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Restriction of picornavirus infection by a host cell mRNA decay protein

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Wendy Elizabeth Ullmer

Dissertation Committee:
Professor Bert L. Semler, Chair
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Professor Klemens J. Hertel
Professor Emiliana Borrelli
Professor Peter Kaiser

2018

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DEDICATION

To

my Mother

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ABSTRACT OF THE DISSERTATION

Restriction of picornavirus infection by a host cell mRNA decay protein

By

Wendy Elizabeth Ullmer

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2018

Professor Bert L. Semler, Chair

The cellular mRNA decay protein AUF1 acts as a restriction factor during infection by picornaviruses including poliovirus, coxsackievirus, and human rhinovirus. AUF1 relocalizes from the nucleus to the cytoplasm during infection by these viruses due to the disruption of nucleocytoplasmic trafficking by viral proteinases. Previous studies have demonstrated that AUF1 binds to poliovirus and coxsackievirus B3 (CVB3) RNA during infection, with binding shown to occur within the internal ribosome entry site (IRES) of the 5' non-coding region (NCR) or the 3' NCR, respectively. Binding to different sites within the viral RNA suggests that AUF1 may negatively regulate infection by these viruses using different mechanisms. The work presented in this dissertation addresses the mechanism of AUF1 inhibition of the replication of poliovirus and CVB3. It is demonstrated that AUF1 knockdown in human cells results in increased viral translation, RNA synthesis, and virus production and that negative regulation of some picornaviruses may occur in a cell type- or species-specific manner. AUF1 is shown to negatively regulate translation of poliovirus and CVB3 IRES reporter RNAs during infection, but not in uninfected cells. This inhibitory

activity is not mediated through destabilization of viral genomic RNA; however, it does require virus-induced relocalization of AUF1 from the nucleus to the cytoplasm during the early phases of infection. All four isoforms of AUF1 are shown to inhibit poliovirus replication when expressed individually; however, endogenous AUF1 may associate with the poliovirus 5' NCR in an isoform-specific manner during infection. These findings suggest that AUF1 restriction of poliovirus and CVB3 replication uses a common mechanism through the viral IRES, which is distinct from the canonical role that AUF1 plays in regulated mRNA decay in uninfected host cells.

CHAPTER 1

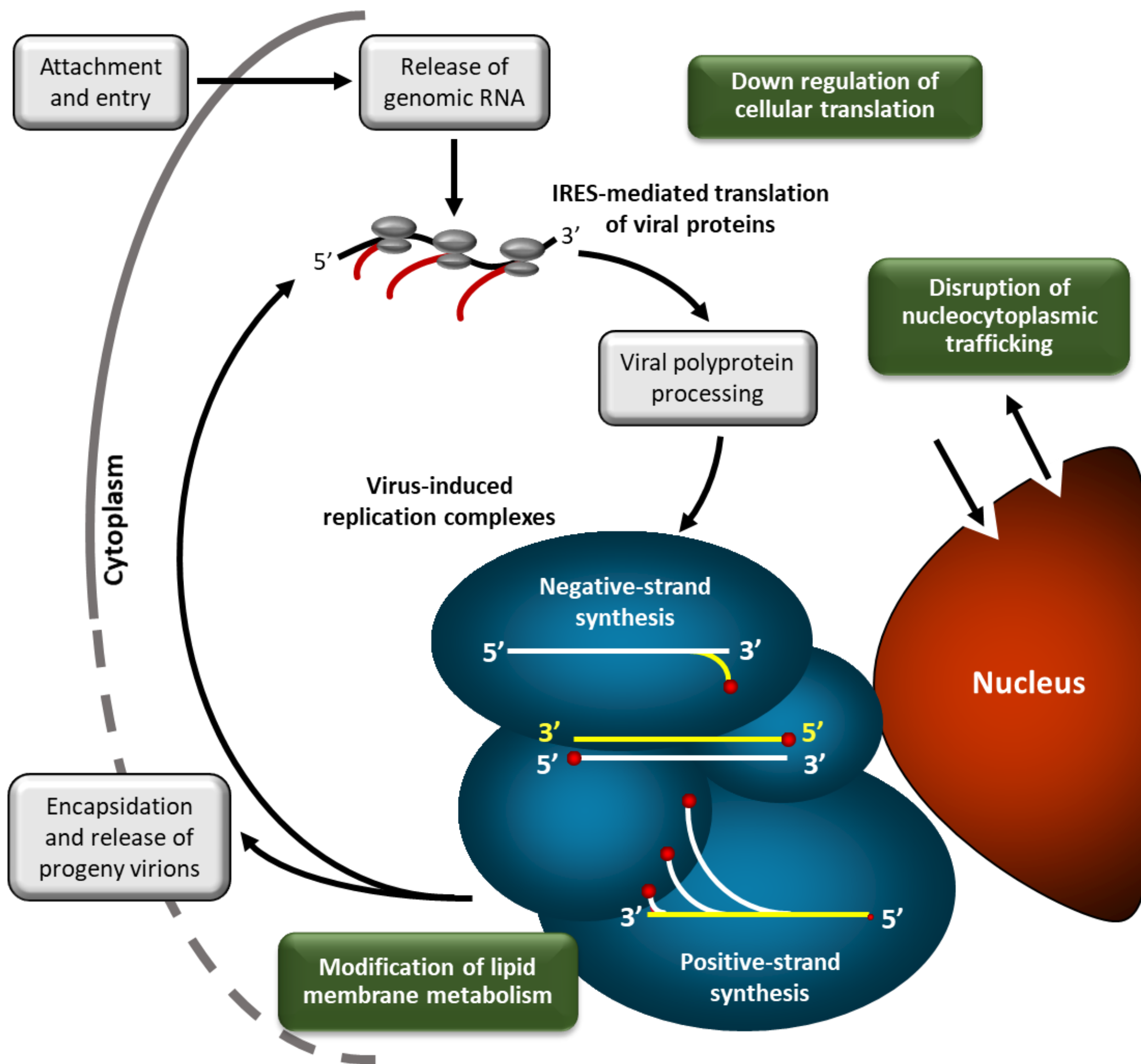
Introduction

I. Introduction to picornaviruses

As a family, *Picornaviridae* is composed of at least 29 different genera which include many significant human and animal pathogens causing a range of illnesses and economic burden. The Enterovirus genus of *Picornaviridae* includes the causative agents of paralytic poliomyelitis (poliovirus), hand, foot, and mouth disease (coxsackievirus A16 [CVA16] and enterovirus 71 [EV71]), and the common cold (human rhinovirus [HRV]). Other severe symptoms of enterovirus infection include meningitis, encephalitis, myocarditis, and pericarditis, which can arise from infection by subtypes of coxsackievirus A (CVA), coxsackievirus B (CVB), enterovirus (EV), or echovirus. The lone member of the Hepatovirus genus, hepatitis A virus (HAV), infects the liver and in rare cases, causes acute liver failure. The Cardiovirus genus includes encephalomyocarditis virus (EMCV), Theiler's murine encephalomyelitis virus (TMEV), and Saffold virus (SAFV). EMCV and TMEV are largely non-human pathogens that cause symptoms that include myocarditis, encephalitis, and for EMCV specifically, reproductive failure in pigs. SAFV is a recently discovered human cardiovirus that does not yet have clearly defined pathological features but has been linked to acute flaccid paralysis, meningitis, and cerebellitis. Foot-and-mouth disease virus (FMDV), a member of the Aphthovirus genus, is one of the most economically important livestock viruses. Infection by FMDV causes painful vesicles in the feet, mouth, and teats of cloven-hoofed animals, reducing their productivity and requiring significant eradication measures.

