Identification of an Antiviral Signaling Variant Demonstrates Immune Regulation Through Alternative Translation

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Identification of an Antiviral Signaling Variant

Demonstrates Immune Regulation Through Alternative Translation

A dissertation presented

by

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Demonstrates Immune Regulation Through Alternative Translation

Abstract

Innate immune signaling pathways initiate host defenses against viral pathogens. Receptors specific for viral nucleic acids activate these pathways culminating in cell-to-cell communication and/or cell death. In mammals, this cell-to-cell communication is achieved through the production of interferons and pro-inflammatory cytokines, which activate antiviral defenses in uninfected neighboring cells and instruct adaptive immune responses. The production of these signaling molecules is essential for the defense against viral infection, but must also be tightly regulated to prevent unnecessary inflammation. As an antiviral defense, cell death is also an effective mechanism to limit viral replication and spread but comes with the cost of tissue damage and inflammation. Therefore, regulating these antiviral responses is critical for controlling the spread of infection as well as preventing unnecessary pathologies related to excessive signaling. Hundreds of genes are involved in controlling these immune responses and a wide variety of mechanisms are utilized to regulate them. One mechanism to regulate gene function is the generation of protein variants through alternative translation. While polycistronic transcripts are a common feature of bacterial and viral gene expression, the process of alternative translation as a means to regulate gene function is not a feature

generally attributed to mammalian mRNA. This dissertation describes a novel regulator of antiviral signaling that is generated through alternative translation. Expression of the transcript encoding the antiviral adaptor protein, MAVS, results in the production of two proteins: the full-length MAVS adaptor and a truncated variant, miniMAVS. Production of these proteins is in part regulated by cis-acting elements that control translation initiation. Production of miniMAVS regulates antiviral signaling by limiting interferon production induced by full-length MAVS, whereas both MAVS variants positively regulate cell death. To identify other examples of alternative translation in mammalian cells a genome-wide ribosomal profiling technique was used to generate a candidate list of antiviral truncation variants. This dissertation therefore demonstrates that protein variants generated through alternative translation of polycistronic mRNAs can be an effective mechanism for immune regulation and may be more common than previously understood.

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Abbreviations

CARD	caspase activation and recruitment domain
CTD	C-terminal domain
ER	endoplasmic reticulum
FL	full-length
IFN	interferon
IRES	internal ribosomal entry site
IRF	interferon regulatory factor
ISG	interferon stimulated gene
LGP2	laboratory of genetics and physiology 2
MAVS	mitochondrial antiviral signaling protein
MDA5	melanoma differentiation-associated gene 5
MEF	mouse embryo fibroblast
ORF	open reading frame
PAMP	pathogen associated molecular pattern
PRR	pattern recognition receptor
RLR	RIG-I-like receptors
RIG-I	retinoic acid inducible gene I
RIG-I sv	RIG-I splice variant
TLR	toll-like receptor
TRAF	TNF receptor associated family
uORF	upstream open reading frame

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"And death shall have no do**mini**on"

Dylan Thomas

Chapter 1

Introduction

Pattern Recognition by the Innate Immune System

The innate immune system detects the presence of microbes and initiates mechanisms to eliminate potentially infectious threats. Microbial detection is achieved through germline encoded pattern recognition receptors (PRR) that survey both the extracellular and intracellular space for conserved determinants of microbial and viral origin [1]. These conserved determinants are recognized as indicators of microbial infection. The model of microbial pattern recognition was proposed by Charles Janeway Jr. and describes two feature of innate immunity: the ability to distinguish infectious non-self molecules from self-molecules and the ability to activate adaptive immune responses to the former [2]. In this model, the distinction between self and infectious non-self was predicted to rely on fixed receptor specificity for conserved molecular patterns common among pathogens. In addition, the activation of these receptors was predicted to initiate signaling events culminating in an effective immune response. Since Janeway made his prediction, many aspects of innate immune signaling have since been characterized and fit within the framework of his original model [3]. Microbial ligands ranging from structural components of bacteria, fungi, and viruses to biosynthetic molecules such as nucleic acids activate PRRs and induce the innate immune responses that protect us from infectious threats.

A major component of an innate immune response is transcriptional and generates the production of pro-inflammatory cytokines and interferons (IFN) [4]; these chemical messages are critical for initiating innate immune defense mechanisms as well as initiating adaptive immune responses. The activation of

PRRs also initiates non-transcriptional responses such as the induction of phagocytosis, cell death, autophagy, and cytokine processing [5-7]. These transcriptional and non-transcriptional innate immune responses are linked to PRR-mediated microbial detection by tightly controlled signal transduction pathways [1]. The coordination of these signaling pathways orchestrates immune responses, which contain the spread of an initial infection as well as direct the appropriate adaptive response [8].

The majority of PRRs can be classified into one of five families based on protein domain homology. These five families consist of the Toll-like receptors (TLR), C-type lectin receptors (CLR), nucleotide-binding domain, leucine-rich repeat containing (or Nod-like) receptors (NLR), the Rig-I-like receptors (RLR), and the AIM-2-like receptors (ALR) [1]. These families can be separated into two main classes: the membrane-bound receptors and the unbound intracellular receptors. The former class consists of the TLRs and CLRs, which are found at the cell surface or on the membranes of endocytic compartments. These receptors survey for the presence of microbial ligands in the extracellular space and within endocytic vesicles. The NLRs, RLRs, and ALRs form the later group being expressed within the cell cytoplasm where they survey for the presence of intracellular pathogens. While the PRRs are divergent in sequence, ligand specificity, and cellular localization, together they coordinate to protect the host from a range of eukaryotic, prokaryotic, or viral pathogens. However, this dissertation is specifically concerned with the regulation of antiviral innate

immune responses; therefore it focuses on the characteristic responses to these infections and the subset of PRR pathways that regulate them.

Antiviral Innate Immune Responses

The innate response to viral infection is initiated by a subset of PRRs and employs generalized mechanisms that broadly respond to the threat of viral infection [9]. Unlike an adaptive response, the innate antiviral response is not based on memory and is not tailored to a specific virus. It is classically defined by the production of type I interferon (IFN), which is a set of cytokines originally identified and named for their ability to "interfere" with viral replication [10-12]. The IFNs have been classified based on the receptor through which they bind and signal (type I, type II, and type III). Type I IFNs, which are the predominant form generated by PRR signaling, activate the IFN-alpha receptor and include IFN-alpha and IFN-beta.

Knockout mice with deficiencies in PRR signaling demonstrate the link between PRR signaling and the production of type I IFN [13-18]. When challenged with a wide range of viral pathogens, these mice are highly susceptible to morbidity and mortality. In addition to the production of type I IFN, antiviral PRRs also induce the production of other pro-inflammatory cytokines [9]. Together these signaling molecules coordinate several aspects of the antiviral response. For example, IFN signaling increases the expression of several antiviral PRRs. This change in expression increases the likelihood of viral detection as the infection spreads to uninfected cells. Furthermore, IFNs signal in an autocrine and

paracrine fashion to induce the expression of interferon stimulated genes (ISG) [11]. Many ISGs function as direct effectors against infection by interfering with viral mechanisms of genome replication, capsid assembly, or shedding. Therefore, the expression of ISGs at the site of infection creates an antiviral state as a countermeasure to prevent the spread of infection. Finally, the IFNs and pro-inflammatory cytokines produced during an antiviral response activate hematopoietic immune cells, which aid in pathogen clearance and activate adaptive immune responses [8, 11]. In particular, type I IFN production is important for recruitment of NK and cytotoxic T cells to the site of infection as well as the proliferation and maintenance of NK cell and CD8$^+$ memory T cell populations [19, 20].

The antiviral PRRs that induce the production of type I IFN almost exclusively recognize nucleic acids [21, 22]. This may have evolved due to the fact that viruses have few invariant structural determinants and a high mutation rate. In this case nucleic acid detection serves as a suitable alternative to recognizing the structural features of a pathogen. While nucleic acids are a common feature among viral pathogens, they are also a biosynthetic component of the host cell biology. Therefore, a means for distinguishing nucleic acids as self or non-self is required to prevent autoimmune activation [23]. To this end, some of the receptors have specificity for features of nucleic acids unique to the genomes, gene expression, or replication intermediates of viruses. This allows PRRs to reliably classify non-self viral nucleic acids as a viral infection and initiate antiviral defenses.

There is a large and growing list of innate immune receptors that recognize nucleic acids[23]. The best-characterized examples include members of the TLR and RLR families. The nucleic acid sensing TLRs survey endolysosomal compartments for the presence of both RNA and DNA molecules whereas the RLRs survey the cytoplasmic space for the presence of viral RNA. Several receptors responsible for detecting cytosolic DNA have also been described; with the enzyme cyclic GMP-AMP synthase (cGAS) being the only DNA sensor whose function is supported by genetic evidence in mice and humans[24-26]. cGAS and perhaps other DNA sensors signal via the ER-localized protein STING which activates IFN production in response to cytosolic DNA[27-29]. While each of the nucleic acid-sensing PRRs have a unique role in the contribution to antiviral defense, this dissertation will be focusing specifically on the regulation of the RLR signaling pathway.

The RIG-I-like Receptor Family

A family of three helicase domain-containing proteins make up the RLRs involved in cytosolic RNA detection[30]. The founding member, retinoic acid inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) have been most thoroughly characterized. Nucleic acid ligand recognition by RIG-I requires a 5' triphosphate, present on many viral genomes, as well as secondary structure such as a panhandle caused by base pairing[31-34]. There is also evidence for some RIG-I sequence specificity based on recognition of the poly-U/UC region within the HCV genome[35, 36]. The ligand specificity for MDA5 is

less well understood, but this receptor will respond to aggregated forms of long double stranded RNA [37-39]. These features such as a 5' triphosphate or double strandedness are not common among host RNAs and allow for the distinction between viral and host RNA within the cytosol.

Both RIG-I and MDA5 share a similar domain structure and some regulatory features. These receptors contain two caspase activation and recruitment (CARD) domains at their N-terminus, a centrally located DExD/H box helicase domain, and a C-terminal domain (CTD) [40]. The helicase and CTD are involved in viral RNA binding, whereas the tandem N-terminal CARD domains are required for the interaction with the downstream mitochondrial antiviral signaling adaptor (MAVS). At steady state the receptors are maintained in a closed conformation with the CARDs bound to the helicase domain to prevent aberrant signaling. RNA binding results in conformational changes that make the tandem CARDS accessible. The CARDs are then post-translational modified and/or interact with the adaptor MAVS [41].

The post-translational modifications regulating the activity of the RLRs include the addition of K63-linked ubiquitin chains and the removal of a phosphate group [42]. The E3 ubiquitin ligases Tripartite motif-containing 25 (TRIM25) and Riplet (also known as RNF135) both modify RIG-I with K63-linked ubiquitin chains and are required for RIG-I activation [43-45]. Trim25 produces K63-linked ubiquitin chains that interact with Lys 172 within CARD2 whereas Riplet modifies a region within the C-terminus of RIG-I [43, 44]. While K63 ubiquitin chains are required for signaling, RIG-I in conjunction with unanchored K63 ubiquitin

chains induce MAVS activation *in vitro* indicating that the covalent linkage to RIG-I is not a requirement [46]. The presence of K63-linked chains supports structural stabilization of a CARD tetramer which can activate the function of MAVS [47]. At resting state the RLRs each have phosphate group modifications that must be removed for the receptor to be activated. The protein phosphatases 1 alpha and 1 gamma are involved in this step of RLR activation. In addition to these post-translational modifications involved in regulating RLR activity, RIG-I and MDA5 form filamentous structures that are capable of inducing MAVS activation [48, 49]. However, ubiquitin modification and filament formation are individually sufficient for RIG-I to activate MAVS, but these two mechanisms may function together synergistically [47]. Taken together, we can appreciate that a number of regulatory steps are involved in controlling RLR activation and the induction of antiviral immune responses.

The role of the third RLR family member, Laboratory of Genetics and Physiology 2 (LGP2), in antiviral signaling has been somewhat controversial. Similar to RIG-I and MDA5, LGP2 contains a DExD/H box helicase domain and a CTD [30]. As this receptor lacks the N-terminal CARD domain required for interacting with the downstream adaptor MAVS, it was initially hypothesized and demonstrated to function as a negative regulator of antiviral signaling [30, 50]. Since the generation of LGP2 knockout mice however, reports indicate a positive role for LGP2 in RIG and MDA5 mediated antiviral signaling [51]. Some of the confusion behind LGP2 function may have been due to the use of non-physiological conditions including transient gene expression and activation using synthetic

ligands. Specifically, LGP2 is dispensable for the IFN response to synthetic ligands but is required for the response to infection from a number of viruses. While the role of LGP2 in antiviral signaling requires further clarification, it is not likely that this receptor exerts an effect through direct interaction with the downstream adaptor MAVS because it lacks a CARD domain.

The Antiviral Signaling Adaptor MAVS

In 2005, four groups individually identified an antiviral gene that encodes the adaptor protein MAVS (also referred to as IPS-1, Cardif, and VISA) [52-55]. As mentioned above, both RIG-I and MDA5 require the signaling adaptor MAVS for IFN production making it a critical component of antiviral defense. The importance of MAVS in antiviral defense has been demonstrated with the generation of MAVS deficient mice that are highly susceptible to infection from a number of RNA viruses [15, 16]. The domain organization of MAVS consists of a single N-terminal CARD domain, a proline-rich region (PRR), and a C-terminal transmembrane (TM) domain (Figure 1.1).

MAVS

CARD	PRR		TM
10-77	103-153		514-534

Figure 1.1 The domain structure of MAVS

The N-terminal caspase activation and recruitment domain (CARD) and a C-terminal transmebrane (TM) domain are required for the MAVS dependent IFN response. Some reports indicate that the proline-rich region (PRR) is required for some protein-protein interactions.

In addition to providing a platform for CARD-mediated RLR interactions, the CARD domain of MAVS is required for self-activation and IFN production. Upon activation MAVS undergoes polymerization creating prion-like aggregates that induce signal transduction [56]. These aggregates share some features with classically defined prions and require the CARD domain for propagation. MAVS aggregation activates a set of cytosolic kinases (TBK1, IKK) and transcription factors (IRF3, NF-κB) culminating in the expression of type I IFNs, pro-inflammatory cytokines, and ISGs (Figure 1.2) [57].
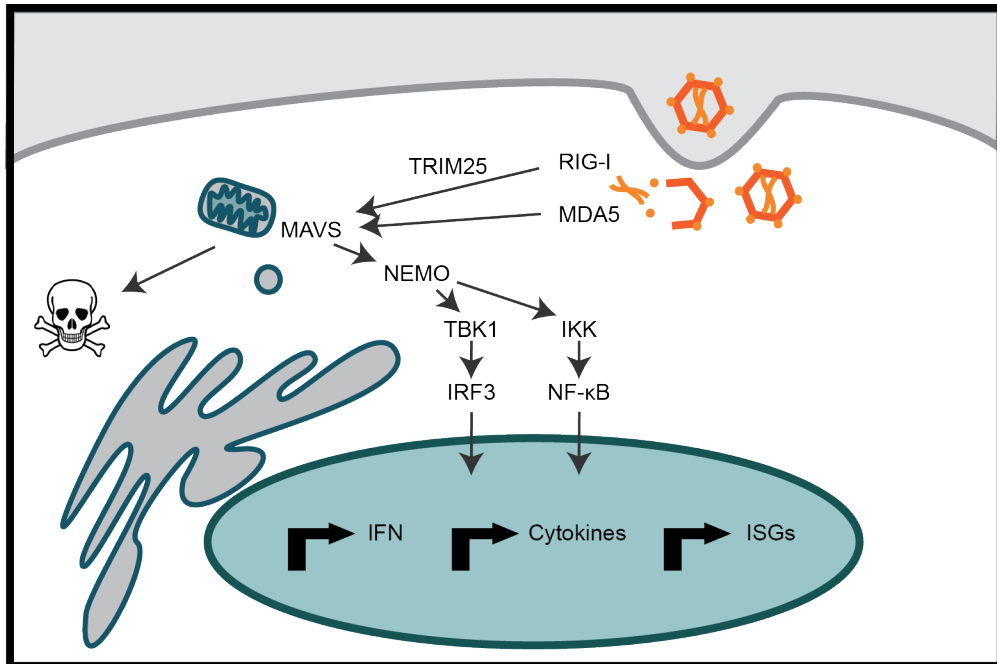
Figure 1.2 MAVS dependent antiviral responses

Viral RNAs within the cytosol activate the innate receptors, RIG-I and MDA5, which signal through MAVS. This antiviral signaling adaptor is required for the activation of downstream transcription factors that orchestrate the production of IFN, pro-inflammatory cytokines, and ISGs. In addition, MAVS induces cell death in an IFN-independent manner.

A defining feature of MAVS function and regulation is dictated by its subcellular localization. When MAVS was initially identified, Seth and colleagues noted that the C-terminal transmembrane domain (Figure 1.1) resembled that of other tail-anchored mitochondrial proteins[52]. The authors demonstrated that the adaptor co-localized and signaled from mitochondria and justly named the

adaptor mitochondrial antiviral signaling protein. In addition to mitochondria, MAVS is also localized on and signals from peroxisomal and mitochondrial associated membranes (MAMS) of the endoplasmic reticulum (ER) [58, 59]. During viral infection, RIG-I and TRIM25 are delivered to these sites of signaling by the mitochondrial chaperone protein 14-3-3ε [60]. MAVS variants that either lack the transmembrane domain or are genetically targeted to other subcellular locations have impaired function indicating a localization requirement for the adaptor [52, 58]. Even though these inactive variants retain the regions necessary for interacting with downstream signaling proteins, they are incapable of inducing an interferon response.

While MAVS requires proper membrane localization to one of these sites of signaling, it is important to note that the MAVS-induced signaling outcome can differ from these sites. Specifically, MAVS signaling from mitochondrial and peroxisomal membranes initiates distinct transcriptional profiles [58]. Signaling from the mitochondrial membrane induces the expression of type I IFN, which results in the subsequent expression of ISGs. In contrast, peroxisomal signaling induces the expression of ISGs independently of type I IFN expression. The mechanisms at work that regulate organelle specific signaling and the biological advantage this system offers have yet to be determined. It is tempting to speculate that factors unique to each organelle are responsible for dictating organelle specific signaling. An interesting feature of mitochondrial and peroxisomal biology is that these organelles interact through MAMs, which form a specialized subdomain of

the ER. This synaptic interface between mitochondria and peroxisomes coordinates an effective MAVS-dependent response to HCV infection [59].

