and 3 µL of Mirus Trans-IT 2020 reagent. After transfection the cells were harvested at 24 hours post-transfection. The media was aspirated off the plate from each well and each well was washed with 1 mL of cold phosphate buffered solution (PBS). Cells were removed from the plate using a cell scraper, then centrifuged at 14,000 rpm at 4°C for 30 seconds. After the PBS was removed, the cells were suspended in 100 µL of Death Effector Domain (DED) lysis buffer (buffer (140 mM NaCl, 10 mM Tris [pH 7.2], 2 mM EDTA, and 1% NP-40) with protease inhibitors (Sigma Aldrich). The cells were incubated on ice for 30 minutes, then centrifuged at 14,000 rpm at 4°C for 10 minutes. The lysates were removed from the pelleted cell debris.

To each sample 20 µL of sample buffer [6X SDS-PAGE (0.375 M Tris pH 6.8, 12% SDS, 60% glycerol, 0.06% bromophenol blue)] with 5% 2-Methylcaptoethanol was added and placed in a heat block between 95°C-100°C for 5 minutes and boiled. The lysates were resolved on a 10% SDS-PAGE. In each sample well 12.5% of prepared sample was added. In the first well 10 µL of precision protein ladder (Bio Rad) was loaded. The gel was run using 1X SDS-PAGE (0.25 mM Tris, 1.92 mM glycine, 0.001% SDS, and deionized water). The gel was run at 120 Volts for 45-60 minutes. The proteins were transferred to a nitrocellulose membrane using transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) and transferred at 90V for 60 minutes.

After the membranes were blocked between 30 minutes and 24 hours in 1X Tris buffered saline with Tween® 20 (TBST (0.01 M Tris pH 7.5, 0.15 M NaCl, 0.05% Tween-20® (USB)) and 5.0% milk (Carnation Non-Fat Dry Milk).The blocking solution was removed and primary antibody, monoclonal mouse anti-HA (Sigma Aldrich) was diluted 1:5000 in 1X TBST with 0.5% milk and blotted for 1 hour while rocked (BioRocker). All mutant MC160 proteins express a HA-epitope tag at the N-terminus of the protein. After an hour the primary antibody was removed and the membrane was washed with 0.5% milk 1X TBST three times for 10 minutes. The secondary antibody, goat anti-mouse (Sigma Aldrich) was diluted 1:2500 in 0.5% milk 1X TBST and blotted for 1 hour while rocked. The membrane was washed three times with 0.5% milk 1X TBST for 10 minutes after the antibody solution was removed and rocked. The membranes were then

removed from the 0.5% 1X TBST solution and blotted dry prior to being placed in ECLC solution (Pierce) to soak. The bands were visualized using chemifluorescence on a STORM860 scanner and ImageQuant software.

Results

Protein alignment of MC159 and MC160

The RxDL motif has been shown to be required in MC159 to inhibit apoptosis (Garvey et al., 2002B). The RxDL motif of MC160 was analyzed in this thesis in IFN and NF-κB signaling pathways. The amino acid residues of the MC159 and MC160 DED regions were aligned using Clone Manager (Figure 6). The charged triad is highly conserved between DED-containing proteins. MC159 and MC160 both contain the RxDL motif.

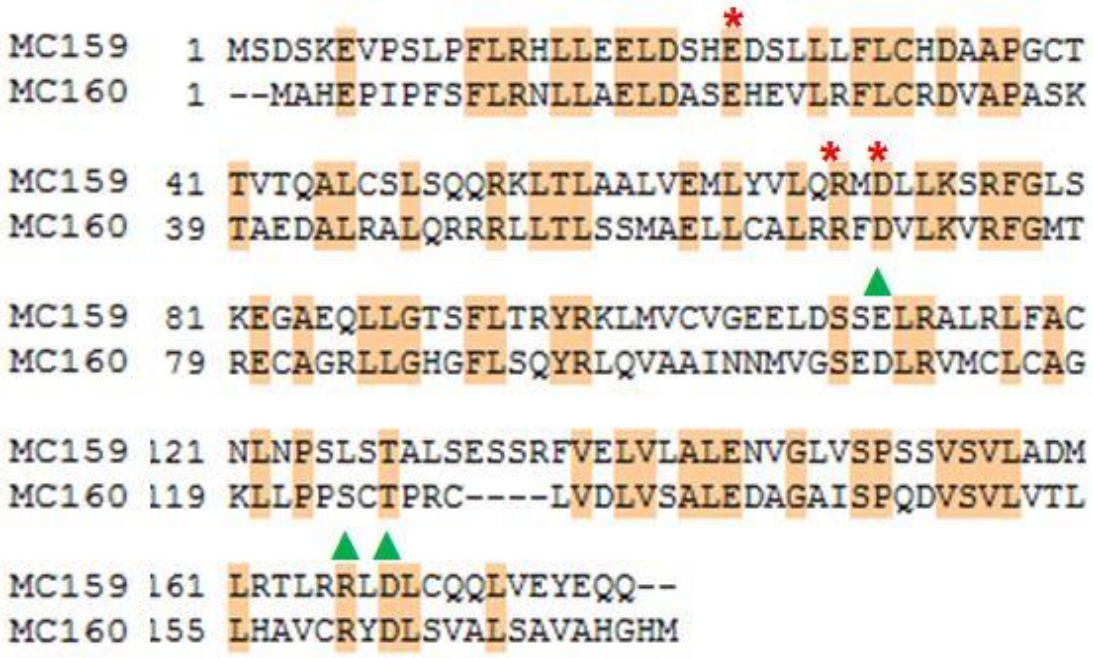

Figure 6: The protein alignment of MC159 and MC160, DED-containing amino acid residues. The RxDL motif for DED 1 are indicated with (*) and DED 2 are indicated with (Δ).

Generation of RxDL mutants by site directed mutagenesis

The RxDL motif has previously been shown to be involved in the immune evasion of pHA-MC159 (Garvey et al., 2002B). In the previous study by Garvey (2002B) changing an arginine/aspartate in the RxDL motif to alanine resulted in a loss of function in apoptotic signaling. Due to the homology between pHA-MC159 and pHA-MC160 and the conservation of the RxDL motif amongst DED containing proteins it was hypothesized this mutation would also result in a loss of function in pHA-MC160 in the IFN-β and NF-κB signaling pathways.

The pHA-MC160 RxDL mutants (R67AD69A, R160AD162A and double mutants) were generated using site directed mutagenesis by PCR. After which, the mutants were sent to Genewiz, Inc for sequencing to verify the nucleotide sequence. The sequencing data was analyzed based on protein alignments and chromatograms. The mutants were sequenced using a pCIseq and two internal primers (Table 2). The chromatograms for pHA-MC160 RxDL mutants all showed the desired changes in the DNA, shown by the low background and high intensity peaks (Fig. 7). The first change underlined (GCT) was the nucleotide sequence changed in the DNA to switch arginine/aspartate to alanines. There was a hairpin structure in the primer for the second DED that was removed by changing A to C and C to T on the RxDL mutants. The second change was done in order to remove a hairpin structure that would have complicated the process of creating the mutant. The wild-type sequences were displayed above the chromatograms.