Picornaviruses induce extensive modification of cellular processes to complete their replication cycle (Figure 1.1). Upon attachment and release of a single-stranded, positive sense RNA genome into the cytoplasm of a host cell, viral proteins are translated by a cap-independent mechanism using an internal ribosome entry site (IRES) located in the 5' non-coding region (NCR) of the viral RNA. Picornavirus genomic RNAs are linked to a small, virus-derived protein at their 5' termini (VPg), possess a 3' poly(A) tract, and encode a single open reading frame that is translated into one polyprotein. Viral proteinases process the viral polyprotein into precursor and mature proteins, resulting in the formation of 4 structural proteins and 7-8 mature non-structural proteins, depending on the virus. Viral proteins induce membrane rearrangements to form replication complexes, which are sites for RNA synthesis by the viral RNA-dependent RNA polymerase, 3D. Newly synthesized RNAs undergo further rounds of translation/replication or become packaged into progeny virions. To redirect host resources toward virus replication, many picornaviruses rapidly shut down cap-dependent translation and disrupt nucleocytoplasmic trafficking to relocalize nuclear proteins required for replication into the cytoplasm (1). Modification of the cellular landscape is largely accomplished through the actions of non-structural proteins, particularly the viral proteinases 2A, 3C, and L (L is encoded by FMDV) (2, 3).

Figure 1.1. Picornavirus replication cycle. Following attachment to a cell surface receptor, picornaviruses release their genomic RNA into the cytoplasm of the infected cell. The single-stranded, positive sense RNA ([+] RNA) genome acts as an mRNA and is translated by a cap independent mechanism using an internal ribosome entry site located within the 5' non-coding region. The polyprotein produced from a single open reading frame is proteolytically processed by viral proteinases into precursor and mature proteins. Once processed, viral proteins induce the formation of membranous replication complexes where viral RNA is synthesized by the virus-encoded RNA-dependent RNA polymerase. Newly synthesized (+) RNAs either undergo further rounds of translation and replication or are packaged into progeny virions and released from the cell. To successfully complete their life cycle, picornaviruses modify many cellular processes to favor virus replication. Examples of these modifications include down regulation of cellular translation to redirect resources toward viral translation, modification of lipid membrane architecture and metabolism to form replication complexes, and disruption of nucleocytoplasmic trafficking to bring nuclear proteins required for replication into the cytoplasm.


Attachment and entry
Release of genomic RNA
Down regulation of cellular translation
IRES-mediated translation of viral proteins
5′
3′
Disruption of nucleocytoplasmic trafficking
Viral polyprotein processing
Virus-induced replication complexes
Cytoplasm
Negative-strand synthesis
5′
3′
3′
5′
5′
3′
Nucleus
Encapsidation and release of progeny virions
3′
5′
Positive-strand synthesis
Modification of lipid membrane metabolism

The disruption of nucleocytoplasmic trafficking during infection is a necessary process to bring host proteins required for virus replication into the cytoplasm. As an unintended consequence of this disruption, nuclear proteins having a negative impact on virus replication may also relocate into the cytoplasm. AUF1, the focus of this dissertation, is an example of a predominantly nuclear host protein that is mislocalized to the cytoplasm during infection and inhibits replication of several picornaviruses. The identification of AUF1, most often described as an mRNA decay protein, as a picornavirus restriction factor provided evidence that RNA decay pathways may be involved in limiting picornavirus infections. This chapter will review picornavirus interactions with RNA decay pathways and describe host restriction factors identified for picornaviruses.

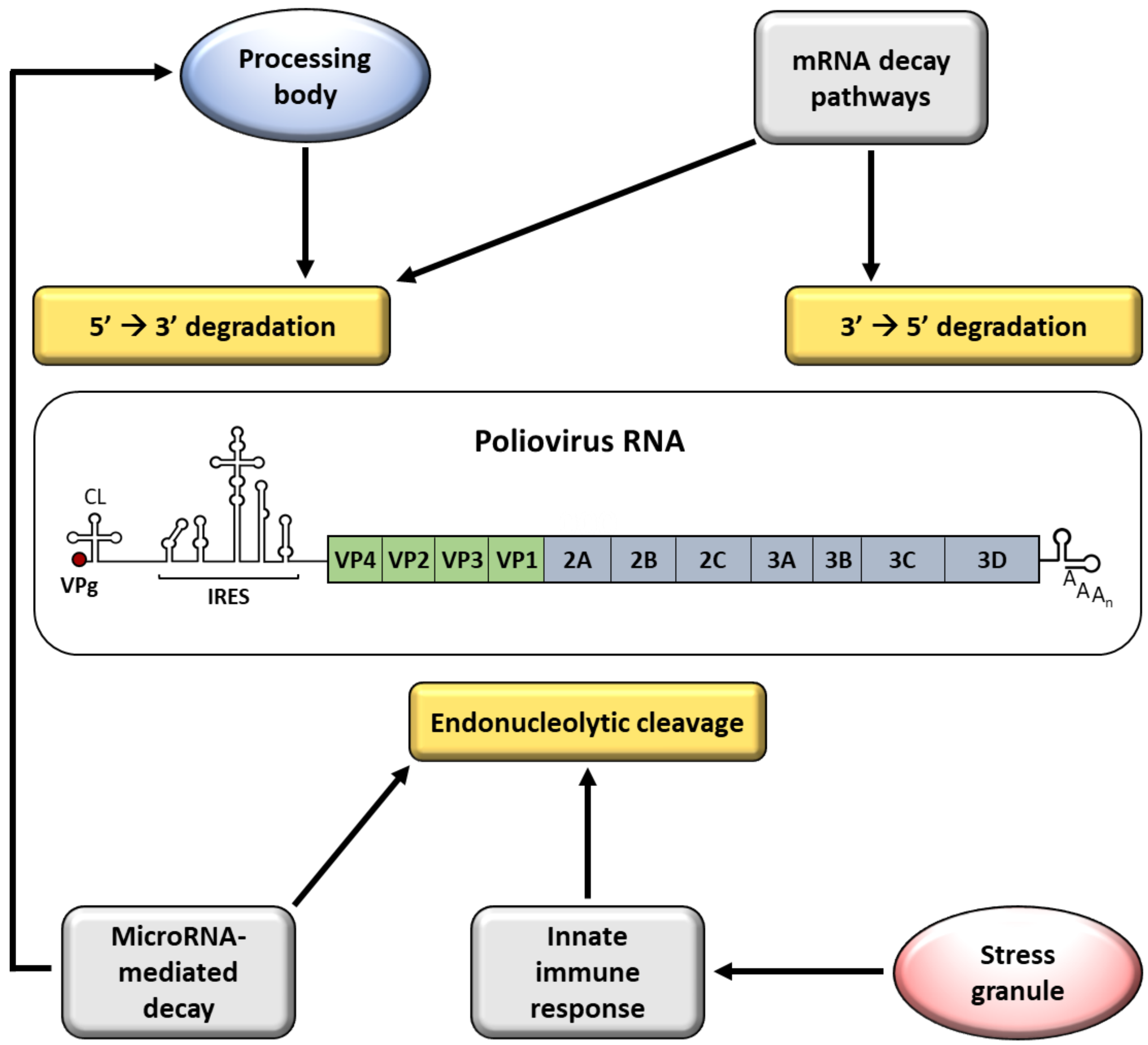
II. Diverse strategies used by picornaviruses to escape host RNA decay pathways