Signaling Downstream of MAVS

The RLR/MAVS signaling pathway is extensively regulated by ubiquitin modifications, and similar to the RIG-I/ubiquitin signaling upstream of MAVS, downstream signaling also requires the ubiquitin system. The TNF receptor associated family (TRAF) proteins are a set of E3 ubiquitin ligases that are involved in the regulation of TNF receptor signaling as well as other innate immune signaling pathways [61]. The enzymatic activity of the TRAFs are involved in attaching ubiquitin to a targeted substrate, and several members of the TRAF family are involved in IFN and cytokine production [62]. When MAVS was first identified, it was noted to contain several TRAF binding domains that interact with TRAF2 and TRAF6 [52, 55]. Other TRAF family members, TRAF3 and TRAF5, also interact with MAVS [63-65]. As noted above, the aggregation of MAVS prion-like polymers is critical for the induction of downstream signaling. Interestingly, these aggregates are capable of specifically recruiting TRAF2 and TRAF6 in response to viral infection [56]. Mutating the TRAF binding sites results in the formation of MAVS polymers that are incapable of recruiting TRAF proteins or inducing the production of IFN [57]. This demonstrates that the TRAF signaling function is downstream of the aggregation of MAVS polymers. The number of TRAFs found to associate with MAVS and the fact that several TRAFs are partially required for signaling is indicative of functional redundancy [52, 55]. This

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redundancy for TRAF2, TRAF5, and TRAF6 demonstrates a requirement for the E3 ligase activity of these TRAF proteins [57] and indicates that a downstream signaling molecule may subsequently sense ubiquitin modifications made by them.

The NFκB essential modulator (NEMO) is a ubiquitin sensor that regulates the activity of the kinases IKK and TBK1, which regulate the transcription factors NFκB and IRF3 respectively [62, 66]. The ubiquitin binding domains of NEMO are required for activation of IRF3 in response to RNA viral infection [67]. Furthermore, NEMO forms a complex with MAVS, TRAFs, and TBK1 as part of the response to RNA viruses [57, 68]. Therefore, the TRAF ubiquitin ligases are required for the formation of a signaling complex containing NEMO and MAVS polymers. Other signaling molecules may also be present in this complex, which ultimately activates the cytosolic kinases IKK and TBK1 (Figure 1.2). The activity of the IKK complex and TBK1 phosphorylates the inhibitor of NFκB (IκBα) and IRF3 respectively [69, 70]. IκBα phosphorylation indirectly activates the transcription factor NFκB by releasing it for translocation to the nucleus. Conversely, phosphorylation directly activates the transcription factor IRF3, which forms homodimers prior to nuclear translocation and transcriptional regulation [71-74]. Together these transcription factors function to coordinate the expression of antiviral genes.

IFN Independent MAVS Mediated Cell Death

While the majority of MAVS research has focused on IFN production, it is worth noting that the RLR pathway can also limit viral replication by initiating cell death (Figure 1.2) [75-79]. Similar to IFN production, control of this cell biological process is MAVS dependent and also requires correct localization of the adaptor [76]. However, the induction of cell death is independent of MAVS induced IFN signaling [80, 81]. The signaling events downstream of MAVS controlling cell death have yet to be fully characterized, but a few reports have identified a subset of genes that are involved in the process. For example, caspase inhibition limits MAVS induced cell death indicating that caspase activation is a component of this process. A few distinct forms of cell death such as apoptosis or necrosis can be induced by caspase activation, yet the specific form induced by MAVS remains to be defined [82]. Several proteins with known cell death functions can interact with MAVS and contribute to the regulation of MAVS-mediated cell death. Some of these include, VDAC1, TRADD, and caspase 8 [81, 83, 84]. TRADD has been shown to interact with MAVS and recruit the proapoptotic proteins FADD and RIP1 [53, 84]. In addition, MAVS mediated SeV-induced death requires the kinases c-Jun N-terminal kinase 2 (JNK2) and MAPK kinase 7 (MKK7) [85]. While much remains to be characterized in the activation of cell death by MAVS, it is interesting to note that cell death does not rely on IFN signaling as demonstrated by cell death competent IFN incompetent MAVS constructs [76, 86].

In addition to not requiring the IFN inducing capacity of MAVS, it is interesting that cell death can be induced independently of the RLR receptors.

For example, SeV-induced death occurs independently of RIG-I [85], and VSV-induced cell death does not require the CARD domain of MAVS [81]. If MAVS mediated cell death does not require its CARD domain or the upstream RLR receptors, how does this critical adaptor receive a signal indicating infection? Do other receptors exist that induce MAVS-mediated cell death? Future studies will be needed to address these questions and characterize how MAVS regulates the process of virus induced cell death.

MAVS Regulation

The antiviral IFN and cell death responses described above must be tightly regulated. The inability to control these responses can make a host more susceptible to infection if the levels of IFN production or cell death are insufficient [15, 16]. Conversely, excessive antiviral responses can be detrimental to the host and incur pathology at the cost of inflammation and cell damage [87]. Most of the regulators that directly target MAVS have been characterized in the context of IFN production, and are discussed below. While MAVS induced cell death and IFN responses are independent of each other, it is possible that some these regulators affect both responses.

The self-aggregation of MAVS might be classified as the first regulator of MAVS function, however there are a number of mechanisms at work that additionally regulate the function of MAVS. Some of these regulatory mechanisms include protein-protein interactions, post-translational modifications, mitochondrial dynamics, autophagy, as well as protease cleavage. Several

regulators can either inhibit or potentiate MAVS signaling by direct protein-protein interaction. NLRX1, UBXN1, and TSPAN6 each physically interact with MAVS and negatively regulate the production of IFN [88-91]. These interactions may inhibit IFN production by preventing the binding of signaling or interfering with MAVS polymerization. The role of NLRX1 in MAVS dependent signaling has been a matter of a debate with some groups claiming that it does not function as an inhibitor of IFN production [92, 93]. However, as discussed below, a more recent publication proposes that NLRX1 may regulate autophagy in addition to IFN production [94]. Positive regulators of MAVS dependent signaling, such as Tom70 and IFIT3, also interact with the adaptor [95, 96]. In both cases these regulators facilitate MAVS interaction with the downstream signaling kinase TBK1 to promote IRF3 activation. While protein-protein interaction is one proposed mechanism for controlling MAVS signaling, several other proteins interact with MAVS and impart their regulatory effects through modification of the adaptor.

Both ubiquitination and phosphorylation are signal-induced post-translational modifications made to MAVS that regulate its function. While K63-linked polyubiquitin chains are involved in RIG-I mediated activation of MAVS, covalent ubiquitin modification of MAVS is primarily a form of negative regulation. A number of genes participate in this negative regulation including: PSMA7, PCBP2/AIP4, PCBP1/AIP4, Ndifp1/Smurf1, and Smurf2 [97-101]. With the exception of PSMA7 (due to a lack of experimental evidence) [97], these regulators specifically attach K48-linked ubiquitin chains to MAVS. This is somewhat expected because K48-linked ubiquitin modification is commonly used to target

proteins for proteolysis, whereas K63-linked ubiquitin coordinates other processes such as the RIG-I mediated activation of MAVS described earlier. Interestingly, there is one example of K63-linked ubiquitin modification of MAVS. This modification at lysine 500 mediates recruitment of IKKε to the adaptor and negatively regulates NF-κB activation and IFN production [102]. This is the only reported form of ubiquitin mediated MAVS regulation that is independent of proteasomal degradation. The E3 ubiquitin ligase responsible for this K63-linked modification at lysine 500 remains to be identified. In addition to ubiquitin modification, phosphorylation of MAVS can affect its function following viral infection. Extensive tyrosine phosphorylation of MAVS occurs with specific phosphorylation at tyrosine 9 required for signaling and IFN production [103]. The tyrosine kinase c-Abl targets MAVS and positively regulates IFN production, but it remains to be determined whether it is the kinase responsible for tyrosine 9 modification [104]. Together, these post-translational modifications regulate MAVS function as part of a coordinated antiviral response.

As mentioned above MAVS is localized to a set of organelles that interact with each other and are highly dynamic. Changes in the mitochondrial network occur following activation of the RLR pathway, and perhaps not surprisingly mitochondrial dynamics appear to regulate MAVS signaling [105]. Mitochondrial fusion is one such event that plays a role in this form of regulation. Cells deficient in regulators of mitochondrial fusion, Mitofusin 1 or Mitofusin 2, display impairments in mitochondrial fusion and IFN signaling in response to viral infection [106, 107]. This indicates that mitochondrial fusion events regulate the RLR

signaling pathway. One possible mechanism of this regulation could be that mitochondrial fusion contributes to the aggregation of MAVS for its polymerization. While several reports provide evidence in agreement that mitochondrial fusion positively contributes to MAVS activation, there is one report that Mitofusin 2 functions alternatively to inhibit MAVS through protein-protein interaction. In support of this hypothesis, increased IFN signaling was observed in Mitofusin 2 knockout cells [108]. Thus Mitofusin 2 may function as positive or negative regulator of IFN production through mitochondrial fusion or direct interactions respectively. Regardless, mitochondrial dynamics have some regulatory function in MAVS signaling, but it remains to be determined whether regulators of peroxisome and ER/MAM physiology also affect the signaling outcomes of the RLR pathway.

In addition to altered mitochondrial dynamics, viral infection is also associated with the induction of autophagy. This catabolic process of cell physiology is utilized for the maintenance of cellular health. It degrades intracellular components at baseline levels, but can be induced in response to cellular stresses such as organelle dysfunction and viral infection. While autophagy has been implicated in the immune response to viral infection as a form of targeted pathogen elimination [109], it is not clear that this process is regulated by RLR signaling. Rather, autophagy may negatively regulate MAVS dependent IFN responses by targeting mitochondria resulting in higher rates of viral replication [110]. Mediators of this process include Atg5, Atg12, NLRX1, TUFM, and COX5B [94, 111, 112]. Interfering with these regulators inhibits the formation of

autophagosomes resulting in higher levels of IFN production and decreased viral replication. The question of how autophagy functions to negatively regulate MAVS signaling requires further characterization. Some proposed mechanisms include the removal of damaged mitochondria, the degradation of aggregated MAVS complexes, and the reduction in reactive oxygen species. Identifying the cellular biological events that control the MAVS-autophagy axis will provide further insight into the regulation of this innate signaling pathway.

As a way to subvert host-mediated antiviral measures, viruses have also evolved their own tactics of regulating the function of MAVS. Targeting MAVS for proteolytic cleavage is an immune evasion strategy employed by many viruses. This connection was made with Hepatitis C Virus (HCV) in one of the original papers that identified MAVS[54]. The HCV encoded protease NS3/4A blocks IFN production during viral infection by cleaving MAVS. These authors also generated a protease resistant variant of MAVS with a point mutation altering the cysteine at amino acid 508 of human MAVS to an alanine[54]. Several other viral proteases also cleave MAVS and as a result decrease IFN production during infection. Some of the viruses encoding these proteases include GB virus B, Hepatitis A Virus, Human Rhinovirus 1a, Coxsackievirus B3 (CVB3), and Enterovirus 71 (EV71) [113-117]. The 3ABC protease precursor encoded by Hepatitis A virus targets the cysteine protease 3C^{pro} to mitochondria, which can cleave MAVS near its C-terminus as demonstrated by a Q428A protease resistant mutant[114]. Similar to NS3/4A, this MAVS proteolysis disrupts interferon signaling and antiviral defense. Rather than targeting the C-terminus for

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cleavage, both CVB3 and EV71 encoded cysteine proteases target the proline-rich region of MAVS [116, 117]. A Q148A mutation in MAVS disrupts cleavage by 3C^{pro} of Coxsackievirus B3, whereas the Enterovirus 71-encoded protease 2A^{pro} may have multiple targets and requires glycine to alanine mutations at residues 209, 251, and 265 of MAVS to completely block proteolysis. Neither the respective N-terminal or C-terminal cleavage products generated by these proteases are capable of inducing an IFN signaling response. While the MAVS C-terminal fragments retained mitochondrial localization, the loss of the N-terminal CARD domain was sufficient to disrupt IFN signaling function. Therefore a large number of virus-employed evasion strategies have evolved to manipulate MAVS signaling. However, many of these virus-encoded proteases can serve multiple functions by targeting several host-encoded proteins. For example NS3/4A of HCV and 3C^{pro} of CVB3 can also cleave TRIF to interfere with TLR driven antiviral responses [116, 118]. Therefore, it can be difficult to determine the relative importance of each target during viral infection. Further characterization of these viral immune evasion tactics will not only provide insight into MAVS regulation but may also highlight potential antiviral therapeutic targets.

In conclusion, the function of MAVS is dynamically regulated by a number of different mechanisms. The strategies employed in MAVS regulation range from direct protein interaction to regulated cell processes such as mitochondrial fusion. However, in addition to host-encoded regulation, viruses can also regulate the function of this antiviral adaptor. In future studies it will be important to dissect how these regulators function in context with each other and to

specifically understand how these regulators function in the context of MAVS polymerization.

Regulation of Innate Immune Responses Through Protein Diversification

The number of MAVS regulators described in the previous section demonstrates not only the extent but also the range of mechanisms available for controlling antiviral gene function. However, one regulatory mechanism yet to be described is the ability to generate protein diversity from within a single genetic locus. For example, one gene can generate a diverse set of protein variants through the mechanism of alternative splicing. This is a form of mRNA processing can dictate gene function by removing or altering the protein domains encoded on a transcript. In addition to generating a diverse set of transcripts from a single gene, a diverse set of proteins can be produced from a single transcript through alternative translation. These two processes offer an effective way to alter protein activity by swapping in and out domains to diversify the function of a single genetic locus.

The first form of protein diversification, alternative splicing, is a form of mRNA processing that is restricted to eukaryotic organisms. There are many genes that encode alternative splice variants including the gene encoding RIG-I [119]. Compared to the full-length protein, the RIG-I splice variant (RIG-I sv) lacks a short region within the first CARD domain corresponding to amino acids 36-80 of RIG-I. This region is required for TRIM25 binding and without it the RIG-I sv is not capable of activating the pathway. Since the expression of RIG-I sv is

induced following viral infection and can inhibit RIG-I-mediated IFN production, it appears to resolve antiviral signaling via its dominant negative function. Many other innate immune genes encode splice variants that regulate signaling including the genes that encode MAVS, MyD88, and TRAM [120-122].

Alternative translation is a second process of generating protein diversity, which occurs when protein synthesis is initiated from more than one start codon on a single transcript. In contrast to alternative splicing, which is exclusive to eukaryotic organisms, alternative translation is a feature more common to bacterial and viral transcripts [123]. In fact, the scarcity of alternative translation examples described in higher organisms has led to the consensus that it is not a generalized mechanism for creating protein diversity in mammalian cells [124]. The few examples that do exist have instead been considered exceptions to the rules of gene regulation in mammals [125-132]. However, genome-wide ribosomal profiling studies now suggest that polycistronic mRNAs are more prevalent in eukaryotes than previously appreciated [133]. Ribosomal profiling is a technique that identifies the footprint, or position, of a ribosome on a transcript by sequencing mRNA associated with it [134]. In conjunction with the drug harringtonine, ribosomal profiling can be used to identify translation initiation sites from endogenous mRNA transcripts [135, 136]. Harringtonine is a natural compound that inhibits the elongation step of translation and stalls ribosomes at sites of initiation. Ribosomal profiling studies have revealed that thousands of transcripts may contain more than one site of translation initiation. Future studies are required to determine

whether translation occurs from these newly identified start sites and whether the products of this synthesis are functional.

All of the known examples of protein diversification that regulate the MAVS antiviral innate signaling pathways fit within the first class of alternative splicing. To date, no example has been described within the context of alternative translation. This dissertation describes the first example of an antiviral innate immune signaling variant generated by alternative translation. The transcript encoding the antiviral signaling adaptor MAVS is bicistronic and encodes FL MAVS and a truncated variant, miniMAVS, through alternative translation. Therefore, the regulation of antiviral signaling depends on the translation of a variant from a downstream start codon within the MAVS transcript. However, certain regulatory mechanisms must be in place to allow for translation events at downstream start codons.

Cis-encoded Regulatory Mechanisms of Translation Initiation at Downstream Start Codons

At the onset of translation the 40s ribosomal subunit binds to the 5' cap of an mRNA transcript and begins scanning, or processing in a 3' direction until it encounters an optimal start codon [137]. During initiation at this start codon, the 60s ribosomal subunit is recruited to the complex prior to the elongation step of protein synthesis. Based on this model of ribosomal scanning, it is not intuitively clear how the translation machinery reaches downstream start codons to initiate protein synthesis. Leaky ribosomal scanning is one of the mechanisms described

to regulate translation at these downstream start codons [138]. In this process, ribosomes "leak" or scan past start codons that are suboptimal. This insufficiency in ribosome initiation is dictated by the nucleotide sequence directly surrounding a start codon and is referred to as the translational context at a start site [139]. Start sites with a strong translational context are optimal for protein synthesis, whereas weak translational start sites have suboptimal conditions for initiation. Marilyn Kozak made this distinction and characterized the consensus sequence for optimal translation initiation on eukaryotic mRNA [140]. Therefore, a weak translation context at upstream start codons may allow for leaky ribosomal scanning and the alternative translation from downstream start codons.

A second mechanism that allows for translation from downstream start sites is through the use of internal ribosomal entry site (IRES) elements. These cis-encoded sequences within the RNA can directly recruit ribosomal subunits independently of a 5' cap [141]. It has been hypothesized that ribosomes are recruited to secondary or tertiary structures formed by these nucleotide sequences. While RNA transcripts encoded by viruses commonly use IRES elements, the existence of eukaryotic encoded IRES elements is controversial because they are not as common and are comparatively less efficient [142].

In addition to leaky ribosomal scanning and IRES elements, translation initiation at small upstream open reading frames (uORFs) within the UTR can regulate translation at downstream start codons [143]. A number of uORF related factors can contribute to downstream start site regulation. For instance, the translational context at uORFs may regulate whether ribosomes continue

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scanning down to the canonical ORF of a transcript. Furthermore, the number of uORFs within the UTR as well as the distance between a uORF termination site and a canonical ORF start site can affect the expression of the canonical ORF. A particularly interesting model for regulating downstream start sites has also been proposed for uORFs that overlap other translational start sites [125, 143]. Translation of the overlapping uORF offers a mechanism for a ribosome to pass a canonical ORF start site (Figure 1.3). Once a translating ribosome terminates translation of the overlapping uORF, it may resume scanning and then re-initiate translation at the start codon of a downstream ORF (Figure 1.3) [144]. One parameter controlling the efficiency of re-initiation is the intercistronic length between termination and a second start site.
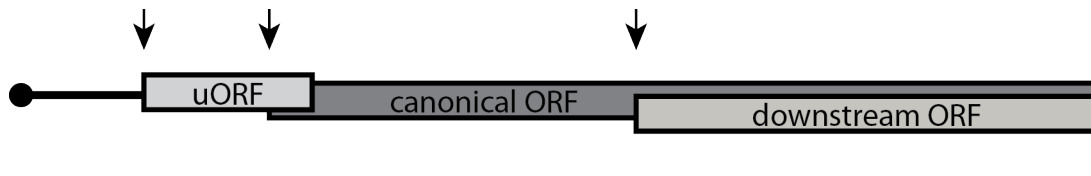

Figure 1.3 A mechanistic model for how an overlapping uORF may regulate translation of downstream ORFs

Shaded boxes highlight the open reading frames present on this cartoon depiction of an mRNA transcript. The arrows indicate the translational start site locations on the transcript. Ribosomes that are translating the uORF can pass the start site for the canonical ORF and re-initiate at the downstream ORF start site.