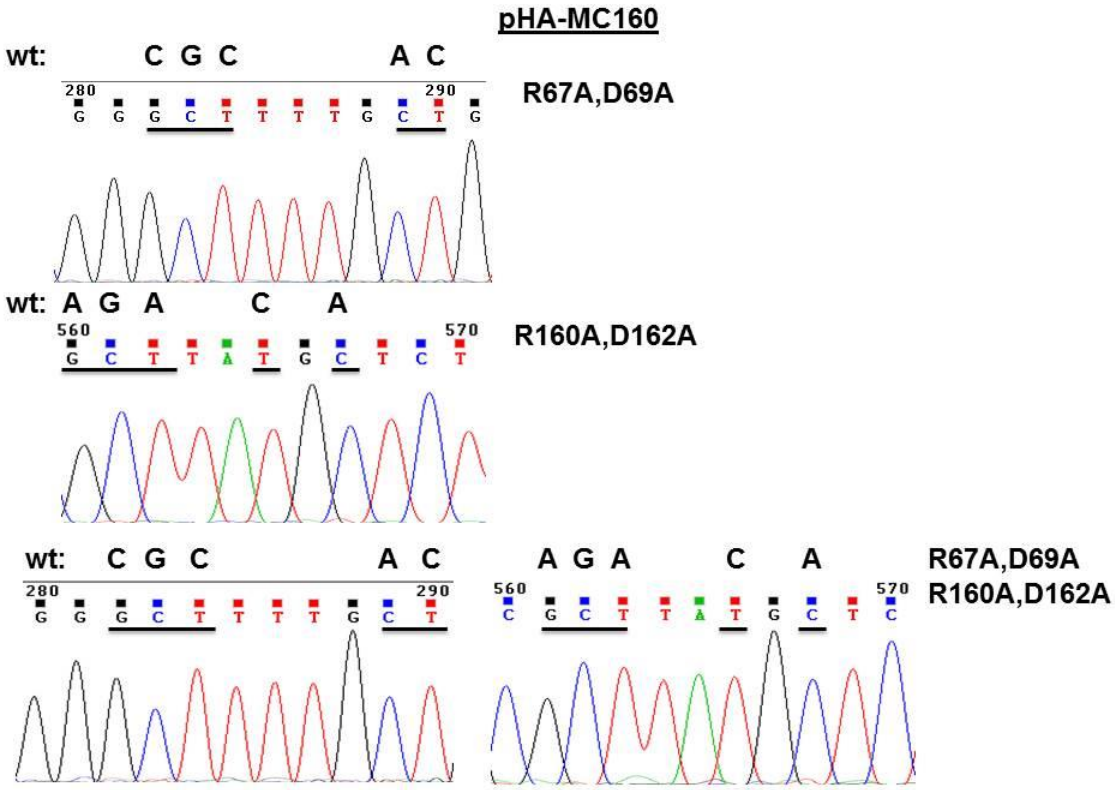

Figure 7: The chromatograms for the mutated pHA-MC160 to verify the nucleotide sequence was changed to code for a neutral alanine rather than a positive arginine on the charged triad, RxDL motif for the first DED R67AD69A, the second DED R160AD162A, and the double mutant with both DEDs changed. The second change was done in order to remove a hairpin structure that would have complicated the process of creating the mutant. Above all of the chromatograms the wild-type (wt) that were changed using the primers listed in Table 2 and sequenced using the primers listed in Table 3 sequences are displayed in bold.

The C-terminus of pHA-MC160 has activity in some of these signaling pathways such as IFN-β and NF-κB (Shisler and Nichols, 2009). To distinguish the activity of only the DED-containing N-terminus from the C-terminus additional mutants were generated using pHA-MC160N which is the DED-containing N-terminus of pHA-MC160. The pHA-MC160N RxDL mutants (R67AD69A, R160AD162A and double mutants) were generated using site directed mutagenesis by PCR. The RxDL mutant DNA samples were sent to Genewiz Inc for sequencing. The mutants were sequenced using a pCIseq and two internal primers (Table 2). The sequencing data was analyzed based on protein alignments and chromatograms. The chromatograms for pHA-MC160N RxDL mutants all displayed the desired nucleotide changes as indicated by the high intensity peaks and low background (Fig. 8). The first underlined change (GCT) shows the nucleotide sequence changed in the DNA to change the amino acids from arginine/aspartate to alanines (Fig. 11 and 12). There was a hairpin structure in the primer for the second DED that was removed by changing A to C and C to T on the RxDL mutants. The second change was done in order to remove a hairpin structure that would have complicated the process of creating the mutant. The wild type (wt) sequences were displayed above the chromatograms.

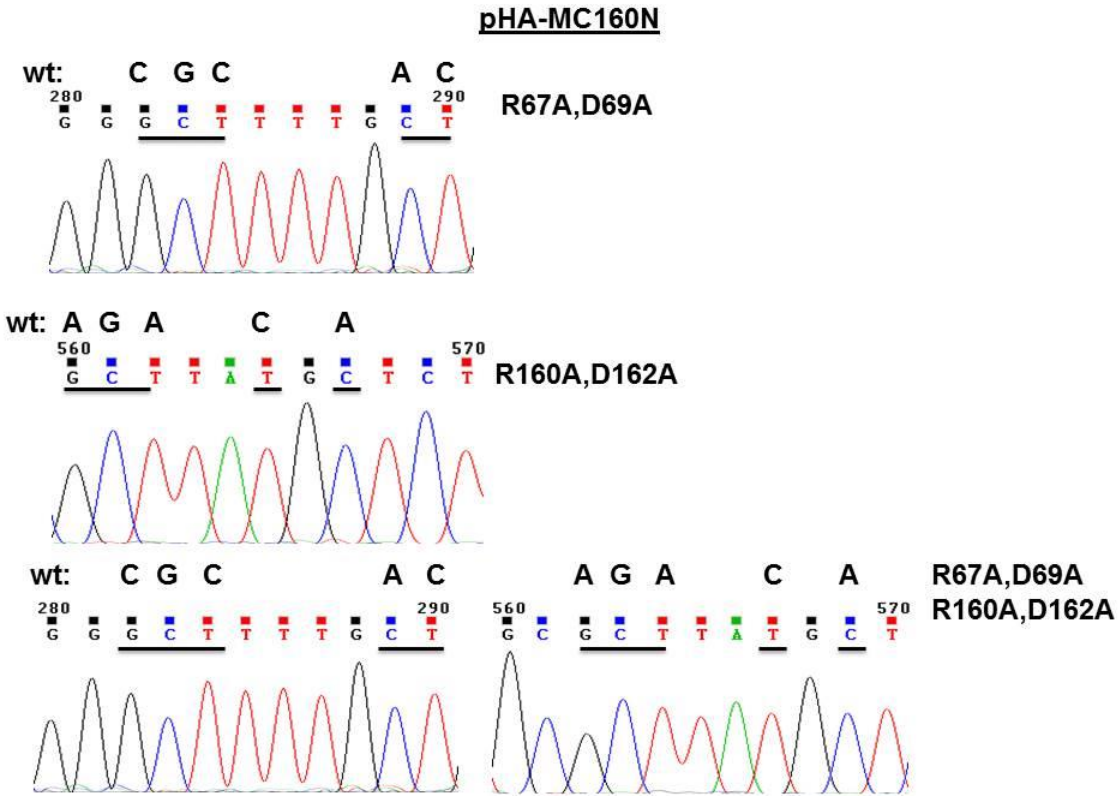

Figure 8: The chromatograms for the mutated pHA-MC160N to verify the nucleotide sequence was changed to code for a neutral alanine rather than a positive arginine on the charged triad, RxDL motif for the first DED R67AD69A, the second DED R160AD162A, and the double mutant with both DEDs changed. The second change was done in order to remove a hairpin structure that would have complicated the process of creating the mutant. Above all of the chromatograms is displayed the wild-type (wt) sequences that were changed using the primers listed in Table 2 and sequenced using the primers listed in Table 3 is displayed in bold.

After the nucleotide sequences were verified using Chromas Lite, the sequences were translated into amino acids using Clone Manager. The amino acid sequences were then aligned with the pHA-MC160 wild-type protein sequence to verify the changes were present in the RxDL motif. In the R67AD69A region arginine/aspartate were changed to alanines (Fig.9A). The R160AD162A was aligned with wild-type and the amino acid changes of arginine/aspartate to alanines were verified (Fig. 9B). The double mutant with both DED 1 and DED 2 changes were made in order to ensure the unchanged DED was not compensating for the change to the RxDL motif of the other region (Fig. 10). Both DEDs can independently inhibit host innate immune responses with DED 1 involved in IFN-β signaling and DED 2 involved in NF-κB signaling (Shisler & Nichols, 2009; Yang et al., 2005).

A

pHA-MC160

R67A,D69A

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HA-MC160 protein       1 MYPYDVPDYAAHEPIPFSFLRNLLAELDASEHEVLRFLCRDVAPASKTAE
DED1_R67A,D69A         1 ....................................................

HA-MC160 protein      51 DALRALQRRRLLTLSSMAELLCALRRFDVLKVRFGMTRECAGRLLGHGFL
DED1_R67A,D69A        51 ..........................A.A.......................