Introduction

Cytoplasmic RNA viruses encounter a myriad of host defense mechanisms that must be countered by a small arsenal of viral proteins. Preserving the stability and integrity of viral RNA is of fundamental importance to the virus to ensure successful generation of progeny virions. Throughout the replication cycle, viral RNAs encounter multiple potentially destabilizing host cell pathways or processes, from degradation as a result of activation of the innate immune response to regulated mRNA decay (Figure 1.2). Given their cytoplasmic replication cycle, picornaviruses avoid nuclear RNA surveillance mechanisms but instead are susceptible to mRNA decay pathways that function in the cytoplasm, such as AU-rich element-mediated decay (AMD). To ensure that viral RNAs are not targeted by RNA decay machinery, picornaviruses disrupt these processes at multiple levels. The strategies that picornaviruses employ to disrupt the innate immune response

have been researched more extensively than picornavirus involvement in mRNA decay pathways. This section focuses on picornavirus interactions with pathways or processes associated with RNA decay, largely highlighting enteroviruses, for which the most experimental evidence exists.

Figure 1.2. Picornavirus encounters with host RNA decay pathways. Picornavirus RNAs are exposed to degradation machinery from multiple RNA decay pathways and processes including 5'→3' degradation in processing bodies, 5'→3' or 3'→5' degradation by mRNA decay pathways, and endonucleolytic cleavage as a result of the activation of the innate immune response or microRNA-mediated decay. Poliovirus RNA serves as a model for the picornavirus genome. Genomic RNA is linked to a small virus-encoded protein, VPg, at the 5' terminus. The 5' non-coding region (NCR) contains a cloverleaf structure (CL) and an internal ribosome entry site (IRES). A single open reading frame codes both structural (VP1-4) and non-structural (2A-3D) proteins that are proteolytically processed into precursor and mature viral proteins. The 3' end of the genome encodes a 3' NCR and poly(A) tract.


Processing body
mRNA decay pathways
5' → 3' degradation
3' → 5' degradation
Poliovirus RNA
CL
VPg
IRES
VP4
VP2
VP3
VP1
2A
2B
2C
3A
3B
3C
3D
AAAn
Endonucleolytic cleavage
MicroRNA-mediated decay
Innate immune response
Stress granule

Genome stabilizing features for picornaviruses

Picornavirus RNAs have acquired several stabilizing features as a form of protection from cellular RNA decay machinery. At the 5' end of the genome, the first barrier to degradation is a small, virus-encoded protein covalently bound to the 5' terminal nucleotide of the viral RNA, called VPg (Viral Protein, genome-linked) (4, 5). VPg is used by the viral RNA polymerase, 3D, as a protein primer for RNA synthesis, resulting in viral RNAs linked to VPg instead of a 7-methylguanosine (m7G) mRNA cap. Lacking a m7G cap, picornavirus RNAs are protected from cellular decapping enzymes like Dcp1 and Dcp2, whose activity initiates 5'→3' RNA degradation. While cellular decapping enzymes are unable to hydrolyze the VPg-RNA bond, a cellular 5'-tyrosyl-DNA phosphodiesterase, TDP2, is capable of cleaving this bond and "unlinking" VPg from viral RNA (6). Cleavage of VPg may not result in destabilization of viral RNAs since unlinked viral RNA has been found to be associated with actively translating ribosomes and therefore protected from degradation, although the stability of unlinked viral RNA has not yet been measured (7-9). As an additional protection, the major 5'→3' exonuclease, Xrn1, is degraded during poliovirus infection, preventing 5'→3' digestion of unlinked viral RNAs (10) (discussed further in Processing bodies section).

For some picornaviruses, viral RNA can inhibit endonucleolytic cleavage directly through association with the endoribonuclease, RNase L. RNase L activity is stimulated by the interferon response and contributes to the cellular defense against infection by degrading viral RNA (11) (discussed further in the Interferon-mediated viral RNA degradation section). The antiviral activity of RNase L has been demonstrated for picornaviruses including EMCV and CVB4 (12, 13). However, poliovirus RNA is resistant to

cleavage by RNase L through a structured RNA element located in the 3C proteinase coding sequence. This structured RNA binds the endoribonuclease domain of RNase L, which inhibits its activity (14). This RNA element is conserved in group C enteroviruses, which include poliovirus and several types of CVA, among others (15, 16). The element is not present in group A, B, or D enteroviruses, of which CVB3 is a member and was found to be sensitive to RNase L. The protection of this RNA element from RNase L activity is not complete, however, as it was discovered that RNase L is still capable of cleaving the poliovirus genome at distinct locations (17).