Therefore, cis-encoded regulatory elements within mRNA transcripts, such as the translational context at a start codon, IRES elements, and uORFs, may help dictate start codon usage. In addition, translation initiation factors and other trans-regulatory elements can control gene expression by regulating how and when protein synthesis is initiated from different start sites. Now that ribosomal profiling has indicated a higher incidence of polycistronic messages than previously appreciated, it will be important to characterize the variants generated in addition to understanding the regulatory mechanisms that control their translation.

Dissertation Objective

Since Charles Janeway's hypothesis that innate immune responses were classified and initiated by a set of receptors with fixed specificity, our understanding of innate immune signal regulation has grown exponentially [1]. Hundreds of genes and regulatory mechanisms participate in the controlled response to microbial infection. However, many gaps still exist in our understanding of how these pathways are controlled. Recently ribosomal profiling studies have discovered that a significant percentage of mammalian transcripts may have more than one functional site of translation initiation [133]. The products generated from these alternative translation start sites may play an important role in many biological processes. This dissertation describes an example of an antiviral innate signaling variant generated from a bicistronic mRNA. This highlights the use of alternative translation as a means of gene regulation that may be more common than previously appreciated. Chapter two demonstrates that the transcript encoding MAVS is bicistronic encoding the full-length MAVS protein and truncated variant miniMAVS. Then chapter three investigates the regulatory function of miniMAVS in the context of antiviral IFN production and cell death. The results of a genome-wide ribosomal profiling study conducted to identify other regulators of innate immunity generated by alternative translation are described in chapter four. Finally, chapter five provides a general discussion of the results presented in this dissertation, their implications, and directions for future research.

Chapter 2

The Bicistronic *MAVS* Transcript Encodes a Truncated Variant

The substance of this chapter was previously published.

Brubaker, S.W., Gauthier, A.E., Mills, E.W., Ingolia, N.T. & Kagan, J.C. 2014, "A bicistronic MAVS transcript highlights a class of truncated variants in antiviral immunity", *Cell,* vol. 156, no. 4, pp. 800-811.

Contributions: N.T. Ingolia performed and analyzed the ribosomal profiling described in Figure 2.4. All other experiments were designed and executed by S.W. Brubaker.

INTRODUCTION

Chapter one discusses the innate immune signal transduction pathways that induce immune responses to microbial infection. These responses and the inflammation associated with them control the spread of infection, but they can also have detrimental effects on host tissues [87]. Therefore, regulation of innate signaling pathways is critical to maintain immune homeostasis. One form of regulating these immune responses is through the diversification of protein form and function. As mentioned in the previous chapter, diversification can be achieved from a single genetic locus through alternative splicing and/or translation, resulting in the production of multiple proteins with distinct functions. These processes provide an effective mechanism to either remove or add protein domains, which increases the functional diversity of a gene to regulate biological processes such as antiviral signaling.

The antiviral RLR signaling pathway is one such innate immune pathway that induces inflammation and can be regulated through protein diversification [22]. The gene encoding RIG-I, one of the receptors for this pathway, can be alternatively spliced to generate two unique mature RNA transcripts [119]. The full-length transcript encodes RIG-I, which functions to detect viruses containing RNA (and in some instances DNA) genomes in the cytosol of infected cells [145, 146]. Once RIG-I binds to viral RNA, it is activated and engages the adaptor MAVS to induce the expression of type I IFN, pro-inflammatory cytokines, and ISGs. The alternative splice variant, RIG-I sv, is a dominant negative and limits the signaling potential of full-length RIG-I [119]. This form of generating protein diversity

from eukaryotic genes is a common mechanism used to control the activity of innate immune signaling pathways. Other examples of innate immunity genes with encoded splice variants that regulate signaling include MAVS, MyD88, and TRAM [120-122].

On the other hand, the process of alternative translation as a means of generating protein diversity has historically been considered a feature limited to viruses and prokaryotes [124]. While a few examples of alternative translation from eukaryotic transcripts have been described, recent genome-wide ribosome profiling studies in eukaryotes indicate that polycistronic mRNAs may be more prevalent than previously appreciated [133, 147]. Some of the genes that encode the transcripts with alternative start sites may function in antiviral innate immune responses. Based on the function of innate immune variants generated through alternative splicing like RIG-I sv, protein variants generated through alternative translation might also be capable of regulating antiviral signaling.

This chapter describes two regulators of antiviral innate immunity that are generated by alternative translation of the same bicistronic message. The transcript encoding the RLR adaptor protein MAVS produces the well-characterized full-length (FL) MAVS adaptor and a truncated variant called miniMAVS. A second start codon downstream of the FL MAVS start site led to the hypothesis that miniMAVS is translated from this alternative start site. Distinct experimental approaches were designed to demonstrate the bicistronic nature of the *MAVS* transcript. A genetic approach was used to mutate translational start sites and shift the reading frame of the transcript to demonstrate alternative

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translation. In addition, ribosomal profiling was used to demonstrate that ribosomes initiate translation at these unique start codons *in vivo*. Finally, cis-regulatory elements within the transcript were also determined to play a role in controlling the synthesis of these two proteins.

The work described in this chapter is significant in that it characterizes a novel level of regulation operating within the *MAVS* antiviral signaling gene. In addition, this process of alternative translation is currently considered to be a unique feature of very few mammalian genes. This provides a mandate to determine if other examples of alternative translation exist in other "well-characterized" genes.

RESULTS

MAVS Point Mutations and Validation of Translational Start Sites

In 2005, the MAVS gene was identified as an adaptor of the type-I IFN antiviral response to RNA viruses [52-55]. As part of its original characterization Seth and colleagues generated a MAVS-specific antibody raised against a peptide consisting of amino acids 131-291. This antibody detected two MAVS proteins with apparent molecular weights of 50 and 72 kilodaltons (kDa). At the time of publication, it was speculated that the 50kDa variant represented a degradation product or processed version of the 72kDa full-length variant FL MAVS [52]. To date, all antiviral activities of the *MAVS* gene have been attributed to FL MAVS. The origin and function of the smaller protein, miniMAVS, has yet to be characterized. These two MAVS proteins can be detected in a number of different human cell lines, indicating that the expression of both MAVS proteins is ubiquitous and likely of functional relevance (Figure 2.1A and [52]). Alternative mRNA splicing is one process that could explain the existence of this second MAVS variant. However, although several *MAVS* splice variants exist [120], none correspond to the correct size of miniMAVS (~50kDa) (data not shown). Additionally, both FL MAVS and miniMAVS were expressed from the *MAVS* coding region (CDS) by *in vitro* transcription and translation. (Figure 2.1B). Thus two proteins were synthesized by translation from a single transcript in vitro. These data indicate that the two MAVS variants may also be generated from a single transcript in vivo and are therefore not likely to be generated by differential mRNA splicing.
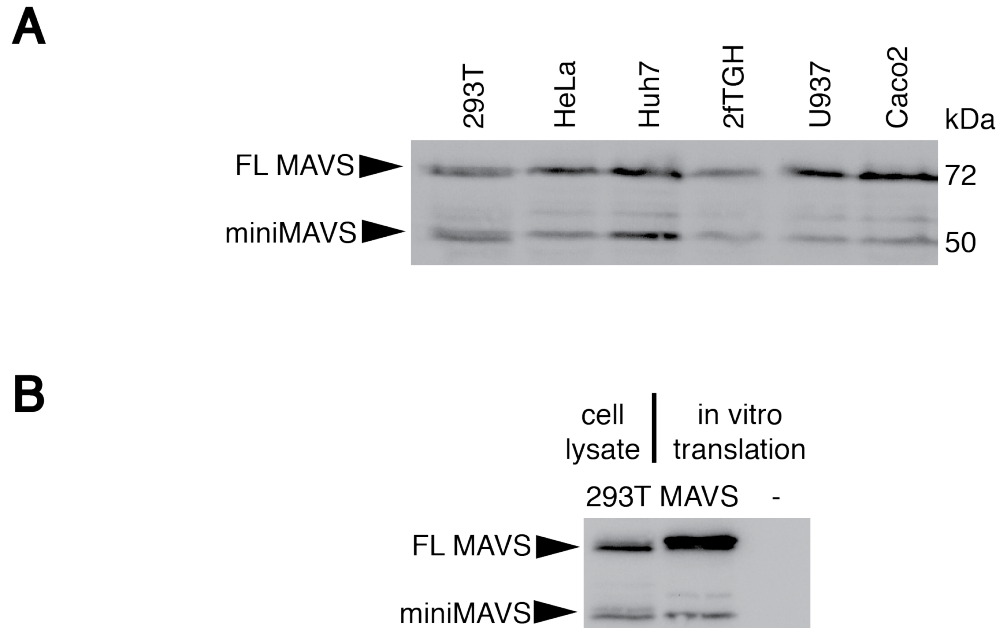
Figure 2.1 Two MAVS variants, FL MAVS and miniMAVS, are observed by western blot

(A) Lysates from several different human cell lines were separated by SDS-PAGE, and endogenous MAVS expression was detected with a MAVS specific antibody.

(B) In vitro transcription and translation of the *MAVS* CDS compared with MAVS expression from 293T cell lysates using a MAVS specific antibody.

The presence of a methionine at amino acid 142 of the *MAVS* CDS led to the hypothesis that miniMAVS expression is the result of translation initiation at an alternative start site (Figure 2.2A). Consistent with this hypothesis, initiation at this putative start codon (Met 142) would generate a protein corresponding to the molecular weight of miniMAVS (approximately 50kDa) and share sequence homology with FL MAVS. To determine if Met 142 was required for the production of miniMAVS, I mutated the corresponding start sites by replacing the methionine with an alanine. Mutation of either the methionine at position 1 or the methionine at position 142 resulted in the respective loss of FL MAVS or miniMAVS expression *in vitro* (Figure 2.2B). Furthermore, *in vivo* stable expression of these mutant alleles in *MAVS* deficient mouse embryo fibroblasts (MEFs) had a similar expression pattern (Figure 2.2C). The putative start site corresponding to Met 142 of human MAVS is conserved among primates and other higher mammals (Figure 2.2D). In contrast, rodent MAVS sequences (*e.g.* Ferret, Guinea pig, mouse, rat and squirrel) do not contain a corresponding Met 142. Thus miniMAVS appears to have evolved later in evolution than the MAVS protein itself. It remains to be determined whether other putative start sites present in rodent MAVS sequences can function to produce variant proteins similar to human miniMAVS. These results suggest that the human *MAVS* transcript is bicistronic and that miniMAVS is the product of a unique open reading frame (ORF) downstream of the FL MAVS start site*.*
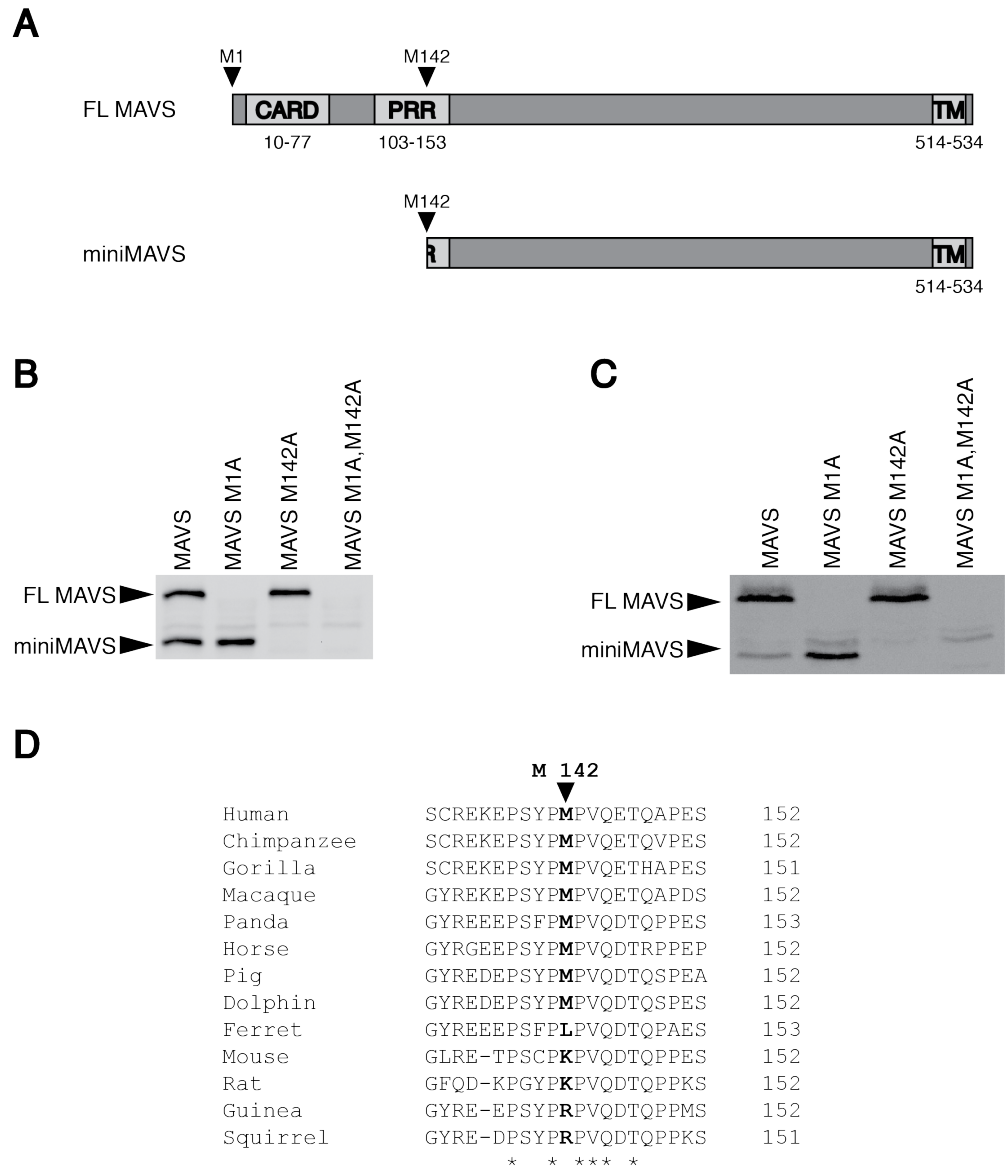
Figure 2.2

miniMAVS is expressed from a second translational start site

(A) Schematic of predicted MAVS translation products FL MAVS and miniMAVS from the start sites corresponding to Met1 and Met142. The major protein domains are shown with corresponding amino acid range below each domain.

Figure 2.2 (Continued)

miniMAVS is expressed from a second translational start site

(B) Point mutations of translational start sites at Met1 and Met142 were made and expressed by *in vitro* transcription and translation assay. The translation products were detected by immunoblot with a MAVS specific antibody following separation by SDS-PAGE.

(C) Point mutations of translational start sites at Met1 and Met142 were made and expressed *in vivo* in MAVS-deficient mouse embryo fibroblasts. The translation products were detected by immunoblot with a MAVS specific antibody.

(D) The region surrounding Met142 of human MAVS was aligned to several other species. Met142 is highlighted in bold and conserved amino acids are highlighted with an asterisk.

Frame-shift Mutation in MAVS Demonstrates Bicistronic Expression From a Single Transcript

The methionine mutations described above suggest that miniMAVS expression is the result of alternative translation of a bicistronic *MAVS* transcript. However, the possibility also exists that miniMAVS is created by the proteolytic cleavage of FL MAVS, and that the methionine at position 142 is necessary for this cleavage event. If the *MAVS* transcript is truly bicistronic, then it should be possible to engineer this mRNA to produce two distinct protein products that share no amino acid homology. To this end, a two-nucleotide insertion was introduced between the FL MAVS and miniMAVS start sites in a *MAVS* construct containing an amino-terminal HA epitope tag (Figure 2.3A). This insertion will shift the reading frame of HA-tagged FL MAVS, resulting in an altered amino acid sequence and a truncated protein called HA-shift. However, since the insertion is upstream of the miniMAVS start site, the reading frame and amino acid sequence of miniMAVS should not be affected. While the HA-shift protein could be detected by antibodies specific for the HA epitope tag, the shift in reading frame rendered the protein undetectable by the MAVS antibody (Figure 2.3B). Interestingly, this transcript still produced miniMAVS, as detected with the MAVS antibody (Figure 2.3B). The expression of these two distinct proteins from the same transcript demonstrates the bicistronic nature of the *MAVS* mRNA. Additionally, the frame-shift mutation rules out the possibility that miniMAVS is generated by post-translational proteolysis of FL MAVS.
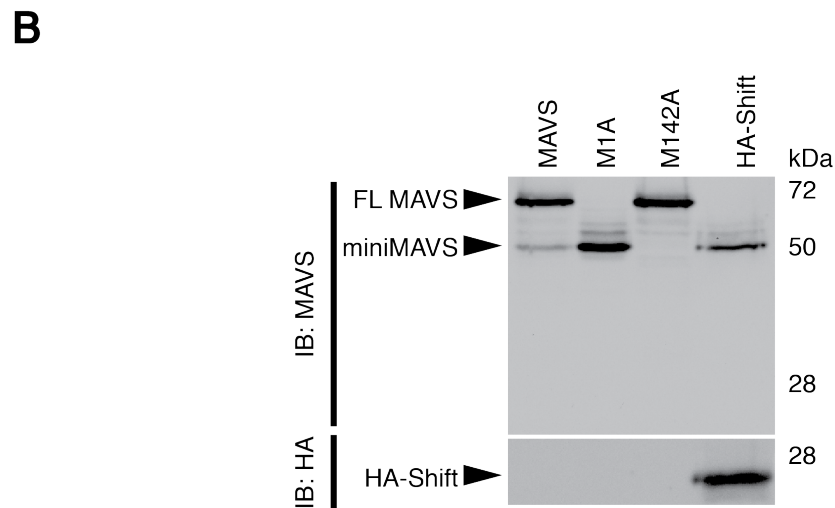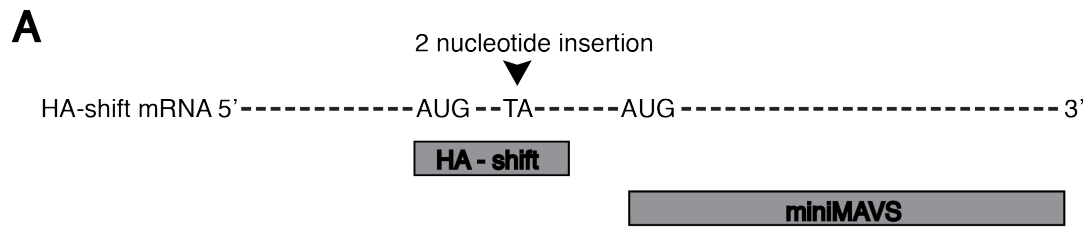
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Figure 2.3 Frameshift mutation demonstrates MAVS is bicistronic

(A) A schematic of the HA-shift expression vector containing a frameshift mutation is displayed with the predicted translation products "HA-shift" and miniMAVS.