HA-MC160 protein     101 SQYRLQVAAINNMVGSEDLRVMCLCAGKLLPPSCTPRCLVDLVSALEDAG
DED1_R67A,D69A       101 ....................................................

HA-MC160 protein     151 AISPQDVSVLVTLLHAVCRYDLSVALSAVAHGHMTVGVGTPVQDEPMDVL
DED1_R67A,D69A       151 ....................................................

HA-MC160 protein     201 EVDDAEPMEATPACDEIGVVKLAGAASAGAPLADGAFAACTSAGKGEDLA
DED1_R67A,D69A       201 ....................................................

HA-MC160 protein     251 TSDLTDSEPEDSVFAVADPVYADVDLSMFVRANATADSSMFVNADAGADS
DED1_R67A,D69A       251 ....................................................

HA-MC160 protein     301 SLVNADAGADSSLVNADAGADSSLVNAVADANSSLMRTTSACTDSEPEDS
DED1_R67A,D69A       301 ....................................................

HA-MC160 protein     351 AGPSCAGMALSMFGRAKSVSSLLLRTKASY
DED1_R67A,D69A       351 ............................
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B

R160A,D162A

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pHA-MC160           1063 EFATMYPYDVPDYAAHEPIPFSFLRNLLAELDASEHEVLRFLCRDVAPASKTAEDALRALQRRRLLTLSS
DED2_RxDL              1 ....................................................................

pHA-MC160           1273 MAELLCALRRFDVLKVRFGMTRECAGRLLGHGFLSQYRLQVAAINNMVGSEDLRVMCLCAGKLLPPSCTP
DED2_RxDL            211 ....................................................................

pHA-MC160           1483 RCLVDLVSALEDAGAISPQDVSVLVTLLHAVCRYDLSVALSAVAHGHMTVGVGTPVQDEPMDVLEVDDAE
DED2_RxDL            421 ..............................A.A.................................

pHA-MC160           1693 PMEATPACDEIGVVKLAGAASAGAPLADGAFAACTSAGKGEDLATSDLTDSEPEDSVFAVADPVYADVDL
DED2_RxDL            631 ....................................................................

pHA-MC160           1903 SMFVRANATADSSMFVNADAGADSSLVNADAGADSSLVNADAGADSSLVNAVADANSSLMRTTSACTDSE
DED2_RxDL            841 ....................................................................

pHA-MC160           2113 PEDSAGPSCAGMALSMFGRAKSVSSLLLRTKASY*RASR
DED2_RxDL           1051 ..............................*....
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Figure 9: The protein alignments for pHA-MC160 and the mutated pHA-MC160 to verify the amino acid was changed from a positive arginine to a neutral alanine on the charged triad, RxDL motif for the first DED R67AD69A (A) and the second DED R160AD162A (B).