Picornavirus RNA is stabilized through association with specific host proteins. Poly(rC) binding protein 2 (PCBP2), an RNA binding protein involved in mRNA stability and translation, binds 5' stem-loop structures in poliovirus RNA to promote genomic RNA stability, viral translation, and RNA replication (18-20). Mutation of one of these stem-loop structures to prevent PCBP2 binding results in diminished ability to form polysomes on poliovirus RNA, rendering the RNA susceptible to degradation (21, 22). PCBP2 also binds the 5' NCR of CVB3, EV71 and HRV RNA to promote viral translation and RNA replication, but it has not yet been determined if this interaction affects viral RNA stability (23-26). Human antigen R (HuR) is a well-characterized mRNA stabilizing protein that was recently found to bind the EV71 5' NCR and act as a positive regulator of translation (27). While the effect of HuR on viral RNA stability has yet to be determined, HuR may have a similar indirect stabilizing effect as PCBP2, whereby promoting translation of viral RNA protects it from degradation. HuR was previously shown to stabilize genomic RNAs of togaviruses, another family of positive sense RNA viruses (28). HuR was also identified as a poliovirus RNA binding protein using thiouracil cross-linking mass spectrometry (TUX-

MS), suggesting that it may have a similar effect on other viral RNAs (29). Host factors that promote picornavirus RNA stability have not been well studied, but it is likely that many of the proteins re-purposed for translation and replication serve a dual purpose in promoting viral RNA stability.

Interferon-induced viral RNA degradation

The earliest defense against virus infection of cells involves activation of the innate immune response, which results in the expression of genes that interfere with virus replication, prevent spread to neighboring cells, and trigger the adaptive immune response. Briefly described, detection of pathogen-associated molecular patterns (PAMPs) by cellular pattern recognition receptors (PRRs) initiates the innate immune response. During picornavirus infections, double-stranded RNAs (dsRNAs) that form during viral RNA replication serve as the PAMP that is recognized by a PRR. PRR bound to viral dsRNA transduces the signal that a viral pathogen has been detected through multiple pathways, leading to the activation of transcription factors which promote the expression of interferon-β (IFN-β). IFN-β production ultimately results in transcription of hundreds of interferon-stimulated genes (ISGs) which collectively contribute to an antiviral state (30, 31).

The IFN response activates multiple pathways to inhibit virus replication, including degradation of viral RNA by RNase L. RNase L is normally expressed in mammalian cells, but remains inactive until infection is detected, resulting in ISG expression. Oligoadenylate synthetase (OAS) is an ISG that activates RNase L by generating 2'-5' oligoadenylates (2-5A), the secondary messenger that induces dimerization and activation of RNase L (11, 32). Some picornaviruses have evolved mechanisms to directly inhibit RNase L activity. As

noted above, RNase L can be directly inhibited by binding to a structured element within the RNA of group C enteroviruses. Additionally, the TMEV L* protein has been shown to bind and inhibit RNase L (33). The L* protein is an alternative, smaller form of L generated by leaky ribosome scanning (34). In an uninfected cell, RNase L is inhibited by the cellular protein known as RNase L inhibitor/ATP-binding cassette, sub-family E member 1 (RLI/ABCE). EMCV infection induces RLI/ABCE expression, which contributes to RNase L inhibition (35). It is not known whether other picornaviruses induce RLI/ABCE expression as well.

Several strategies are employed by picornaviruses to prevent enodonucleolytic cleavage by RNase L prior to the nuclease becoming active. Melanoma differentiation-associated gene 5 (MDA5) is the PRR responsible for detecting the replicative form of picornavirus RNAs (36-42). To avoid detection, MDA5 is cleaved or degraded during infection by poliovirus, CVB3, EV71, EMCV, or HRV1a. For poliovirus, CVB3, and EV71, the 2A proteinase appears to be responsible for cleaving MDA5; however, conflicting reports also indicate that cleavage may occur in a proteasome- or caspase-dependent manner (39, 43, 44). Interestingly, MDA5 degradation is not common to all picornavirus infections, as MDA5 remains intact during HRV16 or echovirus type 1 infection (44). The 3C proteinases of CVA6, CVA16, and EVD68 have been shown to bind MDA5 and inhibit its interaction with MAVS independent of any proteolytic activity (45). Ligand-bound MDA5 assembles with the adaptor molecule mitochondrial antiviral signaling protein (MAVS) at the mitochondrial membrane to transfer the signal downstream. Accordingly, MAVS is also targeted for inhibition during viral infection. MAVS proteolysis has been observed during infection by poliovirus, CVB3, EV71, HRV1a, HAV, and Seneca Valley virus (SVV) (43, 46-

49). Several lines of evidence point toward MAVS cleavage by the 2A and/or 3C proteinases, as well as by caspases. In one study, expression of poliovirus, CVB3, or EV71 2A proteinase alone resulted in MDA5 and MAVS cleavage similar to what is observed during infection (43). FMDV infection also results in reduction of MAVS, but not through its cleavage. Instead, the non-structural protein, 3A, and structural protein, VP3, have both been shown to down-regulate MAVS mRNA expression (50, 51).

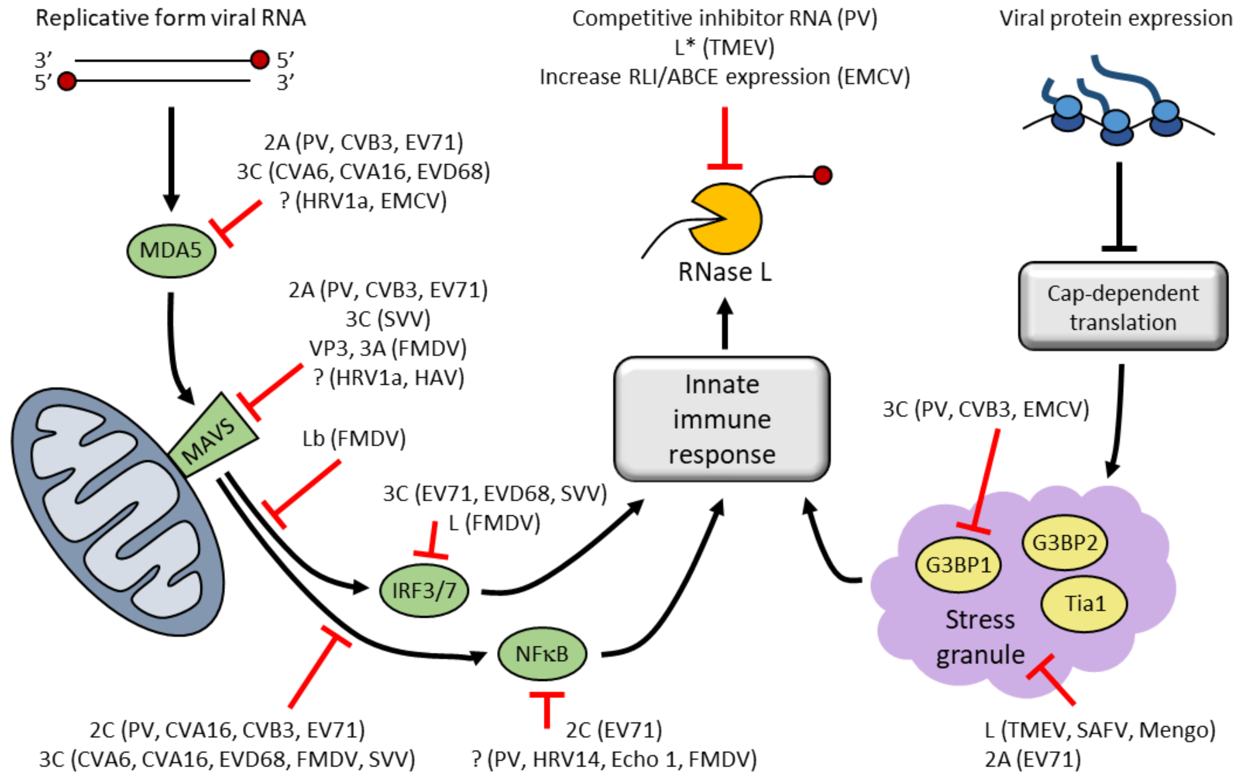
IFN-β expression is induced following recruitment of signaling molecules to MAVS which activates TANK-binding kinase 1 (TBK1), resulting in phosphorylation of the transcription factors interferon regulatory factor 3 and 7 (IRF-3 and -7). Phosphorylated IRF-3/7 proteins translocate to the nucleus to activate transcription of IFN-β. EV71, EVD68, and SVV 3C proteinases cleave IRF-3/7, inhibiting their ability to transactivate IFN-β expression (52-54). FMDV employs a different strategy to inhibit IFN-β expression. The FMDV Lb protein (generated by leaky ribosome scanning, similar to L*) deubiquitinates TBK1 and the signaling molecule TNF receptor-associated factor 3 (TRAF3), thereby inhibiting their activity (55). In addition, the FMDV L protein causes a decrease in IRF-3/7 mRNA levels (56).