(B) Lysates from stable mouse embryo fibroblast cell lines expressing the MAVS and HA-shift constructs were separated by SDS-PAGE and protein expression was determined with MAVS and HA specific antibodies.

Ribosome Profile of the Endogenous MAVS Transcript Confirms Start Site Usage

The experiments detailed above were carried out by genetic manipulation of a cloned MAVS cDNA. To investigate the bicistronic nature of the endogenous human *MAVS* mRNA and determine whether ribosomes initiate translation at these start sites *in vivo*, a ribosome profile of *MAVS* mRNA from HEK293T cells was generated. Ribosome profiling is a strategy that utilizes deep sequencing of ribosome-protected mRNA fragments to investigate different aspects of translation [133, 135, 136]. In conjunction with the drug harringtonine, which stalls ribosomes at initiation codons, this technique allows for the identification of functional translational start sites on endogenous mRNAs. In the absence of harringtonine, ribosomes were found throughout the open reading frame of *MAVS*, indicating active translation and elongation (Figure 2.4). However, in the presence of harringtonine, ribosomes on the *MAVS* mRNA were predominately stalled at the two start sites previously identified to correspond with methionine 1 and methionine 142 of MAVS (Figure 2.4). Therefore, the same translational start sites that are required for FL MAVS and miniMAVS expression *in vitro* are sites of translation initiation on the endogenous *MAVS* mRNA *in vivo*. Taken together, these results establish that the *MAVS* mRNA is bicistronic and encodes for FL MAVS and miniMAVS by alternative translation from two distinct start sites.
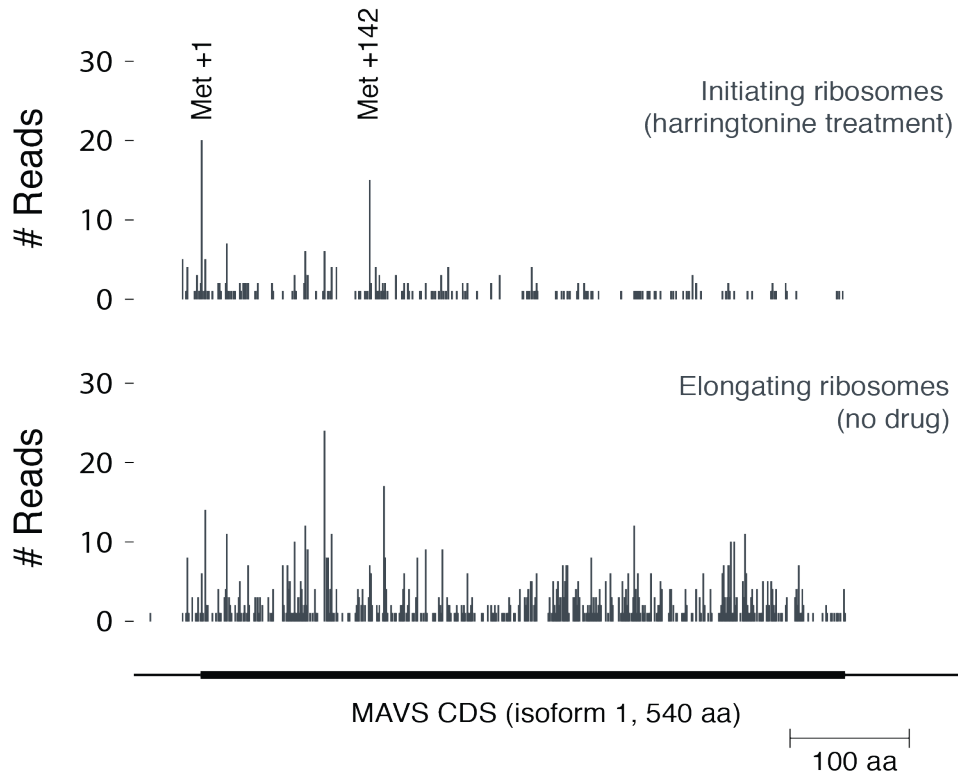
Figure 2.4 Ribosome profiling detects *in vivo* ribosome initiation at the FL MAVS and miniMAVS start sites

Displayed is a pattern of ribosome initiation (harringtonine treatment) and elongation on the endogenous *MAVS* mRNA from 293T cells.

miniMAVS Expression Requires Leaky Ribosomal Scanning

To further understand how the expression of FL MAVS and miniMAVS is regulated, cis-acting elements were identified that control the expression of these variants. One mechanism by which downstream ORFs are expressed from a single transcript involves leaky ribosomal scanning through upstream start codons [138, 143]. Typically, ribosomal scanning begins at the 5' cap of a transcript and translation is initiated at the first optimal start site. Optimal translational start sites were characterized by Marilyn Kozak and depend on the nucleotide context directly surrounding a start codon [123, 139]. Leaky ribosomal scanning occurs when the start site is suboptimal and ribosomes fail to initiate translation [138]. Under these conditions, ribosomes will 'leak' through the initial start site, continue scanning along the mRNA, and initiate at a downstream start site. This mechanism predicts that the expression of downstream proteins is dependent on the translational context of upstream start sites.

If miniMAVS expression requires leaky ribosomal scanning, blocking ribosomal scanning should decrease miniMAVS expression. To test this, new start codons were introduced between the FL MAVS start codon and the miniMAVS start codon to block ribosomal scanning. Translation initiation at a new start codon would block scanning by translating a third protein, 'midiMAVS', thus preventing ribosomes from reaching the miniMAVS start site. Introduction of a new start codon in a position that has a naturally strong start context (L62M) strongly suppressed miniMAVS expression (Figure 2.5A, lane 2). However, artificial start codons with weaker translational start contexts (G67M and E80M)

were leaky, allowing ribosomes to proceed and more efficiently translate miniMAVS (Figure 2.5A, lanes 3 and 4). These results are therefore consistent with the idea that miniMAVS expression relies on leaky ribosomal scanning from the FL MAVS start site to the miniMAVS start site.
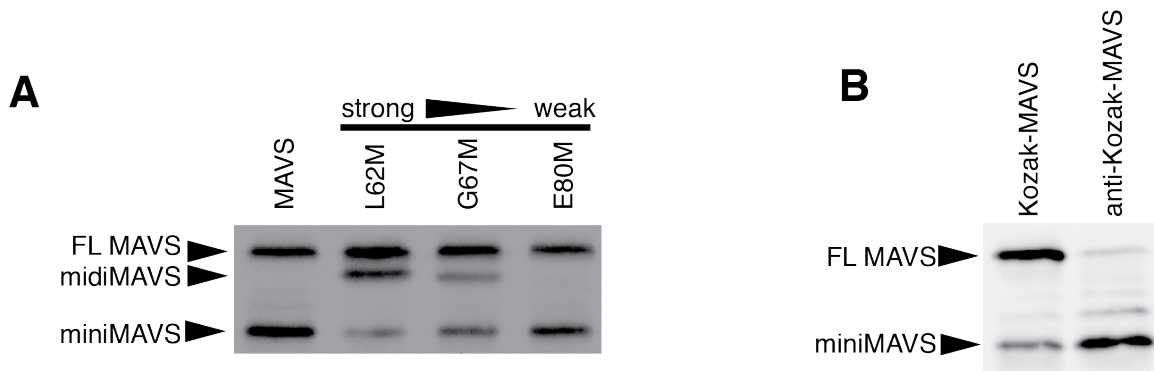

Figure 2.5 miniMAVS expression requires leaky ribosomal scanning

(A) Translational start sites of varying strength were introduced at Leu62, Gly67, and Glu80 of the *MAVS* CDS to block ribosomal scanning between the FL MAVS and miniMAVS start sites. The constructs were expressed in vitro and the resulting MAVS products were detected by immunoblot with a MAVS specific antibody

(B) *In vitro* expression of two *MAVS* CDS constructs containing a strong (Kozak) or weak (anti-Kozak) translational context at the FL MAVS start site.

Based on these data, the translational context of any upstream start site, including the FL MAVS start site, could affect the expression of miniMAVS. This possibility was addressed by placing an artificially strong (Kozak) and weak (anti-Kozak) translational context at the FL MAVS start site [138]. A strong translational context at the FL MAVS start site resulted in the high expression of FL MAVS compared to miniMAVS, whereas a weak translational context resulted in the lower expression of FL MAVS and high expression of miniMAVS (Figure 2.5B). These results establish the translational context surrounding the FL MAVS start site as a cis-regulatory element that controls the expression of miniMAVS.

The above-described experiments all point to an important role for the endogenous 5' untranslated region (UTR) of the *MAVS* transcript in controlling the expression of miniMAVS, as this region contains the natural translational context of the FL MAVS start site. To address this directly, a *MAVS* expression vector containing the endogenous 5'UTR was created. When *MAVS*-deficient MEFs were transiently transfected with this vector, both FL MAVS and miniMAVS were expressed (Figure 2.6A, lane 2) indicating that the endogenous context at the FL MAVS start site is sufficient for miniMAVS expression.
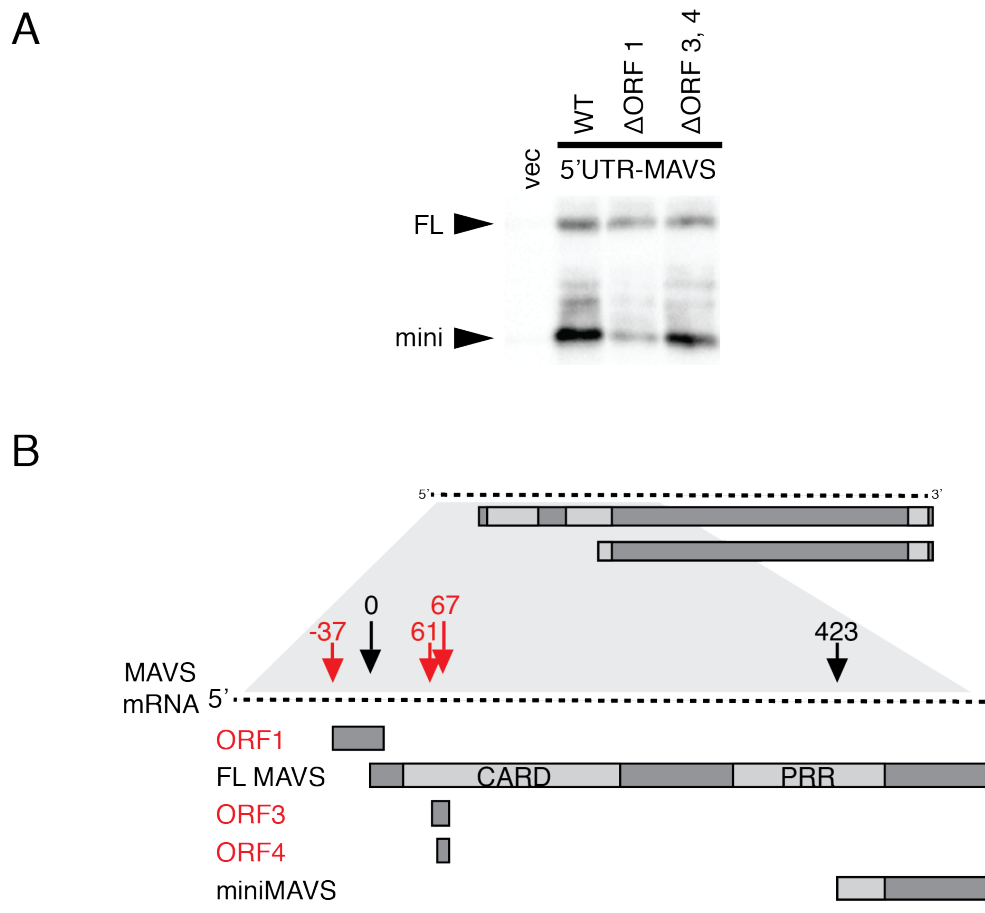
Figure 2.6 miniMAVS expression requires leaky ribosomal scanning

(A) Expression of FL MAVS and miniMAVS in *MAVS*-deficient mouse embryo fibroblasts transfected with expression containing the endogenous 5'UTR of *MAVS* or constructs with mutated start sites for ORF1 or ORF3,4.

(B) Schematic of the *MAVS* mRNA containing the endogenous 5'UTR and highlighting the three ORFs (red) that are out-of-frame with FL MAVS and miniMAVS. Numbers indicate the distance (in nucleotides) that each start site is from the FL MAVS start site.

An Endogenous uORF Can Regulate the Expression of miniMAVS

Examination of all the natural start codons present within the 5'UTR and coding region upstream of miniMAVS suggested a mechanism by which the FL MAVS start site is skipped en route to translating miniMAVS. Three additional start codons are present within this region including one in the 5'UTR (ORF1) coding for an out-of-frame upstream ORF (uORF) (Figure 2.6B). The translation of uORFs in 5'UTRs is emerging as a means by which translation of downstream ORFs can be regulated [125, 143]. For example, if initiation occurs at a uORF that overlaps with the start site of a canonical ORF, the translating ribosome will skip the start codon of the canonical ORF [143]. After termination of uORF translation, the ribosome may resume scanning and re-initiate translation at downstream ORFs. ORF1 is an overlapping uORF, predicted to initiate the translation of a small peptide that overlaps with the coding region of FL MAVS, terminating past its start site (Figure 2.6B). Translation of ORF1 might allow ribosomes to bypass the FL MAVS start site, resume scanning, and re-initiate at the miniMAVS start site. To test this, the start site of ORF1 was mutated, as were the start sites for ORF3 and ORF4, which may create small peptides within the *MAVS* coding region (Figure 2.6B). The resulting constructs were then tested for the expression of FL MAVS and miniMAVS in *MAVS* deficient MEFs. Interestingly, mutating the start codon of ORF1 reduced the level of miniMAVS relative to FL MAVS, whereas mutating ORF3 and ORF4 had a minimal effect on miniMAVS expression (Figure 2.6A). These data suggest that ORF3 and ORF4 are likely bypassed by leaky scanning whereas translation of ORF1 allows ribosomes to

skip the FL MAVS start site and facilitate the translation of miniMAVS, likely by

re-initiation. However, because FL MAVS is expressed when ORF1 is present

(Figure 2.6A), skipping of the FL MAVS start site cannot occur 100% of the time.

Therefore, leaky scanning might occur at the ORF1 start site, allowing for FL

MAVS translation. Consistent with this hypothesis, the translational context at the

ORF1 is suboptimal, suggesting a mechanism by which leaky scanning may

occur (Table 2.1).

Table 2.1 Description of ORF start sites on the MAVS transcript

The strength of each start site was determined by adherence to the
Kozak consensus sequence. Start sites with a purine at position -3
and a guanine at position +4 are considered strong. Start sites with
one of the above properties are considered medium, and those
lacking both are considered weak.

Table 2.1

Start Site Name	Start Site Sequence	Start Site Strength
ORF1	CCACCCTTC**ATG**G	medium
ORF2 - FL MAVS	TGAGCAGCA**ATG**C	medium
ORF3	ATTTTTGCA**ATG**T	medium
ORF4	GCAATGTGG**ATG**T	weak
ORF5 - miniMAVS	AGTTACCCC**ATG**C	weak

It should be noted however, that the mutation at the ORF1 start site could

possibly influence the mRNA in additional ways (*e.g.* changes in secondary

structure), which may contribute to altering the regulation of translation. Overall,

our collective data reveal that cis-regulatory elements in the 5'UTR of the *MAVS*

transcript help explain the relative translation efficiency of FL MAVS and

miniMAVS.

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DISCUSSION

Prior to this work it was well established that the *MAVS* genetic locus has a role in the defense against viral infection. Ubiquitous *MAVS* expression has been reported from many cell types further supporting its biological importance [52]. Interestingly, this expression has repeatedly been demonstrated to control the generation of two proteins from this single locus [52, 56]. The proteins differ in apparent molecular weight and siRNA-mediated knockdown can abrogate their expression [52]. However, the mechanism controlling the generation of these two proteins had yet to be determined. This chapter demonstrates that alternative translation is the mechanism by which the two protein variants, FL MAVS and miniMAVS, are generated from a single mRNA transcript. In addition, cis-regulatory elements within the transcript control the expression of these two variants through translation initiation.

Several lines of evidence support our conclusion that the *MAVS* transcript is bicistronic. (1) The cDNA of *MAVS* can produce both FL MAVS and miniMAVS, and the molecular weight of miniMAVS does not correspond to that of any possible product of alternative splicing. (2) Profiling of ribosomes arrested at translational start sites within the endogenous *MAVS* mRNA revealed two start codons. These start codons are predicted to produce proteins of the sizes corresponding to FL MAVS and miniMAVS. When these start codons were mutated, the resulting transcripts lost the ability to produce the corresponding MAVS variant. (3) Shifting the reading frame of the *MAVS* coding sequence at a site between these two start sites resulted in the production of two distinct protein

products (HA-shift and miniMAVS). Because FL MAVS is not produced under these conditions, the existence of miniMAVS cannot be explained by proteolytic cleavage of the full-length protein. Collectively, the above observations can only be explained by the conclusion that FL MAVS and miniMAVS are produced from a bicistronic mRNA encoded by the *MAVS* gene.

The significance in finding that the *MAVS* transcript is bicistronic is demonstrated by the scarcity of eukaryotic encoded examples of alternative translation. Changes to the process of transcription and RNA maturation have long been considered the predominant form of generating functional diversity from a single genetic locus in eukaryotes. Although alternative translation as a form of gene diversification is commonly observed from viral and bacterial encoded transcripts, it has yet to be considered a common feature of eukaryotic genes [124]. However, the *MAVS* example (in addition to previously described examples of eukaryotic alternative translation) highlights translation as a process that can be used to generate protein variants and diversity from a single transcript in eukaryotes. Some of these examples include transcripts for the genes encoding C/EBP, tetherin, USP18, LAP, p53, osteopontin, and PTEN [125-132]. Both tetherin and osteopontin offer separate examples of immune regulation through alternative translation. The transcript encoding tetherin generates a long isoform l-tetherin and a short isoform s-tetherin. Due to the N-terminal truncation the variant s-tetherin functions as a negative regulator to the NF-κB inducing capabilities of l-tetherin. However, both variants are capable of retaining budding virions, the primary function of the tetherin gene [126]. Alternative translation of

osteopontin generates a full-length secreted isoform, Opn-s, and an intracellular isoform, Opn-i. The differential localization of these variants is dictated by the presence (Opn-s) or truncation (Opn-i) of a signal sequence [131]. These variants are associated with distinct functions between subsets of dendritic cells highlighting cell type-specific control of translation. Specifically, the intracellular form of osteopontin is required for TLR9 driven IFNalpha expression observed in plasmacytoid dendritic cells [148]. These examples support a model where truncated variants generated by alternative translation have distinct functions. Whether the *MAVS* variants have differential function remains to be determined.