pHA-MC160

R67A,D69A R160A,D162A

```
HA-MC160 protein      1 MYPYDVPDYAAHEPIPFSFLRNLLAELDASEHEVLRFLCRDVAPASKTAEDALRALQRRRLLTLSSMAEL
DED1_DED2_RxDL       13 ............................................................

HA-MC160 protein     71 LCALRRFDVLKVRFGMTRECAGRLLGHGFLSQYRLQVAAINNMVGSEDLRVMCLCAGKLLPPSCTPRCLV
DED1_DED2_RxDL      223 .....A.A........................................................

HA-MC160 protein    141 DLVSALEDAGAISPQDVSVLVTLLHAVCRYDLSVALSAVAHGHMTVGVGTPVQDEPMDVLEVDDAEPMEA
DED1_DED2_RxDL      433 ..........................A.A...................................

HA-MC160 protein    211 TPACDEIGVVKLAGAASAGAPLADGAFAACTSAGKGEDLATSDLTDSEPEDSVFAVADPVYADVDLSMFV
DED1_DED2_RxDL      643 ............................................................

HA-MC160 protein    281 RANATADSSMFVNADAGADSSLVNADAGADSSLVNADAGADSSLVNAVADANSSLMRTTSACTDSEPEDS
DED1_DED2_RxDL      853 ............................................................

HA-MC160 protein    351 AGPSCAGMALSMFGRAKSVSSLLLRTKASY
DED1_DED2_RxDL     1063 ............................
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Figure 10: The protein alignments for pHA-MC160 and the mutated pHA-MC160 to verify the amino acid was changed from a positive arginine to a neutral alanine on the charged triad, RxDL motif for the double mutation of the first DED R67AD69A and the second DED R160AD162A.

After the nucleotide sequences were verified using Chromas Lite, the sequences were translated into amino acids using Clone Manager. The amino acid sequences were then aligned with the pHA-MC160N wild-type protein sequence in order to verify the changes were made in the RxDL motif. In the DED 1 region arginine/aspartate were changed to alanine (Fig.11A). Then DED 2 was aligned with wild-type and the amino acid changes were verified, arginine/aspartate to alanine (Fig. 11B). The double mutant with both DED 1 and DED 2 changes were made in order to ensure the unchanged DED was not compensating for the change to the RxDL motif of the other region (Fig. 12). Previous data indicated that the MC160 DED 1 dampens induction of IRF-3, while MC160 DED 2 antagonizes TNF-mediated NF-κB signaling (Randall et al., 2014, Shisler & Moss, 2001).

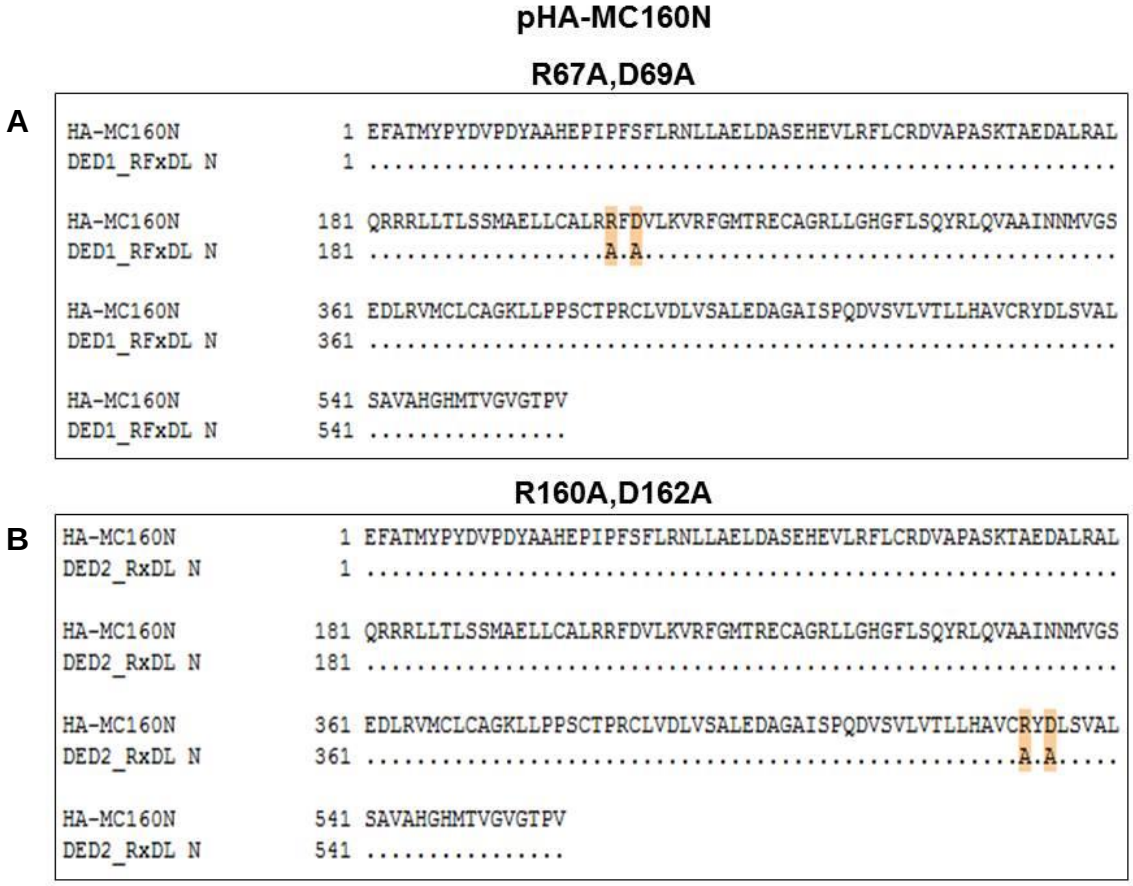

Figure 11: The protein alignments for pHA-MC160N and the mutated pHA-MC160N to verify the amino acid was changed from a positive arginine to a neutral alanine on the charged triad, RxDL motif for the first DED R67AD69A (A) and the second DED R160AD162A (B).

pHA-MC160N

R67A,D69A R160A,D162A

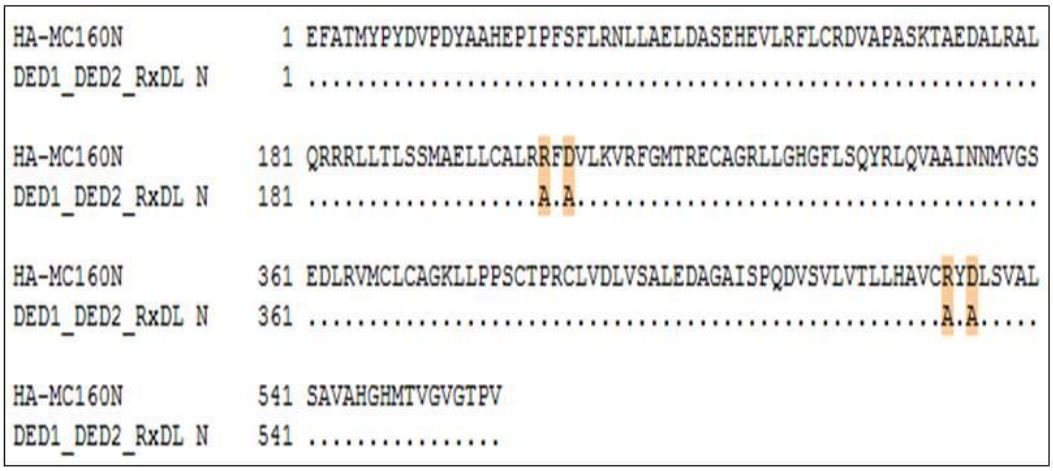

Figure 12: The protein alignments for pHA-MC160N and the mutated pHA-MC160N to verify the amino acid was changed from a positive arginine to a neutral alanine on the charged triad, RxDL motif for the double mutant with the first DED (R67AD69A) and the second DED (R160AD162A).

The expression of RxDL mutants by immunoblotting

After the changes were verified by protein alignment and chromatograms, immunoblotting was performed to verify the protein expression levels (Yang et al., 2005). The pHA-MC160, pHA-MC160N and all RxDL mutants have an HA epitope tag on the N-terminus of the protein allowing for detection using anti-HA antibodies.

The pHA-MC160 R67AD69A and R160AD162A RxDL mutants were detected at similar levels to the wild-type (Fig. 13A). The RxDL mutants were detected at lower levels than wild-type in the R160AD162A mutation for pHA-MC160N while the R67AD69A mutant was detected at similar levels to wild-type (Fig. 13B). However, the double mutants were detected at lower expression levels than the wild-type (Fig. 13C and D) suggesting the RxDL motif might be involved in the conformation and stability of the protein as was the case for MC159 (Garvey et al., 2002B; Twomey et al., 2013).

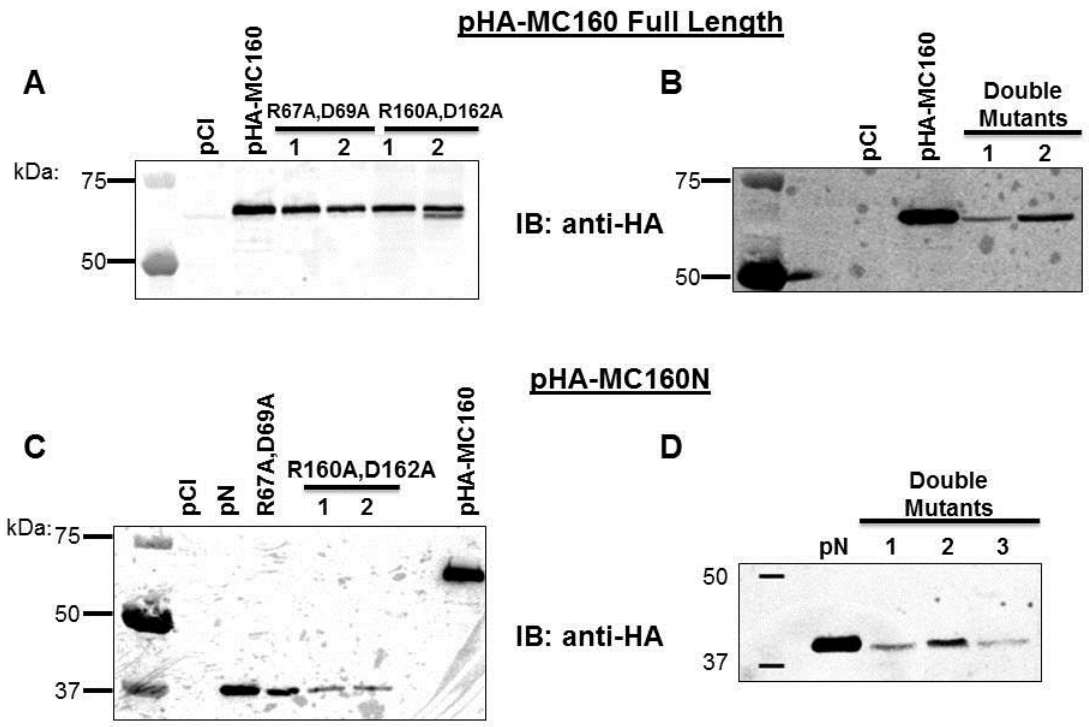

Figure 13: The expression levels of pHA-MC160 proteins and the N-terminus of pHA-MC160 (HA-MC160N or pN) proteins by immunoblotting. The mutation for R160AD162A expressed at weaker levels in comparison to the wild type for both HA-MC160 and HA-MC160N (A) and (C). The double mutants, R67AD69A and R160AD162A, expressed at lower levels than the wild type for both HA-MC160 and HA-MC160N (B) and (D). All membranes were blotted with anti-HA (1:5000) and goat anti-mouse (1:2500).

The MC160 RxDL motif is not required for the inhibition of MAVS-induced activation of IFN-β luciferase