MAVS also activates proinflammatory cytokine expression by the nuclear factor-κB (NFκB) transcription factor. NFκB activation requires phosphorylation of the NFκB inhibitor-α (IκBα) by the IκB kinase complex, IKK (composed of IKKα, IKKβ, IKKγ). Phosphorylation of IκBα releases NFκB, allowing it to translocate to the nucleus and activate transcription. Various strategies are employed by picornaviruses to inhibit NFκB activation. Poliovirus, CVA16, CVB3 and EV71 2C proteins have been shown to inhibit phosphorylation and activation of IKK by recruiting protein phosphatase 1 (PP1) to IKKβ

(57, 58). FMDV inhibits IKK through cleavage of IKKγ by 3C proteinase (59). NFκB is also targeted directly through its p65/RelA subunit. Poliovirus, HRV14, echovirus type 1, and FMDV cleave p65/RelA during infection (60, 61) and the EV71 2C protein has also been shown to inhibit NFκB by binding to p65/RelA (62).

Picornaviruses antagonize the innate immune response at many steps in the pathway, including steps not mentioned here (Figure 1.3). From preventing detection by viral RNA sensors to inhibition and degradation of signal transduction molecules and transcription factors, picornaviruses employ a number of strategies to inhibit the antiviral response. Inhibition of this response promotes picornavirus replication and spread, in part by preventing the activation of RNase L, which poses a significant threat to viral RNA stability.

Figure 1.3. Inhibition of RNase L activation by picornavirus proteins. RNase L is an effector molecule of the innate immune response. In its active form, RNase L endonucleolytically cleaves viral RNA. Several picornaviruses have been shown to inhibit RNase L directly through binding of a viral inhibitor to RNase L or up-regulation of the cellular RNase L inhibitor, RLI/ABCE. Picornaviruses also indirectly prevent activation of RNase L through extensive disruption of pathways that contribute to the innate immune response. The signaling cascade that is initiated by MDA5 following detection of viral double-stranded RNA is disrupted at multiple steps during infection, which prevents expression of interferon-induced genes and activation of RNase L. Additionally, the disassembly of stress granules (SGs) during infection inhibits SG-mediated enhancement of the innate immune response. Simplified pathways are depicted here, highlighting points in the pathway that are inhibited by specific picornaviruses and the viral protein responsible ("?" indicates that the viral protein responsible is unknown). PV: poliovirus; CVA6: coxsackievirus A6; CVA16: coxsackievirus A16; CVB3: coxsackievirus B3; EV71: enterovirus 71; EVD68: enterovirus D68; Echo 1: echovirus type 1; HRV1a: human rhinovirus 1a; EMCV: encephalomyocarditis virus; Mengo: mengovirus (a strain of EMCV); TMEV: Theiler's murine encephalomyelitis virus; SAFV: Saffold virus; HAV: hepatitis A virus; FMDV: foot and mouth disease virus; SVV: Seneca Valley virus.


Replicative form viral RNA
3'
5'
5'
3'
2A (PV, CVB3, EV71)
3C (CVA6, CVA16, EVD68)
? (HRV1a, EMCV)
MDA5
2A (PV, CVB3, EV71)
3C (SVV)
VP3, 3A (FMDV)
? (HRV1a, HAV)
MAVS
Lb (FMDV)
3C (EV71, EVD68, SVV)
L (FMDV)
IRF3/7
NFκB
2C (PV, CVA16, CVB3, EV71)
3C (CVA6, CVA16, EVD68, FMDV, SVV)
2C (EV71)
? (PV, HRV14, Echo 1, FMDV)
Competitive inhibitor RNA (PV)
L* (TMEV)
Increase RLI/ABCE expression (EMCV)
RNase L
Innate immune response
Viral protein expression
Cap-dependent translation
3C (PV, CVB3, EMCV)
G3BP1
G3BP2
Tia1
Stress granule
L (TMEV, SAFV, Mengo)
2A (EV71)

Stress granules

Stress granules (SGs) are a type of cytoplasmic RNA granule that contain non-translating mRNAs and form in response to cellular stress and inhibition of translation (63-65). Unlike processing bodies (PBs), which are RNA granules enriched for mRNA decay proteins, SGs contain many translation initiation factors and form around stalled translation initiation complexes (64, 66-68). SGs can assemble and disassemble continuously and may act as sites for mRNA storage and sorting between a repressed state, active translation on polysomes, or degradation in PBs (69-71). Several mRNA decay factors associate with SGs, including the Xrn1 exonuclease (67), PMR1 endonuclease (72), and the mRNA decay proteins tristetraprolin (TTP), butyrate response factor 1 (BRF-1), and K homology-type splicing-regulatory protein (KHSRP, also known as FBP2) (73, 74); however, SGs likely do not contribute to mRNA decay directly, but instead they promote degradation through their interaction with PBs. SGs and PBs can physically associate, share several protein components, and have been proposed to exchange "cargo," thereby targeting translationally repressed mRNAs for degradation (67, 75). Conversely, SGs are also thought to stabilize non-translating mRNAs by temporarily sequestering them away from components of the decay machinery (76, 77). In the context of viral infection, the major contribution to the destabilization of viral RNA by SGs may be through enhancement of the innate immune response.