Regulating the expression of proteins like miniMAVS from downstream translational start sites is not a trivial matter during the process of translation. The reason for this being that mRNA translation is unidirectional and highly regulated following a set of steps initiated at the 5'terminal end of a transcript [137]. The 40s subunit of the ribosome is recruited to the 5'cap and begins processing in a 3' direction scanning the nucleotide sequence for a translational start site. Once a start site is encountered, translation is initiated and the 60s ribosomal subunit is recruited to form a complete ribosome (80s) complex which commences translation elongation [137]. Thus, for translation to occur at downstream start codons, a mechanism must be in place to allow the ribosome to pass upstream start codons. While some downstream translational start sites rely on direct recruitment of the ribosome complex with an internal ribosomal entry site (IRES), other downstream start sites rely on ribosomes that skip or scan past upstream start sites [138].

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The work described in this chapter supports a model where miniMAVS expression relies on uORF-mediated start codon skipping as well as leaky ribosomal scanning. This proposed model of miniMAVS expression is based on a several pieces of evidence. (1) The expression of miniMAVS is almost completely abrogated when ribosomal scanning is blocked with the introduction of artificial start sites. If miniMAVS translation relied on direct recruitment of ribosomes, then the expression of miniMAVS would have remained constant in these experiments. Therefore, it is not likely that direct recruitment of ribosomes at an IRES element contributes to the expression of miniMAVS. (2) Mutating the start site of the overlapping uORF1 resulted in a loss of miniMAVS expression. Overlapping open frames can mediate start codon skipping by the ribosome complex. During this process, translation can be re-initiated at downstream start codons once translation is terminated from the overlapping reading frame and the ribosome resumes scanning along the transcript [144]. (3) Mutating the start sites for ORF3 and ORF4 had no effect on the expression of miniMAVS. Thus translation initiation does not likely occur at these reading frames, rather ribosomes are likely to pass these start sites by leaky scanning. This hypothesis is supported by the lack of a strong translational context at the start sites for ORF3 and ORF4 (Table 2.1). These findings point to a mechanism where ribosomes initiate at uORF1 to skip the FL MAVS start codon and then scan past ORF3 and ORF4 to then re-initiate at the miniMAVS start site. However, this does not account for FL MAVS expression, which may require some degree of leaky scanning at uORF1.

In addition, there may be other mechanisms that are capable of controlling translation initiation at these two unique start sites. For example, other initiation factors may function in trans to regulate FL MAVS and miniMAVS expression. Global rates of translation and start codon usage can be affected by cellular stresses or viral infection [149, 150]. Therefore, activation or deactivation of translation initiation factors by IFN signaling or viral infection could alter expression of these two proteins. Future research could utilize the technique of ribosomal profiling to identify whether translation regulation occurs on the *MAVS* transcript during different cellular stresses. Other biochemical or genetic approaches would then be required to identify the factors that control such regulation.

Collectively the work described in this chapter demonstrates that the *MAVS* transcript is bicistronic and controls the expression of FL MAVS and miniMAVS through alternative translation. Furthermore, cis-regulatory elements within the *MAVS* transcript help to control the expression of miniMAVS from a downstream start site. While the proposed expression model of these two proteins is based on the experiments described here, this does not exclude the possibility that other factors may also contribute to regulating their translation. For example, the tertiary structure of the *MAVS* transcript (which was not addressed) could be important for regulating translation initiation. It is also likely that trans-regulatory mechanisms contribute to the control of FL MAVS and miniMAVS expression.

Whether or not FL MAVS and miniMAVS expression has more complex regulation will likely be of most interest in the context of their antiviral functions. For this reason, the antiviral function of each variant is systematically addressed in the following chapter.

MATERIALS AND METHODS

Generation of MAVS expression constructs

The *MAVS* CDS from allele BC044952 was a gift of Z.J. Chen (UTSW). Variants were cloned into a pcDNA3 vector containing an N-terminal HA tag. Variants were cloned with (strong translational context) or without (weak translational context) the N-terminal tag. Both used the same C-terminal restriction site XhoI, primer AAAAACTCGAGCTAGTGCAGACGCCGCCGGTACAGC. The strong translational context variants were inserted into the vector with KpnI, fwd primer AAAAAGGTACCGCACCGTTTGCTGAAGACAAGACCTAT. The translational context at the HA start codon of this vector was as follows: AAGCTTACG**ATG**G. The weak translational context variants were inserted with HindIII, which removed the HA tag, fwd primer TTTTTAAGCTTATGCC GTTTGCTGAAGACAAGACCTAT. The translational context at the start codon of this vector was as follows: CCCAAGCTT**ATG**C. For the Kozak and anti-Kozak constructs, the following sequences were placed directly upstream of the FL MAVS start codon: GCCGCCACC and ATATATTTT. The sequence used to generate the 5'UTR *MAVS* constructs is listed in the Ensemble database under transcript ID number ENST00000428216. The HA-shift construct was made by

inserting two nucleotides "TA" at bp number 254 of the *MAVS* CDS with the fwd primer   GTGAGCTAGTTGATCTCGTACGGACGAAGTGGCCTCTGTC.   Stable MAVS cell lines were generated with pMSCV2.2 IRES GFP in *MAVS*-deficient MEF cells. All expression constructs are available for purchase online at www.addgene.org where you can also find sequences and further details about the point mutations that were generated.

MAVS Expression

Endogenous MAVS expression was determined by western blot analysis of cell lysates from several human cell types grown in DMEM containing 10% serum. MAVS in vitro expression was performed using a coupled transcription and translation rabbit reticulocyte lysate kit (Promega) with a T7 pcDNA3 expression vector. MAVS in vivo expression was performed in MAVS-deficient immortalized MEFs cells cultured in DMEM containing 10% serum. MAVS-deficient immortalized MEFs were a gift of Z.J. Chen (UTSW). Stable expression of the MAVS and HA-shift constructs was achieved by genomic integration of a pMSCV2.2 IRES GFP retroviral expression system. Transient expression of the 5' UTR MAVS constructs was achieved by Fugene 6 (Promega) mediated transfection of a pcDNA3 expression vector. The antibodies used for protein detection by western blot were MAVS specific (Bethyl Labs A300-782A) and HA specific (Roche 3F10),

MAVS Alignment

The MAVS amino acid sequences from several different species were aligned using the ClustalW2 alignment software available from the European Bioinformatics Institute online at http://www.ebi.ac.uk/. The amino acid sequences for each species were obtained from the National Center for Biotechnological Information online at http://www.ncbi.nlm.nih.gov/.

Ribosomal Profiling

Ribosomal profiling was done with lysates from 293t cells that were treated or untreated with the drug harringtonine. The profiling and analysis have been previously described [135].

Chapter 3

miniMAVS Regulates Antiviral Signaling Events

The substance of this chapter was previously published.

Brubaker, S.W., Gauthier, A.E., Mills, E.W., Ingolia, N.T. & Kagan, J.C. 2014, "A bicistronic MAVS transcript highlights a class of truncated variants in antiviral immunity", *Cell,* vol. 156, no. 4, pp. 800-811.

Contributions: A.E. Gauthier performed the experiments described in Figure 3.7. All other experiments were designed and executed by S.W. Brubaker.

INTRODUCTION

The *MAVS* gene was originally identified based on the observation that overexpression of the *MAVS* CDS was sufficient to activate the production of type I IFN [52-55]. *MAVS* deficient mice display abrogated IFN responses and are therefore more susceptible to RNA virus infection [15, 16]. In addition to IFN production, the *MAVS* gene is also required for virus-induced cell death that limits viral replication [75, 76]. Thus IFN production and cell death have been established as critical *MAVS* dependent antiviral responses.

The previous chapter established that FL MAVS and miniMAVS are generated through alternative translation of the transcript encoded by the *MAVS* gene. Previous studies reporting on the function of *MAVS* had yet to determine the antiviral signaling function of either protein individually. Therefore, it was of interest to determine the role of each variant in the antiviral activities attributed to the *MAVS* gene. Interestingly, the predicted MAVS cleavage product generated by the CVB3 protease 3C^{pro} resembles miniMAVS and is not capable of inducing an IFN response [116]. This cleavage product spanning residues 149-540 similarly lacks the N-terminal CARD domain, which suggests that miniMAVS may be a host-encoded mechanism for regulating MAVS-dependent antiviral responses.

Changes in the ratio of FL MAVS to miniMAVS can also be detected following viral infection (Figure 3.1). Whereas FL MAVS became less abundant in infected cells over time, miniMAVS levels were not affected (Figure 3.1). Thus as the infection progressed, miniMAVS became the dominant MAVS variant in the cell. This dynamic protein regulation during an antiviral response indicates that

each protein has a unique role during the course of infection, and justifies characterizing the function of each variant.
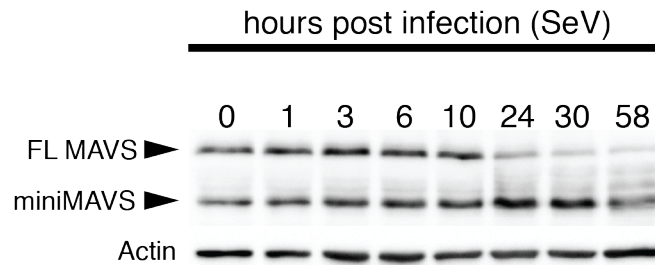
hours post infection (SeV)

Figure 3.1

The ratio of FL MAVS to miniMAVS changes following infection

Western blot analysis of endogenous FL MAVS and miniMAVS expression in 293T cells following SeV infection.

This chapter characterizes the respective signaling functions of FL MAVS and miniMAVS. To address this question, the genetic tools developed in chapter two were utilized. First, the start site mutations (Figure 2.2B and C) were used to test each variant individually for their ability to activate a given cellular response. Second, changes in the translational context of the FL MAVS start site were used to manipulate the expression ratio of FL MAVS to miniMAVS and determine how they function in conjunction. Unlike FL MAVS, data in this chapter demonstrates that miniMAVS is not capable of inducing IFN production on it's own. Rather, miniMAVS expression can interfere with FL MAVS-induced IFN expression. While these two variants are capable of antagonizing one another in the context of IFN production, they are each capable of positively inducing the antiviral cell death response.

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RESULTS

miniMAVS Interferes With FL MAVS mediated IFN production

To determine a role for miniMAVS in the antiviral IFN response, the production of type I IFN was measured following expression of the *MAVS* start site mutants in 293T cells. When only the FL MAVS variant (M142A) was expressed, robust production of type I IFN was observed (Figure 3.2A). Conversely, when only miniMAVS (M1A) was expressed there was no induction of type I IFN. In addition, a miniMAVS deletion mutant lacking the C-terminal localization signal (M1A-500) was not capable of inducing the production of IFN (Figure 3.2A and [52]). The results of this experiment suggest that FL MAVS is sufficient to positively regulate the production of IFN whereas miniMAVS is not sufficient to activate the pathway. However, when the two variants were expressed in conjunction (MAVS) there was a decrease in type I IFN production compared to FL MAVS expression alone (Figure 3.2A). To corroborate these findings, the phosphorylation of STAT1, an indicator of IFN signaling [151], was monitored over the course of 24hrs following transfection. Compared to FL MAVS expression alone (M142A), cells expressing both MAVS variants (MAVS) contained lower levels of phosphorylated STAT1 over time (Figure 3.2B). This difference in signaling activity between cells expressing FL MAVS alone and cells expressing both MAVS variants was not the result of differential expression of FL MAVS. Indeed, western analysis indicated comparable expression of FL MAVS when expressed alone (M142A) or when expression in conjunction with miniMAVS (MAVS) (Figure 3.2B). Taken together, these results suggest that

miniMAVS antagonizes the signaling function of FL MAVS and inhibits IFN production.
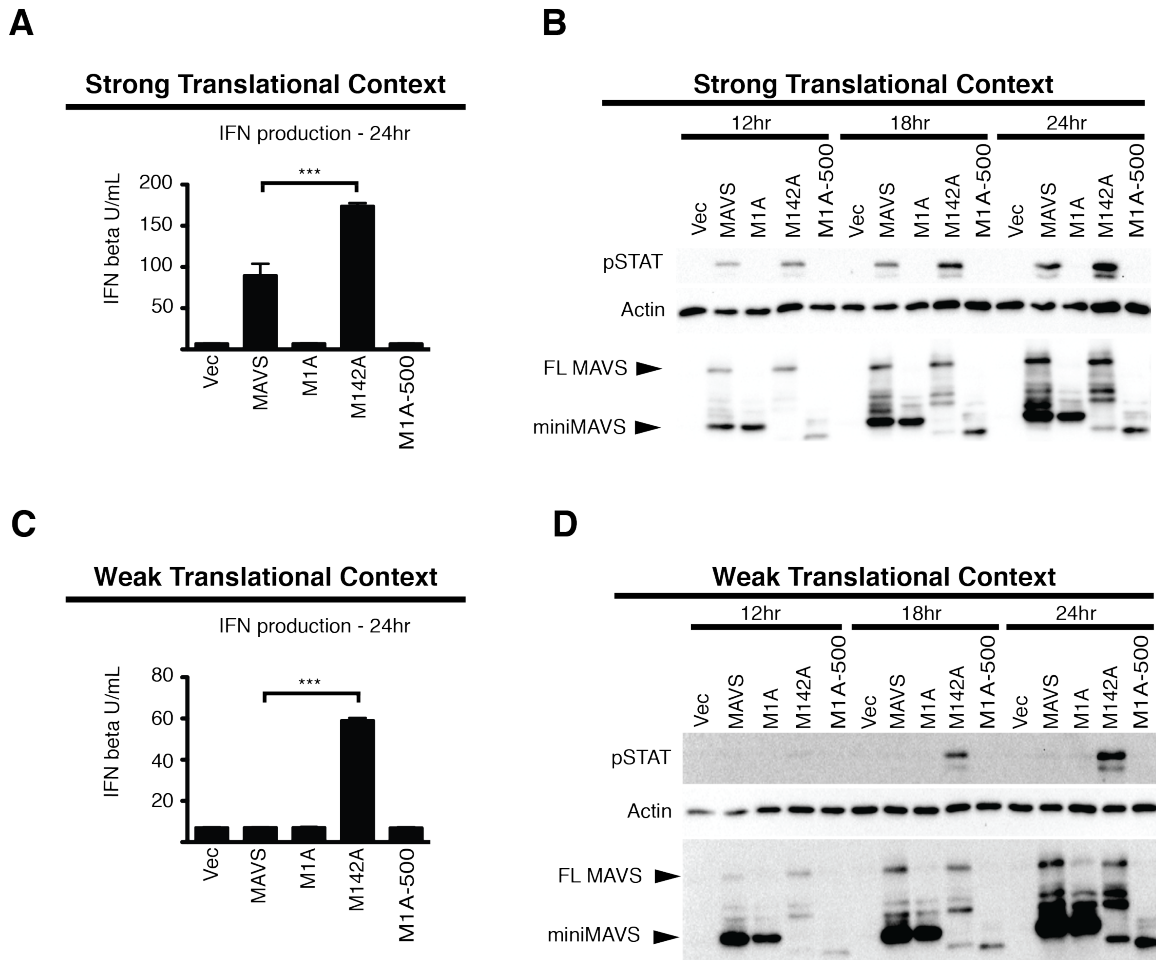
A

Strong Translational Context

IFN production - 24hr

B

Strong Translational Context

C

Weak Translational Context

IFN production - 24hr

D

Weak Translational Context

Figure 3.2 miniMAVS interferes with IFN signaling

(A) Type I IFN production was measured following the expression the miniMAVS alone (M1A), FL MAVS alone (M142A), or the two in conjunction (MAVS). In addition, IFN production from a miniMAVS construct lacking the TM domain was determined (M1A-500).

Figure 3.2 (Continued) miniMAVS interferes with IFN signaling

(B) Following transient transfection of the constructs described in (A), the expression of MAVS variants and STAT1 phosphorylation was monitored over time by western blot.

(C) IFN production and (D) STAT1 phosphorylation were monitored as in (A and B). However the transient expression constructs contain a comparatively weaker translational context at the FL MAVS start site. As a result, there is more leaky scanning and a higher ratio of miniMAVS to FL MAVS expression (compare MAVS conditions from B and D).

***$p < 0.001$ by ANOVA with Tukey's multiple comparison test. Error bars represent standard deviation.

To more directly test the hypothesis that miniMAVS can inhibit the production of IFN, an expression construct with a weak translational context at the FL MAVS start site was characterized. Due to leaky scanning, this weak translational context is predicted to increase the ratio of miniMAVS to FL MAVS in the cell, and this increase in ratio was predicted to further inhibit the production of type I IFN. As expected, the weak translational context resulted in higher abundance of miniMAVS relative to FL MAVS when both variants were expressed (MAVS, Figure 3.2D), as compared to the experiments using MAVS with a strong translational context (Figure 3.2B). Remarkably, when both variants were expressed in conjunction (MAVS) with this weak translational context, the effect was a complete abrogation of IFN production and STAT1 phosphorylation (Figure 3.2C and D). However, under the same conditions, when FL MAVS was expressed alone (M142A), a robust production of IFN and STAT1 activation was observed (Figure 3.2C and D). Taken together, these data reveal miniMAVS as an inhibitor of FL MAVS signaling and that the ratio of FL MAVS to miniMAVS determines whether an antiviral response will occur.

To further test miniMAVS inhibition of IFN signaling, expression constructs that more closely mimicked the natural *MAVS* transcript were examined. Specifically, the activation of IFN signaling following expression from constructs containing the endogenous 5'UTR and *MAVS* CDS were examined. As described in chapter two, ORF1 in the 5'UTR of the transcript can regulate the expression of miniMAVS, and when mutated, there is a decrease in miniMAVS expression (Figure 2.6A). Thus mutating the ORF1 start site would be expected to result in

increased IFN production. Consistent with this idea, when compared to the wild-type (WT) 5'UTR construct, expression of the uORF1 mutant resulted in increased STAT1 activation (Figure 3.3A and B). These data further establish that miniMAVS interferes with the FL MAVS IFN response and identify uORF1 as a regulator of both the expression and function of miniMAVS.
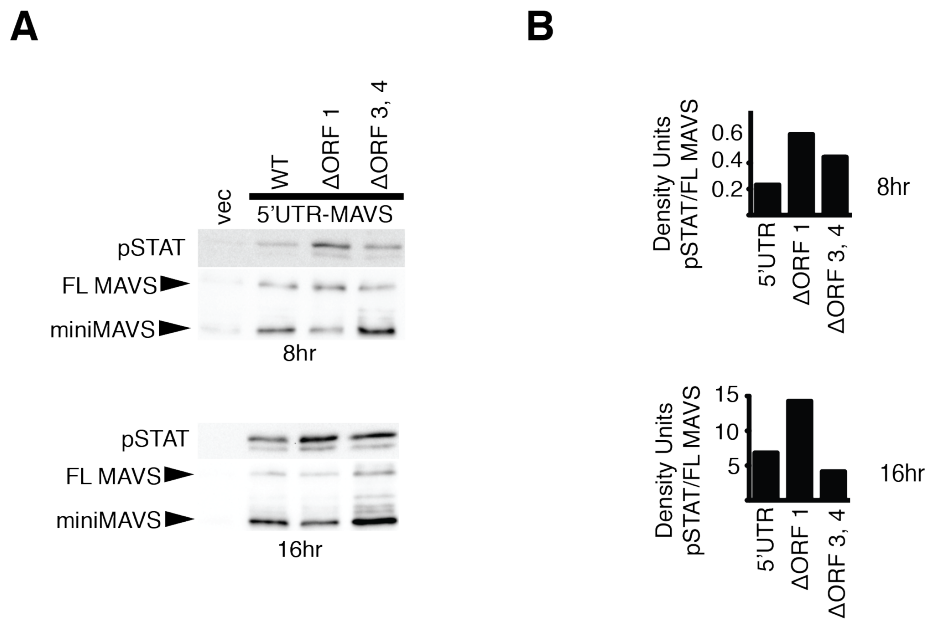

Figure 3.3 STAT1 activation after transient expression of MAVS containing the endogenous 5'UTR and uORF mutations

(A) Western blot analysis of STAT1 phosphorylation and MAVS expression following transient transfection of the indicated MAVS constructs

(B) The ratio of STAT1 phosphorylation to FL MAVS expression was quantified by densitometry. Densitometry is from a representative image of an experiment done in triplicate.