Poxviruses encode a large number of proteins that inhibit innate immune responses. IFN-β can be stimulated by many cellular sensors. For this experiment, overexpression of either MAVS or TBK1 was utilized to activate IFNβ. MAVS is an adaptor protein that will induce formation of the TBK1 and the IKK complex. Overexpression of either MAVS or TBK1 molecules can activate the IFN-β controlled luciferase gene (Seth et al., 2005). It has already been shown that MC159 and MC160 viral FLIP inhibits IFN-β production by binding to the TBK1:IKKε complex (Randall et al., 2014). In this experiment MAVS was used because it has already been shown stimulating with MAVS induces the formation of the signaling complexes and increase IFN-β-luciferase activity in comparison to cells treated with pCI vector alone (Randall et al., 2014). The function the RxDL motif of MC160 has never before been tested on IFN-β-luciferase. However, it was predicated that the RxDL motif mutants would no longer be able to inhibit IFN-β.

When assessing the MAVS inhibitory function of single RxDL mutant MC160/MC160N proteins by luciferase reporter assay, mutated MC160 proteins showed approximately an 8 fold reduction for R160AD162A and 10 fold reduction for R67AD69A for MAVS-induced luciferase activity when compared to pCI-transfected cells (Fig. 14). In contrast the pHA-MC160N RxDL mutants showed approximately a 10 fold reduction for R67AD69A and a 20 fold reduction for

R160AD162A in comparison to pCI stimulated cells (Fig. 14). The R67AD69A RxDL motif mutant inhibited better than pHA-MC160 while the R160AD162A RxDL motif inhibited to similar levels as pHA-MC160. Corresponding to these results R67AD69A and R160AD162A RxDL motif mutants inhibited better than pHA-MC160N. The RxDL mutants showed no loss of function in comparison to the wild-type and inhibited better than or to similar levels of wild-type.

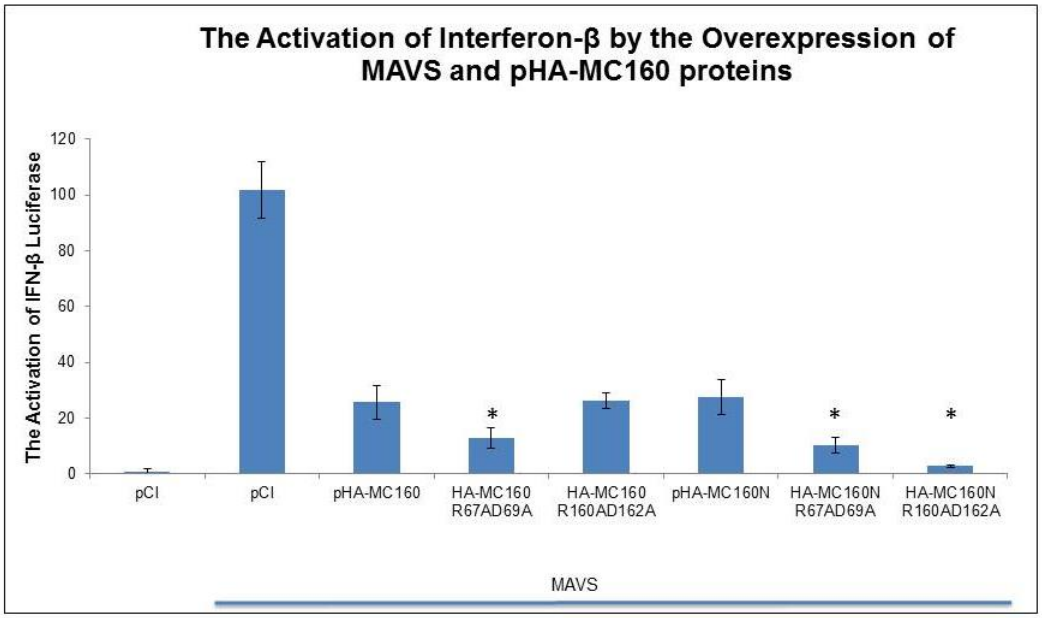

Figure 14: The effect of pHA-MC160 and pHA-MC160N wild type and RxDL mutant proteins on the activation of IFN-β. Subconfluent HEK293T cells were transfected with IFN-β luciferase (225 ng); pRenilla-TK (25 ng); pCI (1000 ng and 500 ng); pHA-MC160 (500 ng), pHA-MC160N (500 ng), pHAMC160 R67AD69A (500 ng), pHA-MC160 R160AD162A (500 ng), pHA-MC160N R67AD69A (500 ng), and pHA-MC160N R160AD162A (500 ng); and MAVS (500 ng). Twenty-four hours later, cells were lysed and firefly and sea pansy luciferase activities were measured. Values are shown as mean ± standard deviations (SD) with significant differences between RxDL mutants and wild-type determined by two tailed t-test with significance set at $p<0.05$ (*) with tests run in triplicate at n=3.

While mutating a single RxDL motif did not result in a loss of function for MAVS-induced IFN-β luciferase activity it was possible that a double mutant would. It was possible that the non-mutated RxDL motif on the DED is compensating for the mutated RxDL motif of the other DED in the signaling pathway. The RxDL double mutants for pHA-MC160 and pHA-MC160N showed approximately a 10 fold reduction of MAVS-induced activation of IFN-β (Fig. 15). The second DED was used as a negative control in this experiment as the second DED is not known to be involved in IFN signaling. Both RxDL mutants inhibit the activation of MAVS-induced IFN-β-luciferase better than the wild-type. The results were consistent with previous studies that DED 1 appears to be involved in inhibiting MAVS signaling through MC160 (Fig. 15) (Shisler, 2014). It can therefore be concluded that the RxDL motif was not required for the MC160 protein inhibition of MAVS-induced IFN-β activity.

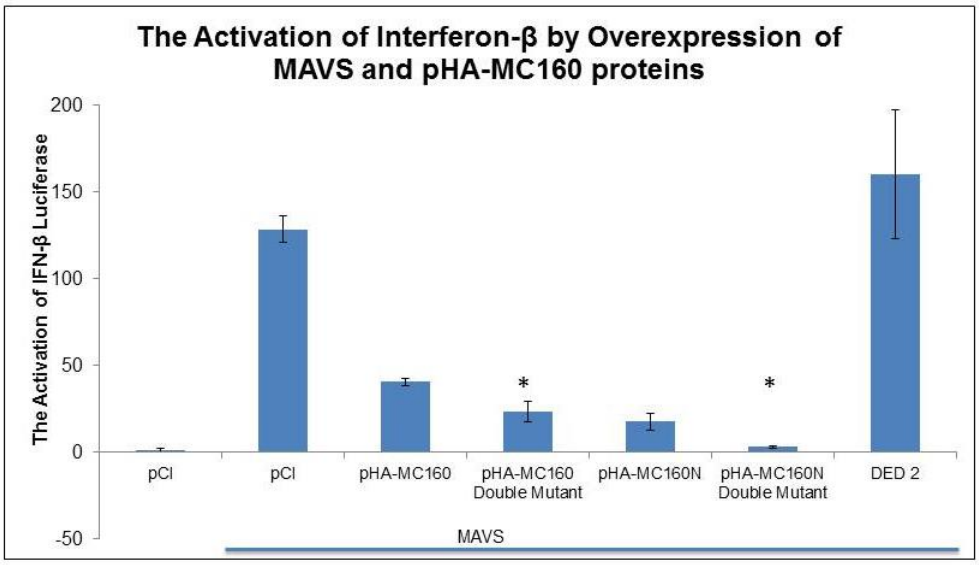

Figure 15: The effect of pHA-MC160 and pHA-MC160N wild type and RxDL mutant proteins on the activation of IFN-β. Subconfluent HEK293T cells were transfected with IFN-β luciferase (225 ng); pRenilla-TK (25 ng); pCI (1000 ng and 500 ng); pHA-MC160 (500 ng), pHA-MC160N (500ng), pHA-MC160N R67AD69A, R160AD162A (500 ng), and pHA-MC160N R67AD69A, R160AD162A (500 ng); and MAVS (500 ng). Twenty-four hours later, cells were lysed and firefly and sea pansy luciferase activities were measured. Values are shown as mean ± standard deviations (SD) with significant differences between RxDL mutants and wild-type determined by two tailed t-test with significance set at $p<0.05$ (*) with tests run in triplicate at n=3.

The luciferase expression tests performed in HEK293T cells all showed no loss of function in RxDL mutants for pHA-MC160 and pHA-MC160N proteins. Therefore, to test if the results were consistent or cell line dependent, the mutants were tested in MEF wild-type cells. The results found in the MEF cells were similar to those in HEK239T cells; none of the RxDL motif mutants resulted in a loss of function. The R67AD69A pHA-MC160 mutant showed approximately a 4.5 fold reduction in comparison to pCI stimulated with MAVS. The R160AD162A pHA-mutant showed approximately a 3.5 fold reduction in comparison to pCI stimulated with MAVS. The pHA-MC160N R67AD69A showed approximately a 5 fold induction and the R160AD162A showed approximately a 4.5 fold reduction in comparison to pCI stimulated with MAVS. However, the values of firefly:sea pansy luciferase activity in the presence of the pHA-MC160N double mutant were similar to the R160A/D162A mutant (Fig. 16). The second DED was used as a negative control as it is not known to inhibit MAVS-induced IFN expression. Consistent with HEK293T data none of the RxDL mutants showed a significant difference in the inhibition of MAVS-induced IFN-β-luciferase when analyzed against the wild-type protein.