Several picornaviruses have been shown to transiently induce the formation of SGs early in infection, which disperse at roughly mid to late times during infection. The transient induction of SGs has been visualized by immunofluorescence for the enteroviruses poliovirus (64, 78, 79), CVB3 (80), and EV71 (81), and cardioviruses EMCV

(82) and TMEV (83). During poliovirus infection, some virus-induced SGs are compositionally unique from stress-induced SGs, containing the splicing factor and viral IRES transacting factor (ITAF), SRSF3 (SRp20) (84). Atypical stress granules have also been identified in cells following infection by EV71 (85, 86). A driving force behind picornavirus-induced SG formation is the shut-down of host cap-dependent translation. This leads to the accumulation of stalled translation initiation complexes which induce the aggregation of SGs. For that reason, expression of poliovirus, CVB3, or EV71 2A proteinases alone, which cleave the cap binding complex component eIF4G, can induce SG formation (80, 81, 86, 87).

Assembly of SGs is mediated by the SG-nucleating proteins Ras-Gap SH3 domain binding protein 1 and 2 (G3BP1 and G3BP2) and T-cell-restricted intracellular antigen 1 (TIA-1), among others (88-90). For poliovirus, CVB3, and EMCV, the disassembly of SGs occurs as a result of G3BP1 cleavage by 3C proteinase (78, 80, 82). Expression of uncleavable G3BP1 prevents the disassembly of SGs during infection, highlighting the importance of 3C cleavage in disrupting virus-induced SGs (80, 82). TIA-1 and G3BP2 remain intact during infection, although that does not exclude cleavage of other SG-nucleating proteins from contributing to the disruption of SGs. Interestingly, G3BP1 cleavage does not contribute to the disassembly of SGs formed following infection by TMEV, which is in contrast to EMCV. Instead, TMEV-induced SGs are inhibited by the L protein through an unknown mechanism. The SAFV and mengovirus L proteins are also capable of inhibiting SGs when expressed in place of TMEV L (83). Other viral proteins may also contribute to the inhibition of SGs. Expression of the poliovirus structural protein coding region P1, 2A proteinase, or 3A alone modestly inhibited SGs induced by oxidative

stress, although it is not understood whether these proteins play a part in inhibition of virus-induced SGs (87).

Picornavirus-induced SGs have previously been implicated in activation of the interferon response, but until recently it was not known how direct activation occurs. SGs induced by influenza A virus infection, a (-) ssRNA virus from the *Orthomyxoviridae* family, contain viral RNA and several antiviral proteins including the viral RNA sensors RIG-I and MDA5, along with OAS and RNase L. Formation of these antiviral stress granules parallel, and potentially activate, the interferon response and concentrate viral RNA in proximity to RNase L (91). Similarly, transient SGs formed during EMCV infection were also associated with an antiviral effect. Expression of uncleavable G3BP1 prior to infection, which prevents the disassembly of virus-induced SGs, resulted in significantly increased levels of IFN-β and other cytokines, as well as decreased virus replication (82). SGs induced by the overexpression of G3BP1 inhibit the replication of CVB3, CVB5, and EV70 and contain proteins involved in the innate immune response, such as OAS2, RNase L, and double-stranded RNA-dependent protein kinase (PKR) (92). These data indicate that a link exists between SGs, G3BP1, and the innate immune response.

Recent investigations have revealed that G3BP1 directly stimulates the antiviral response through recruitment of PKR to SGs. It was shown that G3BP1 directly binds PKR in SGs during mengovirus infection, and in complex with another SG nucleating protein, Caprin1, activates PKR (93). Active PKR then moves into the cytoplasm, where it can mediate the innate immune response through both its kinase activity and its role as an adaptor protein. PKR induces IFN expression through activation of the transcription factor NFκB. PKR activation of NFκB has been shown to occur through indirect phosphorylation

of the NFκB inhibitor IκB, which results in IκB degradation and the translocation of NFκB to the nucleus (94, 95). Interestingly, while PKR is required for IFN-α/β induction during EMCV and TMEV infection, it does not appear to do so through transcriptional activation, but instead through regulation of IFN mRNA integrity. EMCV infection of $PKR^{-/-}$ mouse cells resulted in very little IFN-β protein production despite normal IFN-β mRNA levels. Even though IFN-β mRNA levels appeared normal, the mRNA lacked a poly(A) tract, which resulted in its decreased translation (96).

Given a similar localization of PKR to enterovirus-induced SGs, it would be reasonable to assume that PKR is activated in SGs formed by other picornaviruses, thereby enhancing the interferon response to infection. Even though RNase L was identified as a component of SGs formed following infection by CVB3, CVB5, or EV70, it is unlikely that this localization adds to the degradation of viral RNA (93). The proximity of RNase L to influenza A virus RNA in SGs was suggested as a possible mechanism for inhibition of virus replication; however, picornavirus RNA has not been detected in SGs, which has been tested for poliovirus, CVB3, and TMEV (79, 80, 83). Therefore, any contribution of virus-induced SGs to viral RNA decay can most likely be attributed to the induction of IFNs, which results in activation of RNase L and degradation of viral RNA. The disassembly of SGs during picornavirus infection plays an important part of maintaining viral RNA stability through suppression of interferon-induced RNA decay (Figure 1.3).

Processing bodies

Processing bodies (PBs) are constitutively expressed cytoplasmic RNA granules which are composed of non-translating mRNAs and many proteins, including those involved in mRNA decay such as decapping components (Dcp1, Dcp2, Lsm1-7), 5'→3'

exonuclease (Xrn1), and deadenylation factors (Pan2, Pan3, Ccr4, Caf1, PARN) (67, 97-100). Due to the concentration of mRNA decay machinery in PBs, these granules have been proposed to be involved in 5'→3' mRNA decay (99, 101). However, nonsense-mediated decay (NMD) and ARE-mediated decay (AMD) have both been shown to occur in the absence of PB formation despite localization of NMD or AMD proteins in PBs (74, 102). This suggests that PBs are not required for all types of mRNA decay, but may form as a consequence of mRNA degradation or silencing and serve to enhance the process (103). Not all mRNAs that enter PBs are degraded. mRNAs targeted for miRNA-mediated translational repression can localize to PBs (104-106). Upon relief of repression, these mRNAs may leave the PB and re-enter active translation (107, 108). Given their complex role in mRNA decay and storage, it is not surprising that picornaviruses have evolved ways of disrupting PBs to prevent viral RNAs from aggregating in these granules.