Having established regulatory effects of FL MAVS and miniMAVS on antiviral signaling, the differential expression of the two proteins would also be predicted to affect viral replication. Whereas the expression of miniMAVS alone (M1A) had little effect on the replication of vesicular stomatitis virus (VSV), FL MAVS expression alone (M142A) dramatically reduced VSV replication (Figure 3.4). Interestingly, expression of the two proteins in conjunction (MAVS, as in Figure 3.2D) was less effective at limiting viral replication as compared to expression of FL MAVS alone (Figure 3.4). These data therefore establish that miniMAVS acts to restrict the signaling functions of FL MAVS, the physiological consequence of which is that FL MAVS is less able to create an antiviral cellular state.
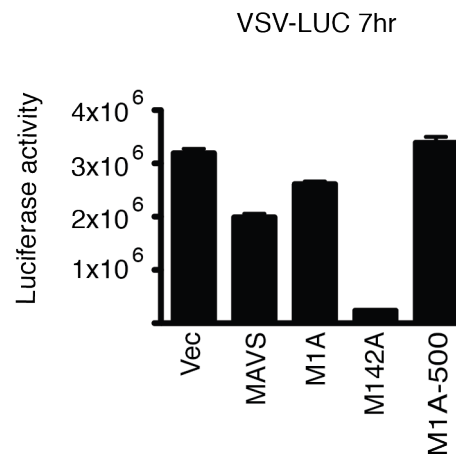

Figure 3.4 The effect of miniMAVS expression on viral replication

293t cells were transfected with the MAVS constructs from Figure 3.2 C. 24 hours post transfection the cells were infected with VSV-encoding firefly luciferase. As a measure of viral replication, the luciferase activity was determined 7 hours following infection. Error bars represent standard deviation of an experiment in triplicate.

miniMAVS Does Not Prevent the Polymerization of FL MAVS

During viral infections, large aggregates of FL MAVS form that recruit downstream enzymes to promote the expression of type I IFNs [56]. It was therefore possible that miniMAVS restricts the signaling functions of FL MAVS by preventing the formation of these large protein aggregates. To address this possibility, FL MAVS was expressed alone or in conjunction with miniMAVS, and FL MAVS aggregates were detected following sucrose gradient ultracentrifugation. For these studies, the expression constructs containing the weak translational context from Figure 3.2 were used, as under these conditions, miniMAVS completely abrogated the production of IFN. When FL MAVS alone (M142A) was expressed, aggregates of FL MAVS could be detected at the bottom of the sucrose gradient (Figure 3.5A). This was expected because the expression of FL MAVS alone results in the production of IFN (Figure 3.2C), and it is thought that IFN signaling is a result of MAVS aggregation [56]. Interestingly, when both FL MAVS and miniMAVS were expressed in conjunction (MAVS), the formation of FL MAVS aggregates was also detected (Figure 3.5A). This was surprising because, under these conditions, miniMAVS completely blocks the production of IFN (Figure 3.2C). These data suggest that miniMAVS cannot block FL MAVS aggregate formation, even under conditions where the signaling functions of FL MAVS are completely prevented. Consistent with the idea that miniMAVS does not influence the aggregate-forming activity of its full-length counterpart; in response to Sendai virus infections, endogenous miniMAVS does

not co-sediment with FL MAVS aggregates (Figure 3.5B). Thus, miniMAVS is neither a component of FL MAVS aggregates nor does it regulate their formation.
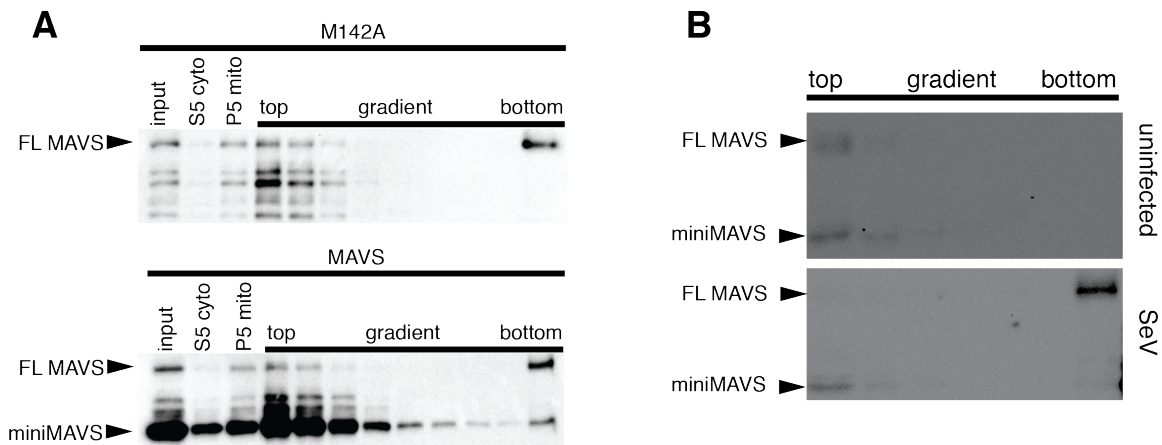

Figure 3.5 miniMAVS does not prevent FL MAVS polymerization

(A) Crude mitochondria (P5) isolated from 293T cells transfected with *MAVS* or the M142A point mutant were separated by sucrose gradient ultracentrifugation. FL MAVS polymers segregated to the bottom of the gradient (right) and were detected by SDS-PAGE followed by immunoblot with a MAVS antibody.

(B) 293t cells were infected with SeV and crude mitochondrial extracts were separated by sucrose gradient centrifugation to detect MAVS polymers by SDS-PAGE and MAVS immunoblot.

miniMAVS Can Interact With the Downstream Signaling Enzymes TRAF2 and TRAF6

Aggregates of FL MAVS promote antiviral signaling by recruitment of the E3 ubiquitin ligases TRAF2 and TRAF6[57]. Because miniMAVS was not capable of blocking FL MAVS aggregation, I hypothesized that it may interfere with signal transduction by interacting with these downstream signaling proteins. To test this, I used a Flag-tagged miniMAVS expression vector and tested Flag-immunoprecipitates for the presence of endogenous TRAF2 and TRAF6. Both endogenous TRAF proteins interacted specifically with Flag-miniMAVS as compared to Flag-tagged RIG-I or a vector control (Figure 3.6A). Flag-tagged TRAF6 formed a modest complex with endogenous TRAF2. Additionally, when Flag-miniMAVS was co-expressed with HA-TRAF6 or HA-TIRAP, TRAF6 was detected in the Flag-immunoprecipitates, whereas the TLR adaptor TIRAP was largely absent (Figure 3.6B). Taken together, these data indicate that miniMAVS forms a complex with TRAF proteins that are known to promote antiviral signaling and IFN production. A possible mechanism of miniMAVS function may therefore be proposed whereby two protein complexes exist that contain MAVS. One complex consists of FL MAVS aggregates and TRAF proteins and is capable of activating type I IFN expression[57]. The second complex consists of miniMAVS and the same TRAFs (Figure 3.6) yet is incapable of activating type I IFN expression. The regulation of the functional competition between these two complexes remains an open area of inquiry.
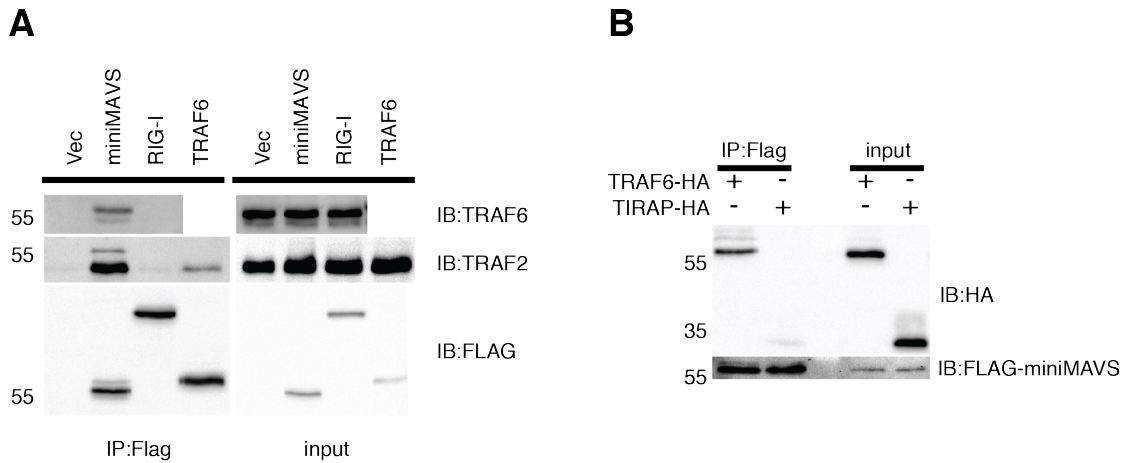
Figure 3.6 miniMAVS interacts with TRAF2 and TRAF6

(A) 293T cells were transfected with Flag-tagged miniMAVS, RIG-I, or TRAF6 and Flag-immunoprecipitates were probed for endogenous TRAF2 and TRAF6.

(B) HA-tagged TRAF6 or TIRAP expression vectors were co-transfected into 293T cells with a 3xFlag-miniMAVS expression vector. Immunoprecipitates were collected with a Flag specific affinity gel. The presence of TRAF6 within the precipitant was determined by an immunoblot with an HA antibody.

miniMAVS Positively Regulates Cell Death

In addition to activating antiviral gene expression, MAVS can promote cell death upon overexpression or in response to certain viral infections [75, 76]. As with the IFN response, the role of miniMAVS in cell death is unknown. To test whether each MAVS variant is sufficient to activate cell death, the variants were overexpressed in 293T cells an monitored for cell death. When both variants were overexpressed in conjunction (MAVS), there were visible signs of cell death compared to cells transfected with a vector control (Figure 3.7A). Interestingly, when miniMAVS (M1A) or FL MAVS (M142A) were expressed alone, cell death was also observed. Quantification of the number of cells that detached from the tissue culture plate revealed that FL MAVS and miniMAVS induce comparable amounts of cell death at 30 hours following transfection (Figure 3.7B, left panel). However, by 48 hours cell death induced by FL MAVS exceeded that of miniMAVS (Figure 3.7B, right panel). The increase in cell death induced by FL MAVS may be the result of secreted IFNs, which can positively influence cell death [152]. Interestingly, a miniMAVS deletion mutant lacking the C-terminal localization domain (M1A-500) did not show signs of cell death compared to the vector control (Figures 3.7). Based on these data, miniMAVS may function to positively regulate cell death in a localization-dependent, but IFN-independent manner.
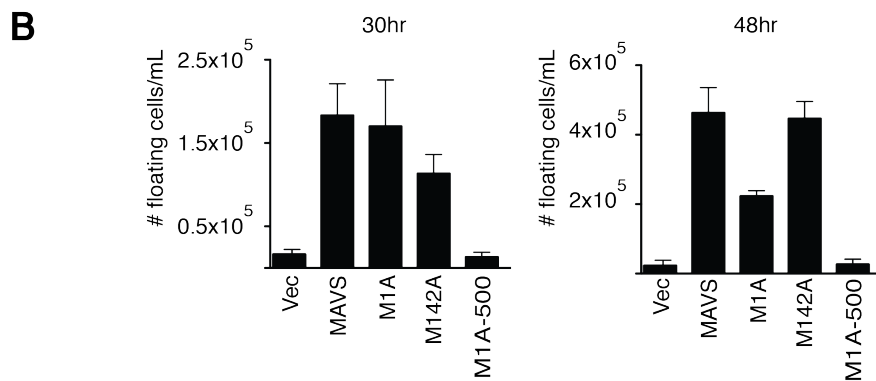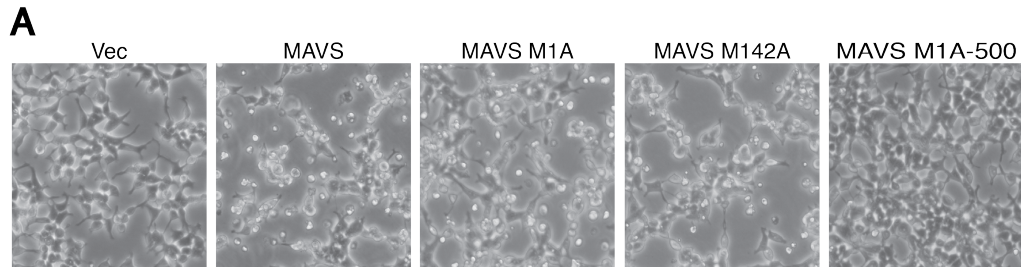
Figure 3.7 FL MAVS and miniMAVS induce signs of cell death

(A) Micrographs of 293T cells 48 hours after transfection with *MAVS* and start site point mutant expression vectors. The MAVS M1A-500 construct lacks the C-terminal transmembrane domain.

(B) The number of floating cells was quantified at 30 and 48 hours following transfection as in (A). Experiment was performed in triplicate and the error bars represent the standard deviation.

To further investigate the induction of cell death by FL MAVS and miniMAVS, two hallmarks of this process were also assessed. Programmed cell death, including apoptosis and necroptosis, is often characterized by the fragmentation of genomic DNA [153]. Both miniMAVS (M1A) and FL MAVS (M142A) induced the fragmentation of genomic DNA following expression in 293T cells (Figure 3.8). In support of our visual observations of cell death, the miniMAVS mutant (M1A-500) lacking the localization signal was not capable of inducing DNA fragmentation. As a control, DNA fragmentation induced by a known regulator of cell death, the TLR adaptor TRIF was also monitored (Figure 3.8) [154, 155].
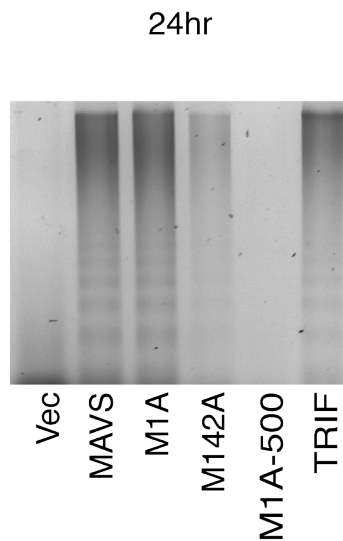
24hr

Figure 3.8 FL MAVS and miniMAVS induce DNA fragmentation

Genomic DNA fragmentation was detected 24 hour following transfection of MAVS and TRIF expression vectors. Genomic DNA was separated on a 2% agarose gel.

Prior to the commitment toward cell death and DNA fragmentation, caspases become activated and subsequently cleave a variety of target substrates to carry out apoptosis [156]. PARP is one of the targets of these activated caspases, making detection of the cleaved product of PARP a reliable marker for cell death. Therefore, to further investigate the induction of cell death by FL MAVS and miniMAVS, the induction of PARP cleavage individually was determined. At several time points following the expression of both variants in conjunction (MAVS), the cleaved product of PARP was observed (Figure 3.9). Again, TRIF was used as a positive control for cell death to monitor PARP cleavage. In agreement with our DNA fragmentation results, PARP cleavage was detected in cells individually expressing either miniMAVS (M1A) or FL MAVS (M142A) but not cells expressing the improperly localized miniMAVS mutant M1A-500. These data indicate that unlike their antagonizing activities toward IFN expression, FL MAVS or miniMAVS can both promote PARP cleavage and cell death. Although the MAVS localization domain directs this adaptor to mitochondria, peroxisomes, and MAMS [58, 59], the central role of mitochondria in programmed cell death led us to speculate that the death-inducing signal from MAVS probably emerges from this organelle [153, 156]. When another mitochondrial protein (NLRX1) [88] was examined in the PARP-cleavage assay, no PARP cleavage was observed (Figure 3.9). Therefore, the observed cell-death phenotype is specific to FL MAVS and miniMAVS and is not a general response to ectopic expression of another mitochondrial membrane protein. Thus, in

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addition to their antagonistic actions in regulating IFN expression, FL MAVS and miniMAVS can each promote the cell death response.
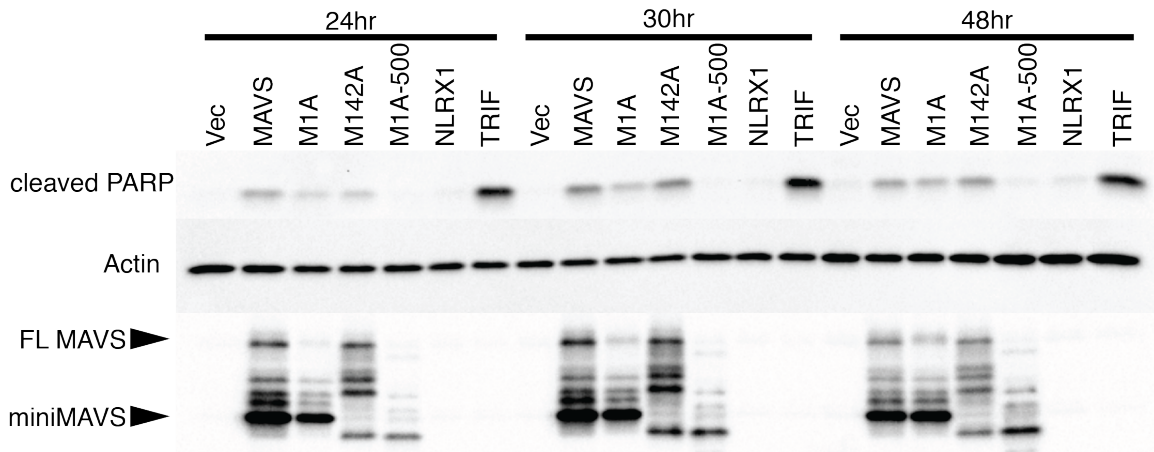

Figure 3.9 FL MAVS and miniMAVS induce PARP cleavage

Cell lysates were collected at 24, 30, and 48 hours post-transfection of *MAVS*, the translational start site point mutants, NLRX1, and TRIF. PARP cleavage and MAVS expression was determined by immunoblot following SDS-PAGE.