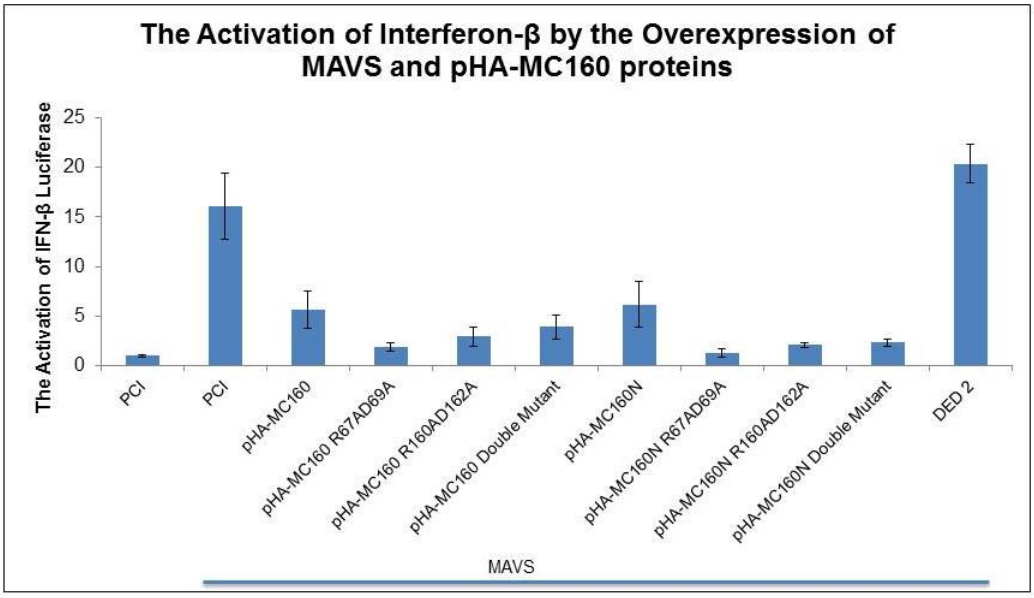

Figure 16: The effect of pHA-MC160 and pHA-MC160N wild type and RxDL mutant proteins on the activation of IFN-β. Subconfluent MEF wt cells were transfected with IFN-β luciferase (225 ng); pRenilla-TK (25 ng); pCI (1000 ng and 500 ng); pHA-MC160 (500 ng), pHA-MC160N (500 ng), DED 2 (500 ng), pHAMC160 R67AD69A (500 ng), pHA-MC160 R160AD162A (500 ng), pHA-MC160 R67AD69A, R160AD162A (500 ng), pHA-MC160N R67AD69A (500 ng), pHA-MC160N R160AD162A (500 ng) and pHA-MC160N R67AD69A, R160AD162A (500 ng); and MAVS (500 ng). Values are shown as mean ± standard deviations (SD) with significant differences between RxDL mutants and wild-type determined by two tailed t-test with tests run in triplicate at n=3.

The MC160 RxDL motif is not required for the inhibition of TBK1-induced activation of IFN-β luciferase

TBK1 is a downstream kinase involved in the activation of IFN-β. TBK1 can directly phosphorylate the IFN-β transcription factor IRF 3. MC160 has not been shown to associate with TBK1. However, MC160 has been shown to inhibit IRF3 but, the molecular mechanism is unknown. Both viral proteins of MCV can inhibit TBK1 activation through DEDs, MC160 DED 1 shows inhibition, while DED 2 does not (Randall et al., 2014).

When assessing the effect of RxDL mutant MC160/MC160N proteins by luciferase reporter assay, the RxDL motif mutants still inhibited TBK1-induced IFN-β luciferase. pHA-MC160 R160AD162A showed a 3.5 fold reduction and R67AD69A a 14 fold reduction for TBK1-induced luciferase activity when compared to pCI-transfected cells (Fig. 17). The pHA-MC160N RxDL mutants showed a 14 fold reduction for R67AD69A and R160AD162A for TBK1-induced luciferase activity when compared to pCI stimulated with TBK1 (Fig. 17). The RxDL mutants showed no loss of function in comparison to the wild-type and inhibited better than or to similar levels of wild-type. However, R67AD69A mutants showed stronger inhibition of TBK1-induced IFN signaling in comparison to MC160/MC160N. The R160AD162A mutant showed less inhibition of TBK1-induced IFN signaling only for pHA-MC160. The pHA-MC160N R160AD162A mutant inhibited to similar levels of wild-type. From these results it can be

concluded that the RxDL motif was not required for inhibition of TBK1-induced IFN signaling by the MC160 protein.

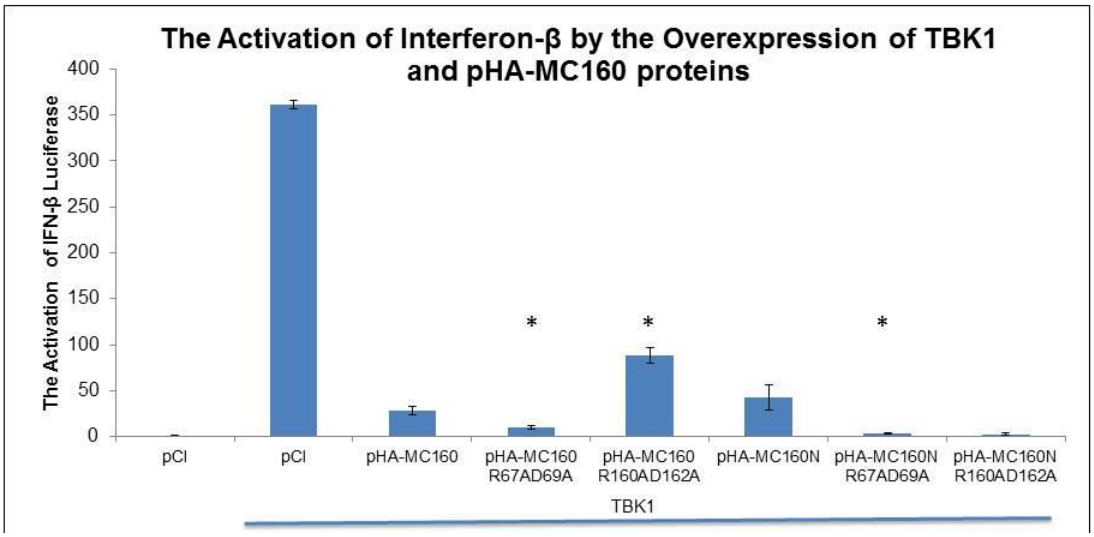

Figure 17: The effect of pHA-MC160 and pHA-MC160N wild type and RxDL mutant proteins on the activation of IFN-β. Subconfluent HEK293T cells were transfected with IFN-β luciferase (225 ng); pRenilla-TK (25 ng); pCI (1000 ng and 500 ng); pHA-MC160 (500 ng), pHA-MC160N (500 ng), pHAMC160 R67AD69A (500 ng), pHA-MC160 R160AD162A (500 ng), pHA-MC160N R67AD69A (500 ng), and pHA-MC160N R160AD162A (500 ng); and TBK1 (500 ng). Twenty-four hours later, cells were lysed and firefly and sea pansy luciferase activities were measured. Values are shown as mean ± standard deviations (SD) with significant differences between RxDL mutants and wild-type determined by two tailed t-test with significance set at $p<0.05$ (*) with tests run in triplicate at n=3.

While single RxDL mutants did not result in a loss of function for TBK1-induced IFN-β luciferase activity it was possible that a double mutant would. The function of the two DEDs is not interchangeable and it was possible that the non-mutated RxDL motif on the DED was compensating for the mutated RxDL motif of the other DED in the signaling pathway The RxDL double mutants for pHA-MC160 and pHA-MC160N both showed approximately an 18 fold reduction for TBK1-induced luciferase activity when compared to pCI stimulated with TBK1 (Fig. 18). Both RxDL double mutants inhibit the activation of TBK1-induced IFN-β-luciferase better than the wild-type. These results were consistent with previous studies that MC160 inhibits TBK1 signaling (Fig. 18) (Randall et al., 2014). It can therefore be concluded that the RxDL motif was not required for the MC160 proteins inhibition of TBK1-induced IFN-β activity.

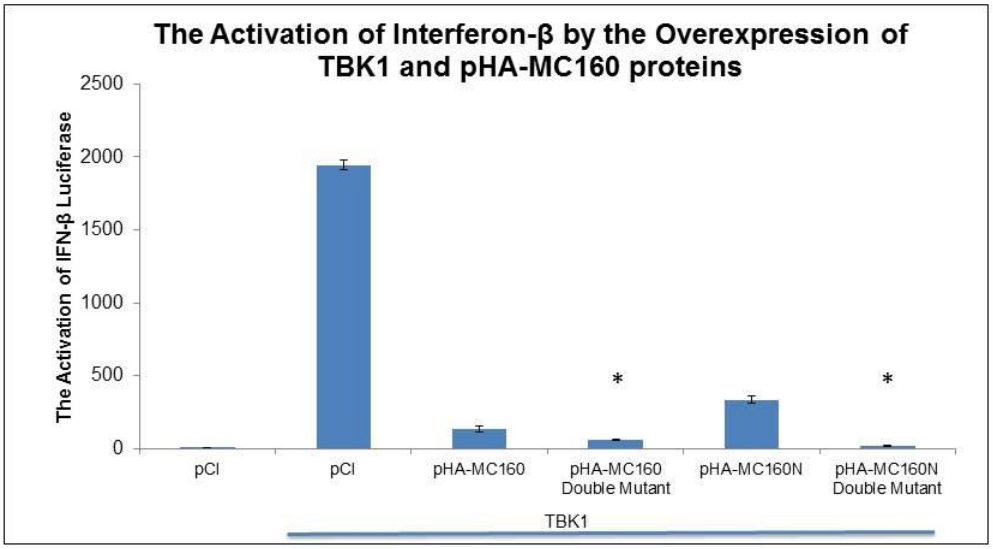

Figure 18: The effect of pHA-MC160 and pHA-MC160N wild type and RxDL mutant proteins on the activation of IFN-β. Subconfluent HEK293T cells were transfected with IFN-β luciferase (225 ng); pRenilla-TK (25 ng); pCI (1000 ng and 500 ng); pHA-MC160 (500 ng), pHA-MC160N (500 ng), pHA-MC160 R67AD69A, R160AD162A (500 ng), and pHA-MC160N R67AD69A, R160AD162A (500 ng); and TBK1 (500 ng). Twenty-four hours later, cells were lysed and firefly and sea pansy luciferase activities were measured. Values are shown as mean ± standard deviations (SD) with significant differences between double mutants and wild-type determined by two tailed t-test with significance set at $p<0.05$ (*) with tests run in triplicate at n=3.