The effect of picornavirus infection on PBs has been studied for two enteroviruses, poliovirus and CVB3. Following infection by poliovirus or CVB3, PB foci were almost completely absent by mid-infection. The PB proteins Dcp1a and Xrn1 are degraded during poliovirus infection. Loss of Dcp1a likely occurs as a result of poliovirus 3C cleavage, while Xrn1 does not appear to be a target of either viral proteinase and is instead degraded through a proteasome-dependent pathway (10). Degradation of Xrn1 may have an additional benefit in protecting non-VPg linked viral RNAs from digestion by this major 5'→3' exonuclease. While cleavage and degradation of Dcp1a and Xrn1 likely contribute to PB disruption, the near complete dispersal of PBs suggests that these granules are targeted through multiple mechanisms.

Deadenylation of mRNA is a necessary first step for PB formation. Inhibition of active deadenylation through the expression of dominant negative Caf1 results in almost complete disruption of PBs. Additionally, components of the deadenylase complexes Pan2-Pan3 and Ccr4-Caf1 localize to PBs, and siRNA-mediated knockdown of either Caf1, Ccr4, or Pan3 disrupt PB formation (100, 109). Therefore, inhibition of deadenylation presents an additional opportunity for virus-mediated disruption of PBs. Upon examination of deadenylase components following poliovirus infection, it was discovered that Pan3 is degraded, possibly as result of 3C cleavage, while poly(A) RNase (PARN), Pan2, Ccr4, and Caf1 remain intact (10). Knockdown of Pan3 has been shown to block PB formation but does not impair deadenylation of mRNAs (100). Thus, poliovirus-mediated degradation of Pan3 may contribute to the disruption of PBs by preventing the association or nucleation of PB components, not through blocking deadenylation.

Several poliovirus proteins promote the dispersal of PBs when expressed individually, although the mechanisms for disruption remain unclear. Expression of either the 2A or 3C proteinases alone significantly reduced the number of PBs formed per cell, with 2A having a more pronounced effect than 3C. Additionally, expression of either 3CD (a precursor of 3C that also possesses proteinase activity) or the viral RNA polymerase, 3D, induced a modest reduction in PBs, although their molecular targets are unknown. The dispersal of PBs by 2A or 3C appears to occur through different mechanisms. Expression of 2A could neither prevent the formation of stress-induced PBs nor disrupt PBs containing exogenously expressed Dcp1a, while 3C could do both (87). These data emphasize the importance of the disassembly and prevention of formation of PBs during poliovirus and

CVB3 infection, a process that likely extends to other picornaviruses. The disruption of PBs highlights the significance of this process in the virus life cycle (Figure 1.4).

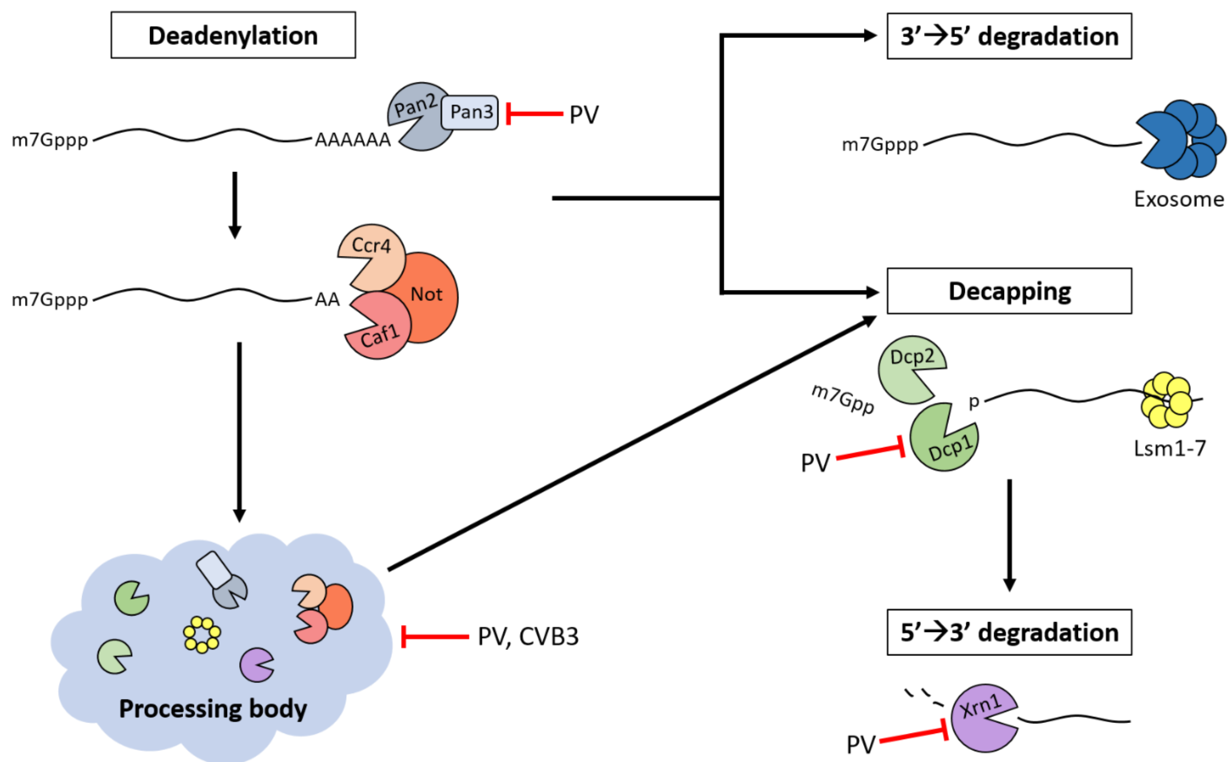


Figure 1.4. Inhibition of processing bodies (PBs) and mRNA decay proteins during picornavirus infection. Deadenylation, decapping, and 5'→3' RNA degradation machinery are targeted during poliovirus (PV) and coxsackievirus B3 (CVB3) infection. The localization of non-translating RNAs and mRNA decay proteins in PBs indicate that these RNA granules may be involved in 5'→3' mRNA decay. PBs are dispersed during poliovirus and CVB3 infection, thereby disrupting their possible contribution to viral RNA degradation. During poliovirus infection, Pan3 and Dcp1a are cleaved by 3C proteinase, and Xrn1 is degraded by a proteasome-dependent mechanism.

ARE-mediated mRNA decay

Regulation of mRNA stability and turnover is a critical component of post-transcriptional gene expression. Both *cis*- and *trans*-acting elements participate in regulating the stability of mRNA. Many mRNAs encode adenylate uridylate-rich elements (AREs), which are sequences often found within the 3' UTR that regulate mRNA stability through their association with the approximately 20 identified ARE-binding proteins (AUBPs). Stabilization or degradation of a transcript depends on which AUBPs are bound. Several AUBPs have been well characterized for promoting ARE-mediated decay (AMD) of target transcripts: ARE/poly(U)-binding/degradation factor 1 (AUF1), KHSRP, TTP, and BRF-1/2. Other AUBPs are better known for either stabilizing (HuR, HuD) or repressing the translation (TIA-1, TIAR) of target transcripts (110).