DISCUSSION

The regulation of innate immune signaling pathways is crucial for the defense against pathogenic infection as well as the maintenance of immune homeostasis [1, 87]. Hundreds of genes and regulatory mechanisms are involved in controlling the response to RNA viruses alone. Previously, the *MAVS* gene has been demonstrated to play a role in the defense against viral pathogens. However, as demonstrated in chapter two, this gene encodes a transcript that produces two alternatively translated variants, FL MAVS and miniMAVS. The role that these variants play in the antiviral response was unknown. This chapter demonstrates that the variants FL MAVS and miniMAVS have differential functions in the context of antiviral response mechanisms. Specifically, FL MAVS functions as a positive regulator of IFN production, which is consistent with many previously published functional studies characterizing the *MAVS* gene. In contrast, miniMAVS cannot activate the production of IFN but it can inhibit IFN production induced by its full-length counterpart. Interestingly, both variants are capable of inducing cell death.

Chapter one discusses regulators of MAVS that are believed to function through direct interaction. While the data in this chapter demonstrates that miniMAVS is a negative regulator of FL MAVS induced IFN production, it does not appear that miniMAVS interacts with polymerized FL MAVS as a mechanism of action. Several pieces of evidence allow us to make this conclusion. (1) miniMAVS does not contain the CARD domain, which is required for homotypic interactions and MAVS polymerization (Figure 2.2). Therefore miniMAVS is not

likely to interact with the polymerized forms of FL MAVS. (2) miniMAVS was not found to associate with the aggregates of FL MAVS polymers following viral activation (Figure 3.5). (3) Co-immunoprecipitation experiments failed to show an interaction between FL MAVS and miniMAVS (data not shown). The possibility remains that miniMAVS and FL MAVS interact with each other at steady state in the absence of infection, however in the context of active IFN signaling, it does not appear that miniMAVS interacts with the FL MAVS polymers.

Since miniMAVS can induce cell death, one could speculate that this process functions to limit IFN production. However, the IFN and cell death responses appear to be completely independent of one another, and the timing of each does not support such a hypothesis. Whereas IFN production can be detected very rapidly, the induction of cell death occurred over a longer period of time. Therefore in this case, the control of IFN production by cell death would be anachronistic and thus not a likely mechanism of miniMAVS-mediated IFN control.

Future studies will be needed to more definitively address the mechanism by which miniMAVS inhibits FL MAVS dependent IFN production. Regardless of the mechanism of action, miniMAVS provides a clear demonstration of immune regulation through alternative translation. Many other examples of immune regulation generated by alternative translation may also exist but have yet to be identified. The following chapter describes a ribosome-profiling screen that was established in an effort to make such identifications.

MATERIALS AND METHODS

MAVS Expression and Type I IFN Bioassay

The MAVS constructs were cloned into pcDNA3 expression constructs. Transient expression was achieved in 293T cells cultured in DMEM (10% serum) by Fugene 6 (Promega) mediated transfection. The type I IFN bioassay was performed as previously described [58]. Briefly, cell supernatants were collected following gene expression and incubated with an IFN-luciferase reporter cell line. Luciferase reporter activity and responsiveness was standardized to known concentrations of recombinant human IFN-beta.

Antibodies

The antibodies used for western blot analysis were as follows: MAVS (Bethyl Labs A300-782A), pSTAT (BD 612132), PARP (BD 611038), HA (Roche 3F10), Flag (Biolegend 637301) TRAF2 Cell Signaling (C192), and TRAF6 Abcam 33915.

Viral Infections

The Cantell Strain of SeV (Charles River Laboratories) was used to infect cells at a concentration of 50 U/ml. A VSV strain containing a firefly luciferase reporter was provided as a gift of Sean Whelan. Cells were infected with the VSV-firefly-luc at a multiplicity of infection (moi) = 1. Cells and virus were incubated in media lacking serum at low volume for 1 hour at the start of each infection.

Co-immunoprecipitations

miniMAVS was cloned into a 3xFlag CMV expression construct and transiently transfected into 293t cells. Flag-vector, Flag-RIG-I, and Flag-TRAF6 were transfected in parallel as controls. Flag immunoprecipitates were isolated with M2-affinity gel (Sigma) and eluted with a FLAG peptide.

Statistical Analysis

Prism (GraphPad) was used to create all figures and perform all statistical analysis. Error bars indicate the standard deviation.

Detection of FL MAVS Polymers by Sucrose Gradient Ultracentrifugation

Sucrose gradient ultracentrifugation was performed as previously described [56]. Briefly, $5x10^5$ 293T cells were plated in 10 cm dishes and transfected with MAVS expression vectors. Ten hours after transfection, cells were lifted and lysed by dounce homogenization. A P5 crude mitochondrial pellet was obtained and solubilized in 1% DDM [56]. Soluble mitochondria were then loaded onto a 30%–60% sucrose gradient and centrifuged for 2 hour at 170,000 g at four degrees Celsius. Fractions were then removed from the gradient with the bottom fraction containing MAVS polymers.

Detection of DNA Fragmentation

Fragmented genomic DNA was observed by agarose gel electrophoresis following phenol chlororform extraction [157]. 24 hours following transient MAVS

expression cells were collected, lysed, and the genomic DNA was isolated and analyzed as described.

Chapter 4

Genome-wide Ribosomal Profiling Predicts a Set of mRNAs that Encode

Regulators of Innate Immunity from Alternative Start Sites

The substance of this chapter was previously published.

Contributions: The data for this chapter was generated by ribosomal profiling experiments carried out by S. W. Brubaker and E. W. Mills. N.T. Ingolia contributed data analysis and figure design.

INTRODUCTION

Based on the work described in Chapter two, *MAVS* can now be added to a small list of eukaryotic genes known to produce bicistronic transcripts. FL MAVS and miniMAVS are two protein variants generated from a single transcript during the process of translation. Recently a new technique termed ribosome profiling has been developed to study the process of translation *in vivo* [135]. This technique utilizes deep sequencing of mRNA transcripts that are protected by the ribosome complex to determine which transcripts are actively being translated. As described in chapter two, in conjunction with the drug harringtonine, this technique can be used to monitor translation initiation and determine start codon usage. In one recent study, global translation initiation and start codon usage was determined in mouse embryonic stem cells [133]. This study indicated that there might be many more examples of eukaryotic transcripts in which translation is initiated from more than one start codon. Therefore alternative translation of mRNA transcripts may be a generalized mechanism for regulating gene function.

We considered the possibility that alternative translation commonly occurs on transcripts encoded by antiviral innate immunity genes. This could be a mechanism for regulating antiviral responses in the face of changes to translation that occur during viral infection [150, 158]. Both virus encoded and host encoded mechanisms can alter the translational landscape during viral infection as a means of ensuring viral protein synthesis or preventing it. Ribosome profiling is an unbiased approach to studying translation globally and an effective way to generate a candidate list of genes that may also encode transcripts that are

alternatively translated. Therefore, we set out to perform a ribosome profiling screen to determine if other antiviral innate immune genes encode transcripts that are alternatively translated.

As mentioned above, a previously published report conducted translational start site analysis by ribosomal profiling of mouse embryonic stem cell transcripts. However, miniMAVS alternative translation was characterized with the human encoded *MAVS* transcript. In addition, the translational start site and methionine at position 142 is not conserved between mouse and human transcripts (chapter 2). Therefore, while many translational start sites are conserved, there is likely to be some specificity between species. For these reasons, the translational start site analysis described in this chapter was performed with transcripts from a human cell type. The choice of cell type within a species was also considered due to differences in transcript expression between cell types. While many antiviral innate immune genes are ubiquitously expressed between cell types, monocytes of hematopoietic lineage, such as macrophages and dendritic cells, have a higher percentage of innate immune gene expression. Thus the monocytic cell line U937 derived from a histiocytic lymphoma was chosen for translational start site analysis by ribosomal profiling [159]. These cells differ from primary human monocytes in that they have transformed into an immortal state and express genes required for immortality. As these cells retain gene expression and phenotypic similarities to primary monocytes, they are a suitable substitute for the study of monocyte protein mRNA translation [159]. However, as described for the gene encoding osteopontin

[131], differences in start site usage can also exist between cell types. Therefore, the start codon usage identified in this study may differ from the translational start codons used in other cell types. This chapter describes the results of this ribosome profiling study done in collaboration with the laboratory of Nicholas Ingolia.

RESULTS

Global Start Site Analysis

In the presence of harringtonine, ribosome profiling identified 14,336 sites of active translation initiation present on 8,893 transcripts expressed in U937 cells (A list of these start sites can be found online in the supplemental data table published here [86]). Therefore, many of the expressed transcripts contain more than one functional translation start site (Figures 4.1A). The predicted protein products generated from these start sites can be classified into different groups relative to the reading frame of the annotated CDS. These groups include start sites for the generation of the canonical CDS, uORFs, internal out-of-frame products, truncations, and extensions (Figure 4.1B). As an example, the human *MAVS* transcript characterized in chapter two has a "canonical" start site responsible for translation of FL MAVS and a "truncation" start site responsible for miniMAVS translation.

One aspect of the host-mediated changes in translation that occur during viral infection can be attributed to the interferon-stimulated expression of protein kinase RNA-activated (PKR) [158]. This is one of four kinases that target the translation initiation factor eIF-2α, which prevents the initiation factor from being recycled and leads to a general inhibition of translation. To determine host-mediated changes in the rate of translation and start site usage, U937 cells were treated with recombinant human IFN beta and ribosome profiles were compared with those of untreated cells. Unfortunately, the results of this comparison

indicated little to no significant change in the rate of translation or in start site usage following IFN treatment (data not shown).
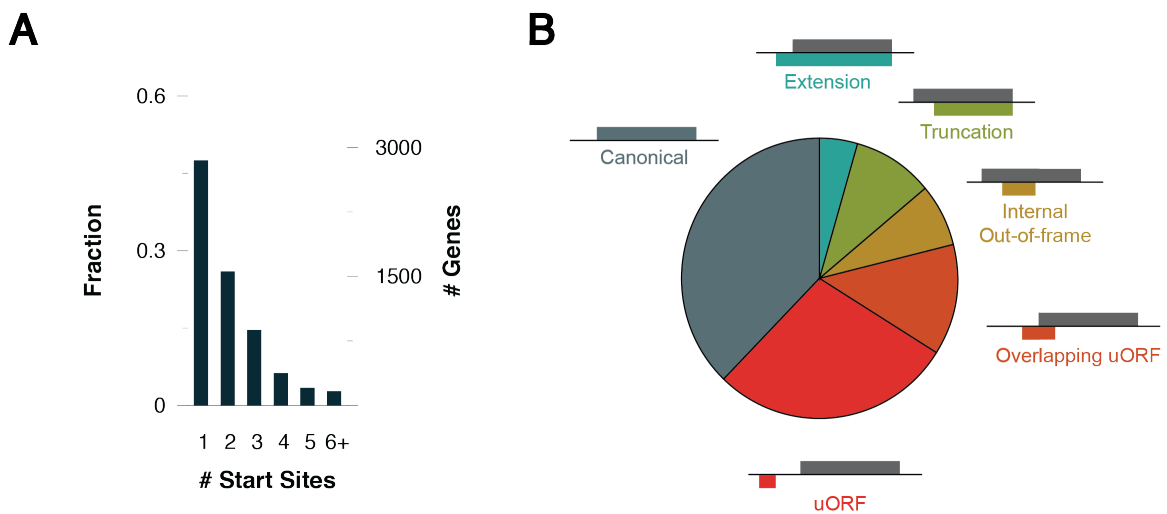
A

B

Figure 4.1 Translation start site analysis by ribosomal profiling

(A) The fraction and number of genes that were detected to have one or more translational start sites

(B) Classification chart of each predicted translation product as it relates to the annotated coding sequence of a gene.

The work on *MAVS* described in chapters 2 & 3 highlighted the importance of protein diversification via alternative translation. Thus transcripts containing start sites predicted to result in the production of variant protein isoforms such as truncations and extensions were of interest because they lose or gain some amino acid sequence relative to the canonical CDS. This can alter the function of the variant as in the case of miniMAVS, which lacks the CARD domain present in the amino terminus of FL MAVS. Our analysis indicated that 14% of the start sites identified in U937 cells are predicted to encode either a truncation or an extension, and a fraction of the genes are predicted to translate more than one isoform in U937 cells (Figure 4.2A). In addition, our profiling data indicate that truncations are more prevalent than would be expected from random chance. Based on the use of triplet codons, a third of possible start codons would be in-frame with the canonical ORF and two-thirds would be out-of-frame. If start-site selection were random, we would expect a 1:2 ratio of truncations to internal out-of-frame ORFs. However, we observed about a 4:3 ratio in the favor of truncations (Figure 4.2B). Additionally, truncations appear to be more frequent than extensions, suggesting that these variants may have more biological significance (Figure 4.2C).
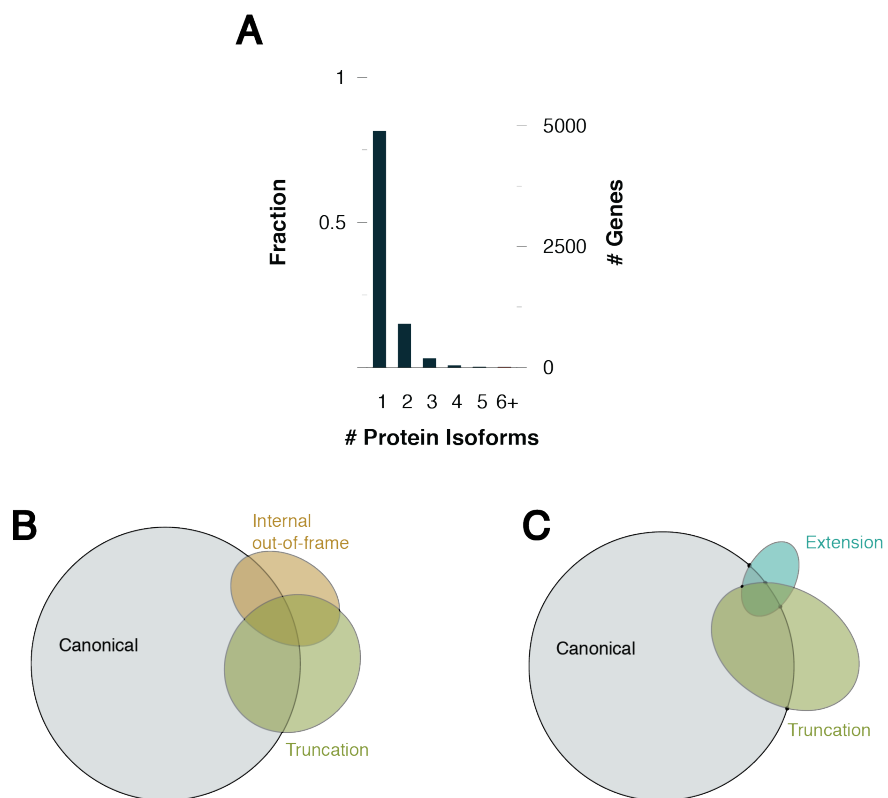
Figure 4.2 Translation start site analysis by ribosomal profiling

(A) The fraction and number of genes that were detected to have one or more protein isoform.

(B and C) Venn diagrams showing genes containing canonical, truncation, and (B) internal out-of-frame or (C) extension start sites.

Innate Immune Genes with Alternative Start Sites

Several genes involved in antiviral innate immunity were identified by our analysis to potentially produce truncated variants from alternative translational start sites. The patterns of ribosomal profiling indicate that, like *MAVS*, IFIH1 (also known as MDA-5), MX2, IFITM2, and TRIM25 ribosomes are stalled at start sites downstream of a respective canonical start site in the presence of harringtonine (Figures 4.3 A-D). In addition to stalled ribosomes, other factors make some of these transcripts good candidates for alternative translation such as the presence of overlapping uORFs or the lack of evidence for splice variants that would explain these start sites. As in the case for IFIH1 and MX2 (Figure 4.3 A-B), the identified overlapping uORFs may help to explain translation initiation from downstream start sites. However, the number of transcripts with potential alternative start sites comprise only a fraction of all the antiviral innate immunity transcripts monitored and most translate only a single canonical protein product, such as DDX58 (RIG-I) and TMEM173 (STING) (Figures 4.3E and 4.3F). In addition to truncation variants, extension variants as well as other non-immune related genes might also be of biological interest. Protein products that are generated from start sites that are out-of-frame with a canonical protein may also be of biological significance. These products however would not share any amino acid sequence with the canonical protein and therefore may be more difficult to characterize with distinct biological implications compared to the canonical protein. A searchable list of all the U937 start sites identified in this study is available online [86].

Figure 4.3 Ribosome profiles of select antiviral genes
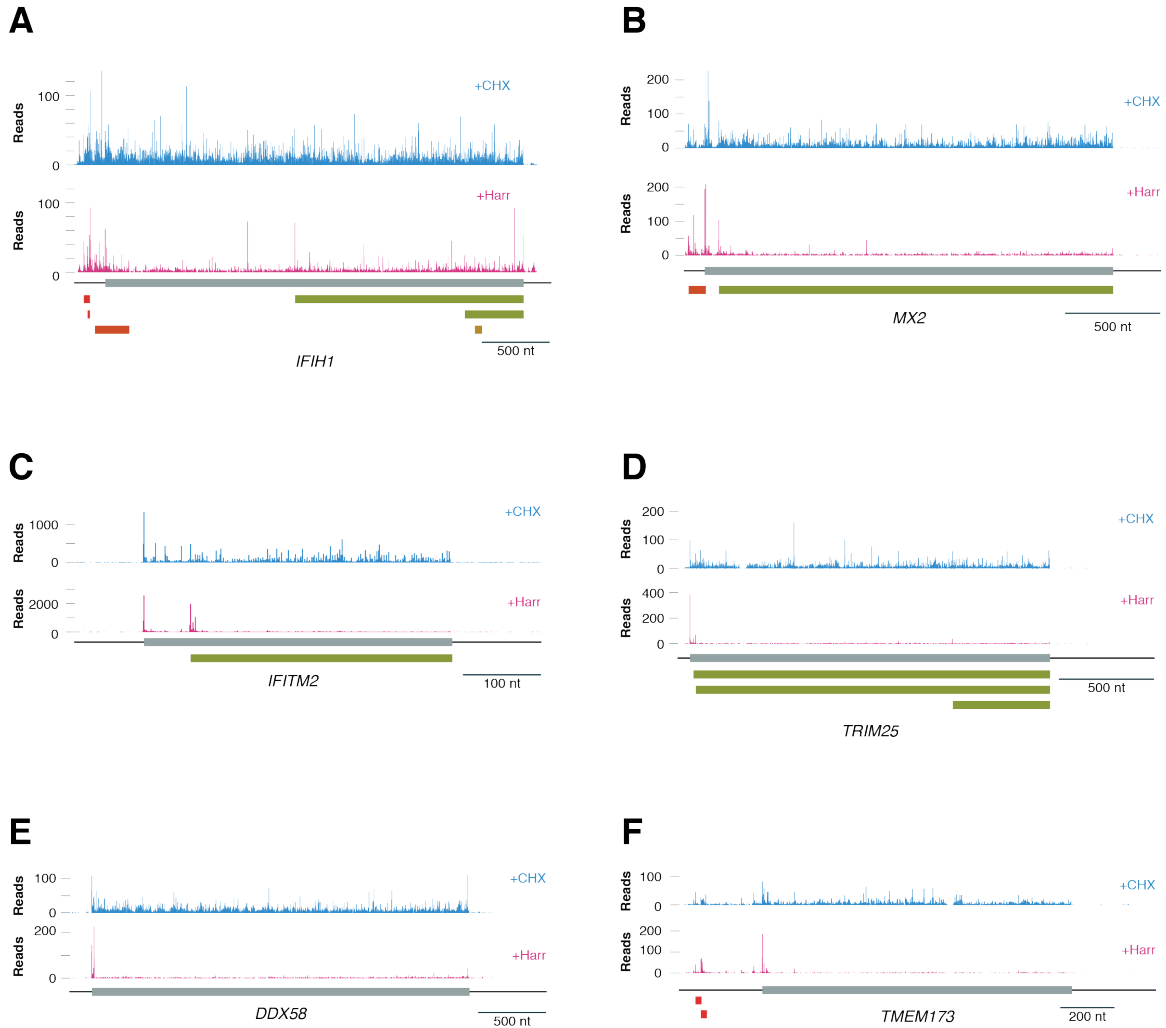
(A-F) The patterns of ribosome elongation (+CHX) and initiation (+Harr) are shown for several transcripts involved in antiviral immunity. The translation products predicted by harringtonine treatment are shown below each profile. Canonical translation products are in grey, truncations in green, uORFs in red, and internal out-of-frame translation products are in orange.