The MC160 RxDL motif is not required to inhibit procaspase-8-induced activation of NF-κB luciferase

Caspase-8 plays an essential role in the regulation of apoptotic and non-apoptotic signaling pathways. Caspase binds to the DED of FADD (Takahashi et al., 2006). When procaspase-8 is activated by proteolytic cleavage it will transmit apoptotic signals through cleavage of various substrates.Caspase-8 can also induce NF-κB activation in response to Fas or antigen receptors (Kruez et al., 2004; Takahashi et al., 2006).The NF-κB:IκB complex that must be phosphorylated and ubiquinated for NF-κB expression. The RxDL motif of the MC160 protein has not been tested for interaction with procaspase-8, but it is known that MC160 associates with procaspase-8.

In conditions with over-expression of C360S, catalytically inactive caspase-8, the NF-κB regulated luciferase activity varied based on the concentration of procaspase-8 to MCV proteins. When procaspase-8 and MC160N and RxDL mutant proteins were present at a 1:1 concentration there was less than a fold difference in the activity of RxDL mutants and MC160N when compared to pCI stimulated cells (Fig. 19A). At a 1:1 ratio the RxDL mutants were unable to inhibit the activation of NF-κB (Fig. 19A). This suggests that at equal ratios of procaspase-8 and MC160 protein the activation of NF-κB was not completely suppressed.

When assessing the procaspase-8 inhibitory function of the mutant MC160N proteins by luciferase reporter assay, mutated MC160N proteins

showed approximately a 10 fold reduction of procaspase-8-induced luciferase activity when compared to pCI-transfected cells (Fig. 19B). When there was a ratio of procaspase-8 to MC160/MC160N/mutants proteins MC160N of 2:1 the luciferase activity for NF-κB was significantly decreased (Fig. 19B). Procaspase-8 levels for the mutated charged triad mutants and the double mutants were found to be inhibiting to the similar levels as wild type (Fig. 19). The data suggests inhibition of NF-κB depends on the levels of viral proteins in relation to procaspase-8 to prevent the activation of proinflammatory cytokine gene expression and that the RxDL motif was not required for inhibition of procaspase-8 induced NF-κB inhibition.

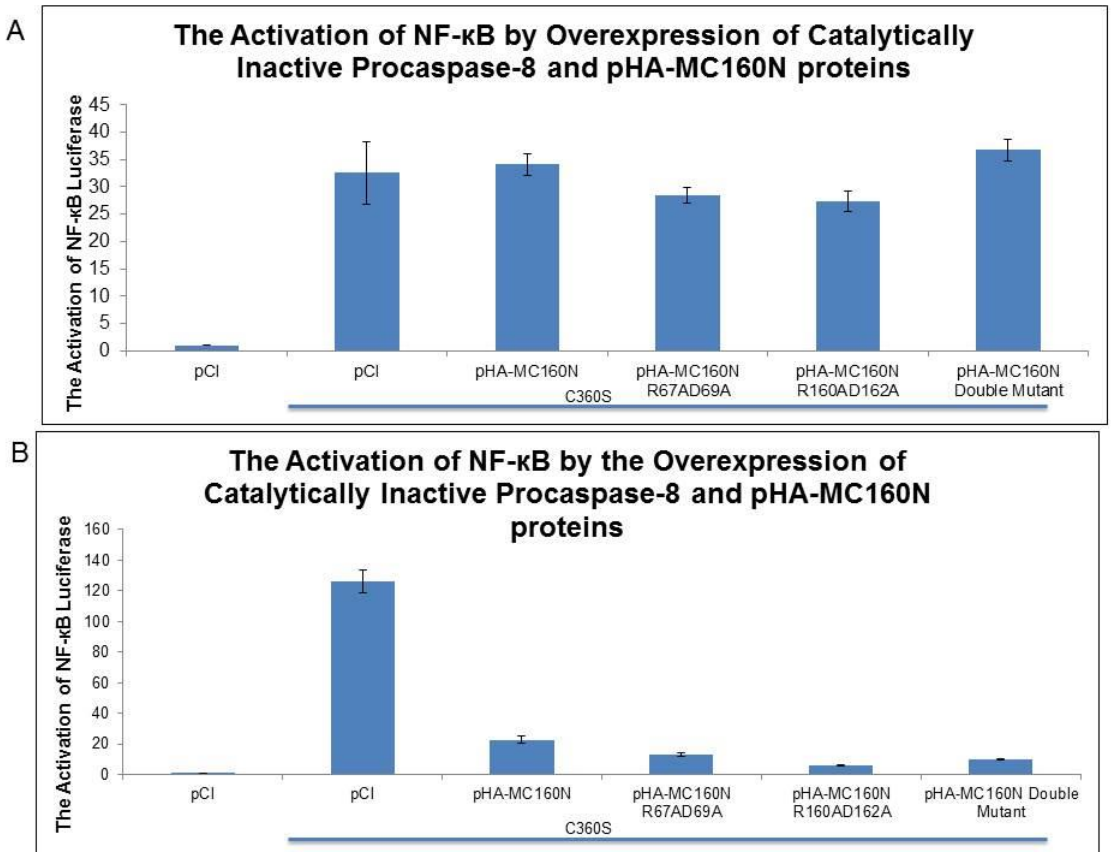

Figure 19: The effect of pHA-MC160N wild type and RxDL mutant proteins on the activation of NF-κB. (A) Subconfluent HEK 293T cells were transfected with NF-κB luciferase (225 ng); pRenilla-TK (25 ng); pCI (750 ng and 325 ng); pHA-MC160N (250 ng), pHA-MC160N R67AD69A (250 ng), pHA-MC160N R160AD162A (250 ng), pHA-MC160N R67AD69A, and R160AD162A (250 ng); and C360S (250 ng). (B) Subconfluent HEK293T cells were transfected with NF-κB luciferase (225 ng); pRenilla-TK (25 ng); pCI (1000 ng and 500 ng); pHA-MC160N (500 ng), pHA-MC160N R67AD69A (500 ng), pHA-MC160N R160AD162A (500 ng), and pHA-MC160N R67AD69A, R160AD162A (500 ng); and C360S (250 ng). Twenty-four hours later, cells were lysed and firefly and sea pansy luciferase activities were measured. Values are shown as mean ± standard deviations (SD) with significant differences between RxDL mutants and wild-type determined by two tailed t-test with significance set at $p<0.05$ (*) with tests run in triplicate at n=3 (A) and tests run in duplicate at n=3 (B).

The MC160 RxDL motif is not required to inhibit RIP-1-induced activation of NF-κB luciferase

Downstream of procaspase-8 in TNFR signaling is RIP-1. Previous studies have shown that MC160 can inhibit RIP-1 (Shisler & Nichols, 2009). DED 1 of MC160 has no effect on RIP-1-induced NF-κB activation (Shisler & Nichols, 2009).The NF-κB regulated luciferase, activity was significantly decreased when MC160N or MC160N mutants were present (Fig. 20). When assessing the RIP-1 inhibitory function of the mutant MC160N proteins by luciferase reporter assay, MC160N RxDL mutant proteins showed a 24 fold reduction of RIP-1-induced luciferase activity when compared to pCI-transfected cells (Fig. 20). RIP-1 levels for the mutated charged triad mutants and the double mutant were found to inhibit better than wild-type (Fig. 20). This suggests that the RxDL motif of MC160 was not necessary for the inhibition of RIP-1-induced NF-κB expression.

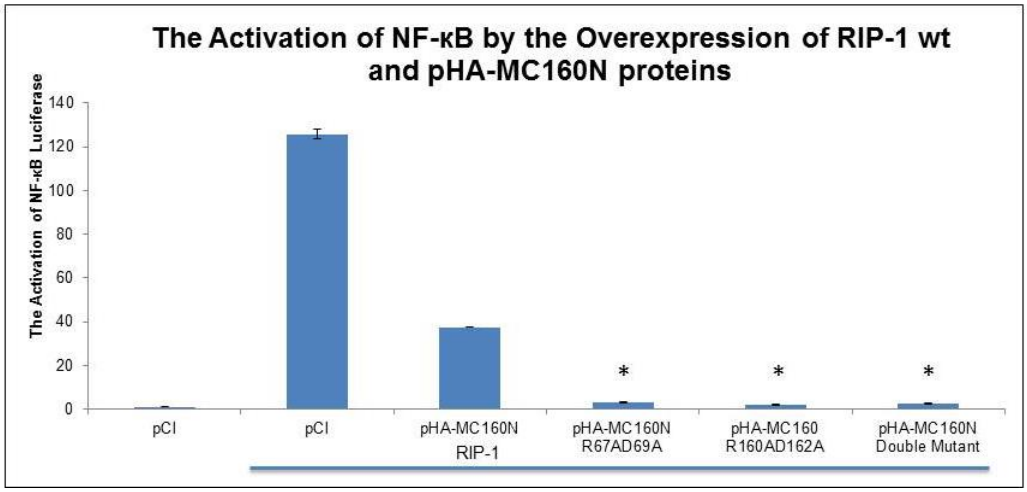

Figure 20: The effect of pHA-MC160N wild type and RxDL mutant proteins on RIP-1 induced activation of NF-κβ. Subconfluent HEK293T cells were transfected with NF-κB luciferase (225 ng); pRenilla-TK (25 ng); pCI (1000 ng and 500 ng); pHA-MC160N (500 ng), pHA-MC160N R67AD69A (500 ng), pHA-MC160N R160AD162A (500 ng), and pHA-MC160N R67AD69A, R160AD162A (500 ng); and RIP-1 wt (500 ng). Twenty-four hours later, cells were lysed and firefly and sea pansy luciferase activities were measured. Values are shown as mean ± standard deviations (SD) with significant differences between RxDL mutants and wild-type determined by two tailed t-test with significance set at $p<0.05$ (*) with tests run in triplicate at n=3.