DISCUSSION

This chapter describes the global analysis of translation initiation from the U937 monocytic cell line. In addition, the effect of IFN signaling on translation was also tested in these cells. Unfortunately, the comparison with IFN treatment yielded no detectable changes in either the rate of translation or in start site usage. This result was somewhat of a surprise based on interferon-inducible PKR-mediated changes to translation during viral infection [158]. However, there are a few explanations as to why no change was observed in our analysis. First, our treatment time (8hr) was based off of IFN responsiveness detected at the transcript level. It is possible that the effect of IFN treatment on translation may be transient, and thus may have resolved prior to our analysis. Secondly, changes in translation associated with viral infection require PKR activation in addition to its IFN induced expression [158]. Furthermore, the kinase activity of PKR requires the presence of a double stranded RNA ligand. Thus in our experimental setup, the absence of a double stranded RNA ligand provides a potential explanation as to why we observed no change in translation.

While the data indicated no significant changes in translation from IFN treatment alone, a global list of steady state translational start sites from U937 monocyte transcripts was generated. This analysis suggests that FL MAVS and miniMAVS are not the only regulators of antiviral innate immunity encoded by a polycistronic transcript. From the start sites identified, a list of candidate antiviral innate immune genes was created (Figure 4.3) that may encode polycistronic transcripts. Additional work is needed to verify that these genes do in fact encode

transcripts that are polycistronic, because it remains possible that these start sites are found on uncharacterized splice variants. Furthermore, each start site should be mutated to determine an effect on protein expression. The abundance, stability, and function of the predicted protein variants will also need further verification to determine whether they are regulators of innate immunity like miniMAVS. Regardless, this analysis highlights the potential existence of a class of bicistronic regulators of antiviral innate immunity.

As discussed above, the usage of translational start sites on transcripts may differ between species, cell type, or even environmental condition. For example the gene encoding Osteopontin provides an example of cell type specific start codon usage [131] however, another example of differences in translation have been observed from the gene encoding ECSIT. The transcript encoding this adapter of toll pathways was identified as bicistronic in mouse embryonic stem cells [133, 160]. However, separate translational start site analyses of ribosome profiling from 293T cells and U937 cells did not identify ECSIT as bicistronic (data not shown). Between these experiments, there are differences in both the species and the cell type analyzed. Therefore, it remains possible that ECSIT functions bicistronically in embryonic stem cells but not in kidney cells or monocytes. Another possibility is that the transcript encoding ECSIT is only bicistronic in cells derived from mice. Regardless, this indicates that the control of translation and start site usage is highly dynamic between species and cell types. Changes in translation that occur during viral infection may also drastically alter start site usage from host-encoded transcripts. Therefore further studies that

reveal changes in translation that occur between cell types and during viral infection may reveal novel insights into the regulation of antiviral defense through translation.

MATERIALS AND METHODS

Cell lysis and ribosome recovery

U937 cells were cultured in DMEM (10% serum) and were incubated with or without 2ug/mL of harringtonine. Cyclohexamide was subsequently added at a concentration of 100ug/mL. Lysis buffer was added to the cells and the lysates were treated with RNase. Following RNA digestion, ribosomes were pelleted on a sucrose gradient by ultracentrifugation.

Linker ligation, reverse transcription, and sequencing

Detailed procedures have been published previously [135]. Briefly, purified ribosome footprints were ligated to linkers for reverse transcription. This DNA can then be circularized and ribosomal RNA specific sequences are depleted. Sequences are then amplified by PCR and barcodes can be added during this step to help with the analysis of sequencing results.

Chapter 5

Conclusion

DISSERTATION SUMMARY

The Bicistronic Nature of the *MAVS* Transcript

The results described in chapter two explain the mechanism responsible for generating the two MAVS variant proteins previously observed by western blot analysis. It was hypothesized that these variants, FL MAVS and miniMAVS, were the product of alternative translation initiation at two unique start sites present on the *MAVS* transcript. Two start codons at methionine 1 and methionine 142 of the annotated MAVS protein sequence were each predicted to generate proteins that corresponded to the relative weight of FL MAVS and miniMAVS. Mutations to these start sites ablated the expression of the respective proteins both in vitro and in vivo (Figure 2.2). These genetic alterations to the *MAVS* cDNA demonstrated that two proteins were being alternatively translated from this single transcript, and suggested that the same mechanism occurred on the endogenous *MAVS* transcript. Ribosomal profiling was then performed to test whether ribosomes initiate translation from these two start codons on the endogenous transcript. In the presence of the drug harringtonine, which stalls the initiation step of translation, ribosomes were enriched at the sites corresponding to the previously identified start sites at Met1 and Met142 (Figure 2.4). These results lead to the conclusion that FL MAVS and miniMAVS are expressed by alternative translation from a single bicistronic transcript.

A defining feature of mRNA translation is that this process has an inherent directionality [137]. Thus, for a ribosome to initiate protein synthesis from a downstream start site requires a mechanism allowing it to bypass upstream start

sites. Cis-regulatory features of the *MAVS* transcript were examined to better understand how the expression of FL MAVS and miniMAVS is controlled. Artificial start sites were introduced upstream of the miniMAVS start codon to test for leaky ribosomal scanning. These start sites blocked scanning ribosomes, which resulted in decreased expression of miniMAVS. This indicates that miniMAVS relies on leaky ribosomal scanning and that ribosomes are not directly recruited by an IRES-like element (Figure 2.5). Furthermore, changes in the translational context of upstream start sites altered the expression of miniMAVS indicating that the *MAVS* transcript evolved to permit some degree of leaky ribosomal scanning. In addition the presence of a naturally encoded overlapping uORF positively regulates miniMAVS expression (Figure 2.6). Based on previously described examples of overlapping uORF gene regulation [125, 143, 161], this suggested that some ribosomes pass the FL MAVS start site by initiating translation of a small uORF peptide. Following uORF-mediated start site skipping, ribosomes may resume scanning and reinitiate at the miniMAVS start site. Therefore, the endogenous *MAVS* transcript contains cis-regulatory elements, which mediate uORF start site skipping and leaky ribosomal scanning to ensure proper miniMAVS expression. Collectively, chapter two demonstrates that the *MAVS* transcript is bicistronic and that translation of the two variants, FL MAVS and miniMAVS, depend on cis-regulatory elements encoded within the transcript.

The Role of FL MAVS and miniMAVS in Antiviral Responses

Chapter three describes the functional role of FL MAVS and miniMAVS in regulating the antiviral IFN and cell death responses. Using point mutations that eliminated the translational start codon for FL MAVS or miniMAVS, it was demonstrated that IFN production is specifically induced by FL MAVS, whereas miniMAVS was incapable of inducing this antiviral response. However, when the two proteins were expressed in conjunction, the level of IFN production was abrogated as compared to FL MAVS expression alone (Figure 3.2). This indicated that miniMAVS functioned by inhibiting FL MAVS induced IFN production. In further support of this hypothesis, IFN production and signaling were completely abrogated when the expression levels of miniMAVS were increased relative to FL MAVS (Figure 3.2). Interestingly, when these variants were expressed either on their own or in conjunction, they were capable of inducing cell death at later time points (Figures 3.6-3.8).

In conclusion, FL MAVS and miniMAVS can antagonize one another, and the strength of antiviral gene expression induced by *MAVS* is the result of the collective actions of these two MAVS variants. A competition may exist within cells at the level of the MAVS proteins, and the relative abundance of each variant may determine the signaling potential of the RLR pathway. Thus, the relative level of miniMAVS protein expression may provide the host with the ability to fine tune antiviral IFN responses. While changes in the ratio of these two variants may control antiviral gene expression, cell death can be induced irrespective of their relative abundance. The ability for both variants to induce cell

death may therefore provide a mechanism to control viral infection in environments where IFN production may be deleterious.

A Search for Alternatively Translated Innate Immune Regulators

The identification of miniMAVS highlights the concept of immune regulation generated by alternative translation, and based on this example other antiviral innate immune genes may also be regulated in a similar fashion. Chapter four describes a global screen to characterize the translational start sites utilized in U937 cells by ribosome profiling. Through this analysis, 14,336 translation initiation sites were identified corresponding to 8,893 transcripts (Figure 4.1). This indicates that some transcripts must contain more than one start site and may initiate synthesis of multiple proteins similarly to the *MAVS* transcript. The bioinformatic analysis of these start sites indicate that the majority of the alternative start sites are uORFs. However, roughly 10% are predicted to generate extension or truncation variations of a canonical protein. Due to the loss or gain of amino acid sequence, it is likely that these variants have differential function compared to the canonical protein function. However, based on amino acid similarities with the canonical protein, these variants most likely differentially regulate the same biological processes the canonical proteins regulate. From the predicted pool of extension and truncation variants a candidate list of antiviral innate immune genes was generated for further characterization (Figure 4.3). Whether these variants are synthesized, stable, and functional remains to be determined.

FUTURE DIRECTIONS

miniMAVS Inhibition of FL MAVS Induced IFN Signaling

The results described in this dissertation indicate that miniMAVS functions to inhibit FL MAVS induced IFN production. Preliminarily, this seems to be independent or downstream of FL MAVS polymerization. The fact that miniMAVS does not participate in polymerization is consistent with other studies demonstrating a CARD requirement for polymerization. If the function of miniMAVS is independent of FL MAVS polymerization, the mechanism by which this inhibition takes place remains to be characterized. It is possible that miniMAVS functions to inhibit signaling by forming a complex with downstream signaling proteins as the interactions with TRAF2 and TRAF6 suggest. In this case, downstream signaling proteins may be part of an inactive miniMAVS complex and are recruited to an active FL MAVS complex for IFN signaling and production. Whether these complexes exist between endogenous proteins at steady state remains to be determined.

If miniMAVS is important for the control of FL MAVS signaling, then one would expect that the loss of miniMAVS would result in uncontrolled IFN signaling. However, testing this hypothesis will prove to be technically challenging for a few reasons. (1) Current siRNA mediated knockdown methods eliminate expression from an entire transcript rather than selectively from a single start site. Therefore, a genetically engineered FL MAVS only construct would need to be re-introduced to test for a loss of miniMAVS phenotype. However, the interpretation of results from an experiment of this type may be complicated,

because the transient expression of FL MAVS is known to activate the signaling pathway. (2) The methionine at position 142 is not conserved in mice. Therefore, using a mouse model to study miniMAVS function may not be an appropriate system. For example, the mechanisms in place that regulate the translation of FL MAVS and miniMAVS in humans may not be the same in mice. Alternatively, the mechanism by which miniMAVS inhibits FL MAVS induced IFN production may require other host factors not present in mice. Therefore, understanding the role of miniMAVS in antiviral defense at the level of the organism may not be possible.

Due to these experimental limitations, the best approach to characterize the mechanism of miniMAVS inhibition will be through biochemical studies of the endogenously expressed human proteins. Purification and biochemical analysis of FL MAVS or miniMAVS can be performed over the course of antiviral signaling and/or infection. Previously identified post-translational modifications to MAVS activate or repress IFN production [97-101, 104]. Characterizing whether these modifications are specific to FL MAVS or miniMAVS and when they occur during the course of an infection will inform how these proteins coordinate IFN signaling. Furthermore, several interacting proteins positively or negatively regulate the IFN potential of MAVS [88-91, 95, 96]. Here again, determining which interactions are specific to each variant and when these interactions occur over the course of an infection will provide insight into how these proteins are regulating the IFN and cell death responses.

Immune Regulation Through Changes in mRNA Translation

Changes in translation have historically been associated with viral infection. These changes are the combined result of viral manipulations to subvert host translation as well as host-encoded responses (e.g. PKR) aimed to control viral protein synthesis [10, 150, 158]. Regardless of intent, the changes in the global landscape of translation during viral infection may provide a mechanism to regulate antiviral immune signaling. Therefore, it will be of interest to study the effects that this regulation has on antiviral signaling and defense mechanisms. While the number of publications reporting on genome-wide transcript analysis has exploded in the past decade, there have been relatively few genome-wide studies of translation [162]. However, with the advent of new technologies to study genome-wide translation its effect on immune regulation may soon be more clearly elucidated.

In the context of *MAVS*, one might expect to observe naturally occurring changes in the ratio of FL MAVS to miniMAVS. For example certain cell types or conditions may require different IFN responsiveness. Changes in the steady state ratio of FL MAVS to miniMAVS may be one mechanism that regulates differences in IFN production. This could be achieved through different rates of translation initiation or through differences in protein stability (discussed below). The rates of FL MAVS and miniMAVS translation initiation during viral infection or under other forms of stress could be determined using ribosome profiling or pulse chase experiments. Once a condition is identified in which translation initiation rates deviate from steady state expression, genetic or biochemical techniques

could be used to identify the regulatory factors responsible. However, *MAVS* is just one example of immune regulation created through the process of translation. One benefit of the tools available to study genome-wide translation is the ability to identify more examples where the process of translation functions to regulate immune responses in an unbiased fashion.

MAVS Regulation Through Changes in Protein Stability

As I alluded to in the previous section, the levels of FL MAVS and miniMAVS could be regulated by changes to protein stability rather than changes in translation. Changes in FL MAVS protein stability may make a cell incapable of inducing IFN production in response to RNA virus infection. The converse of this may also be true, but because miniMAVS shares its amino acid sequence with FL MAVS this would require a mechanism to specifically target miniMAVS. As discussed in chapter one, MAVS can be modified with ubiquitin for targeted proteasomal degradation [97-101]. In some of these studies, the ubiquitin-modified residue has been mapped and thus provides insight into whether FL MAVS or miniMAVS is targeted. However, in some cases the targeted residue is common between the two variants. One way to determine the stability of each variant would be to determine which variants receive these modifications following activation of the pathway or viral infection.

Resolution of Innate Immune Polymers

The use of protein polymers for the activation of signal transduction is an emerging theme in the study of innate immunity. In addition to MAVS, both RIG-I and MDA-5 form polymers or fibrils following ligand binding [47-49]. Thus, the RLR pathway seems to be regulated at several points by the generation of these protein polymers. Additionally, this form of regulation through polymer formation has also been recognized in the activation of the inflammasome. Recently two reports have demonstrated that similarly to MAVS, the adaptor protein ASC forms prion-like polymers upon activation that are self-propagating [163-165]. Many of the proteins that function in these innate immune signaling pathways contain domains that facilitate homotypic interactions (e.g. CARD, death domain (DD), Pyrin domain), which may explain why polymer formation is common among them [166]. The ability of MAVS and ASC to nucleate and propagate a self-polymerization process make these proteins prion-like, and are the first reported examples of prion-like polymerization having a beneficial effect in mammalian cells [56, 163]. This brings up the interesting question of how these polymers are resolved or degraded following activation. The build-up of prion aggregates is associated with several neurological diseases indicating that these aggregates are not easily resolved and can be difficult to remove [167]. Is it possible that unresolved aggregation of MAVS or ASC manifests in disease? Presumably, there are mechanisms in place to reset the cell back to a pre-activation state with the proteins in their original conformation. Understanding the regulatory

mechanisms that control this will be of significant interest in understanding the maintenance of immune homeostasis.

MAVS-Mediated Cell Death

As mentioned in chapter one, *MAVS* mediated cell death has not been as clearly characterized as the *MAVS* dependent IFN response. The factors both upstream and downstream of FL MAVS and miniMAVS need to be identified and characterized in the context of cell death. While one may expect the upstream factors to be common among IFN signaling and cell death, several reports indicate that *MAVS*-mediated virus-induced cell death occurs independently of the RLRs and IFN [81, 85]. Therefore, it remains to be determined how FL MAVS and miniMAVS sense viral infection to induce cell death. Perhaps upstream receptors distinct from the RLRs are uniquely responsible for this process.

The mechanisms downstream of FL MAVS and miniMAVS that initiate cell death also require further elucidation, however it is worth noting that this process likely occurs independent of the formation of MAVS polymers. This is based on the work described here and in reports demonstrating the polymerization of MAVS. miniMAVS does not contain the required CARD domain for self-polymerization and it does not induce FL MAVS polymerization, yet it can still induce cell death [56, 86] (and unpublished observations SW Brubaker). Several other factors participate in caspase activation and *MAVS*-mediated cell death including VDAC1, TRADD, FADD, RIP1, and caspase 8 [53, 81, 83, 84]. It remains to be determined whether FL MAVS and miniMAVS utilize these same mechanisms

to induce cell death, or if there are differences in the way each variant induces cell death. In addition the localization requirements for cell death will also need to be verified. In this report and other reports on *MAVS* mediated cell death, it has been demonstrated that a proper localization domain is required for this process [76, 81, 86]. Because this domain directs the adaptor to mitochondria, peroxisomes, as well as MAMS it remains to be determined which subcellular location is responsible for *MAVS* mediated cell death. Due to the number of cell death regulatory factors associated with mitochondria it is plausible that localization on this organelle may specifically be required for this phenotype.

CLOSING REMARKS

In the 25 years since Charles Janeway Jr. announced his hypothetical model of pattern recognition, there has been an explosion of research characterizing the complex network of innate immune response pathways. These innate immune responses, which are critical for containing infection and initiating adaptive immunity, are often characterized by dramatic changes in transcription. Regulating innate immune responses is not only critical for host defense, but also for maintaining immune homeostasis. The work described here demonstrates just one example of immune regulation that is controlled by the process of translation. However genome-wide ribosome profiling indicates that translation from alternative start sites, as in the case of miniMAVS, may be more prevalent than previously appreciated. Therefore, this dissertation not only provides insight into the regulation of antiviral signaling, but also provides a mandate to consider translation as a layer of regulation within other genes as well.

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