Discussion

The persistence of MCV is believed to be due to the immune evasion proteins. The molecular mechanism of MC159 has been well characterized, while the molecular mechanism of MC160 is poorly understood. It is important to further study and evaluate the immune evasion proteins of MCV in order to understand the viral pathogenesis of MCV.

DEDs play an important role in the immune evasion of MCV to inhibit the innate immune response. The RxDL motif of the DED is believed to be involved in the binding of these immune evasion proteins to inhibit the immune response and is conserved amongst DED containing proteins. Garvey et al. (2002B) has already shown knock out mutations of the RxDL motif in MC159 results in a loss of function in apoptotic signaling. These mutants lost the ability to inhibit apoptosis mediated by Fas and caspase-8. MC160 does not have any anti-apoptotic function, but the RxDL motif is conserved between these two proteins. MC160 is known to inhibit NF-κB signaling and IFN signaling; however the molecular mechanism is unknown (Randall et al., 2014). Due to the highly conserved nature of the RxDL motifs in DED containing proteins it was hypothesized a mutation of the MC160 protein RxDL motif would also result in a loss of function for NF-κB and IFN.

Wild-type MC159 and MC160 inhibit the phosphorylation of TBK1 to block IFN transcription factors (IRF3) expression (Randall et al., 2014). The molecular mechanism by which MC160 inhibits TBK1 is not known. However in this thesis it

was found that both RxDL DED mutants of MC160 and MC160N inhibit TBK1- and MAVS-induced IFN-β expression. Therefore, it can be concluded the RxDL motif of MC160 is not required for either MAVS or TBK1-induced IFN-β inhibition. An accurate comparison cannot be made between the RxDL motif of MC159 and MC160 for these signaling pathways as the MC159 RxDL mutants have not been tested for MAVS- or TBK1-induced IFN expression.

In addition, the RxDL mutants of MC160N do not inhibit procaspase-8-induced NF-κB activation at a 1:1 ratio of MC160N RxDL mutants to procaspase-8. However, at a 2:1 ratio of MC160N RxDL mutants to procaspase-8, procaspase-8 induced NF-κB activation was inhibited. MC160 is known to bind with procaspase-8.The level of caspase-8 induced NF-κB inhibition was found to be dependent on the level of MC160 vector transfected in the cells. The result suggests that MC160 is unable to prevent procaspase-8 signaling when present at equal concentrations as procaspase-8. However, treating the cells with equal concentrations of plasmid DNA does not necessarily result in equal levels of protein. The result suggests the inhibition of NF-κB and reduction of proinflammatory cytokine production were dependent on the levels of MC160 protein present and that the RxDL motif of MC160 is not required for procaspase-8 or RIP-1-induced NF-κB activation.

These findings suggest that unlike other DED proteins the RxDL motif of MC160 is not required for its function. These results suggest the RxDL motif is required for mediating anti-apoptotic function but not for preventing inhibition of

NF-κB and IFN-β. The MC160 and MC160N RxDL mutants were not detected at similar levels as wild-type proteins by immunoblotting suggesting the RxDL motif might be involved in protein folding and stability. As shown in MC159 and MC160 it is likely the immune evasion proteins of MCV have overlapping inhibitory functions on a signaling pathways such as IFN and NF-κB.

The future experiments for this project would be to mutate other residues that might be required for MC160 function such as F25L26 and F122GL123G residues that are required for the interaction of caspase-8 and FADD (Yang et al., 2005). MC160 interacts with FADD and procaspase-8 but the functionality of this interaction is unknown, as MC160 cannot inhibit FADD-mediated apoptotic responses (Shisler & Moss, 2001). F25L26 and F122GL123G residues may be required for procaspase-8 and FADD interaction. Therefore, mutating these residues might yield information on the molecular mechanism of MC160 and determine if FADD/procaspase-8 binding is important for the MC160 protein's ability to dampen host antiviral responses. To prove the RxDL motif has no effect on the MC160 protein function immunoprecipitation must be performed to detect if these mutants are still able to associate with caspase-8 and FADD. In conclusion, the RxDL motif of the MC160 DED-containing protein is not required for inhibition of IFN by MAVS or TBK1 or inhibition of NF-κB expression by procaspase-8 or RIP-1.

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