ARE-mediated mRNA degradation (AMD) occurs in the cell cytoplasm and is initiated by deadenylase digestion of the 3' poly(A) tract, followed by degradation of the body of the mRNA using both 3'→5' and 5'→3' exonucleolytic pathways (111-114). Several decay-promoting AUBPs have been shown to directly interact with components of the RNA degradation machinery, suggesting a mechanism for their initiation of mRNA decay (115, 116). It seems plausible that binding of certain AUBPs to viral RNA could lead to recruitment of RNA degradation machinery and subsequent decay of viral RNA. If so, then AMD could present an additional antiviral strategy utilized by the cell. To date, two AUBPs known for promoting AMD, AUF1 and KHSRP, have been characterized for their roles in the picornavirus life cycle. Both proteins are shown to have a negative impact on virus replication, but apparently not through their expected functions in promoting RNA decay.

AUF1 (also known as hnRNP D) is one of the best described AUBPs involved in ARE-mediated mRNA decay and has been shown to promote the decay of mRNAs encoding oncogenic, inflammatory, and cell cycle proteins, among others (117-120). In addition to its role in promoting mRNA decay, AUF1 has also been shown to stabilize (121, 122) and promote the translation of targeted transcripts (123). AUF1 is expressed as four different isoforms generated through alternative pre-mRNA splicing, named p37, p40, p42, and p45 based on their apparent molecular weights (124). All of the AUF1 protein isoforms are composed of the same two, non-identical RNA recognition motifs (RRMs) and a glutamine-rich domain but display different affinities for ARE substrates and subcellular localization (125, 126). The two largest isoforms of AUF1, p42 and p45, localize primarily to the nucleus, while the smaller p37 and p40 isoforms are predominantly nuclear, but can transit between the nucleus and cytoplasm (127).

A large-scale identification of AUF1 target transcripts using photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) revealed that AUF1 isoforms bind over 3,000 mRNAs as well as many non-coding RNAs (ncRNAs) (128). Pairing PAR-CLIP with RNA-seq and polysome analysis following AUF1 overexpression implicated AUF1 in a range of regulatory events including stabilization and destabilization of both mRNA and ncRNA, promotion and repression of translation, and modulation of alternative splicing. In addition, AUF1 has recently been shown to have a role in the life cycle of DNA and RNA viruses including Epstein-Barr virus (129, 130), HIV (131), hepatitis C virus (132), the flaviviruses West Nile, Dengue and Zika virus (133-135), and several picornaviruses (136-139).

A direct interaction between AUF1 and picornavirus RNA was originally discovered from an RNA affinity screen using the 5' NCR of poliovirus RNA (140). This finding was later expanded to include binding to HRV, CVB3, and EV71 RNA (136, 139, 140). It was subsequently discovered that AUF1 knockout or knockdown in human or mouse cells resulted in increased replication of these viruses, suggesting that AUF1 may act as a host restriction factor to enterovirus infection (136-139). Prior to these discoveries, a study of HRV-16 infection of human airway epithelial cells reported the observation that cytoplasmic levels of AUF1 increased during infection (141). Cytoplasmic relocalization of AUF1 was also observed following poliovirus, HRV-14, CVB3, EV71, or EMCV infection of human cells (136-139). For two of the viruses studied, poliovirus and CVB3, AUF1 was shown to relocalize as a result of the disruption of nucleocytoplasmic trafficking by the viral 2A proteinase (136, 138). Given the cytoplasmic life cycle of these viruses, relocalization of AUF1 to the cytoplasm may contribute significantly to its negative impact on virus replication.

The mechanism by which AUF1 acts as a restriction factor during picornavirus infection has not been fully clarified; however, one report suggested that AUF1 may play a role in promoting viral RNA decay. AUF1 was found to bind to a reporter RNA harboring a CVB3 3' NCR, and knockdown of AUF1 was shown to stabilize that RNA (138). These data suggest that AUF1 may regulate the stability of CVB3 RNA through binding of its 3' NCR; however, the effect of AUF1 on the stability of viral RNA during infection was not measured. Despite this evidence, most reports aimed at determining the mechanism by which AUF1 acts as a restriction factor point toward a negative regulation of viral translation, which will be discussed further in the next section.

Similar to AUF1, KHSRP is another AUBP that has been best characterized for its role in promoting AMD of target transcripts. Additional roles in transcription, alternative splicing, and miRNA maturation have also been described for KHSRP (115, 142-144). Like AUF1, KHSRP was identified in an RNA affinity screen for its association with viral RNA; in this case, as a novel EV71 5' NCR binding protein. KHSRP was shown to relocalize from the nucleus to the cytoplasm during infection and to associate with the EV71 5' NCR, with binding occurring at multiple sites within the viral IRES. KHSRP was not shown to promote the degradation of viral RNA and was instead shown to be a negative ITAF for EV71 using protein pulse-labeling and bicistronic reporter assays (145, 146). Like AUF1, KHSRP is cleaved during infection. However, KHSRP is not a substrate for the viral 2A or 3C proteinases but is instead cleaved and degraded through the activity of caspases and the proteasome and autophagy pathways (146). KHSRP has not yet been shown to act as a negative regulator of other picornavirus infections, but it was identified as a poliovirus RNA binding protein using TUX-MS and thus, may have a similar effect on other enteroviruses (29).

Other AUBPs have been shown to associate with picornavirus RNAs and participate in the picornavirus replication cycle; however, these proteins are not typically linked to AMD. Instead, these AUBPs are often associated with stabilization or translation of mRNA (discussed in the Genome stabilization features for picornaviruses section). hnRNP A1, the AUBP that was shown to compete with AUF1 for binding to the EV71 IRES, is a multifunctional protein involved in transcription, alternative splicing, mRNA localization, translation, and stability (147). hnRNP A1 has been reported to destabilize mRNAs bearing AREs (148) or an ARE-like motif (a motif which was identified in ~7% of mRNAs) (149).

However, instead of destabilizing EV71 RNA, hnRNP A1 is re-purposed by the virus as a positive regulator of translation (150). Among its many functions, hnRNP A1 has also been shown to act as an ITAF for cellular IRESs, and it is this function that is utilized to promote virus replication (150-152).

MicroRNA-mediated decay

MicroRNAs (miRNAs) are small, regulatory RNAs produced in eukaryotic cells that bind to complementary sites in mRNA and act to translationally repress or signal the degradation of target transcripts (153). Biogenesis of miRNA begins in the nucleus, where precursor miRNAs (pre-miRNAs) are cleaved from hairpin structures within primary miRNA transcripts by the RNase III nuclease, Drosha (154). Following export to the cytoplasm, pre-miRNAs are further processed to mature miRNAs by Dicer, another RNase III nuclease (155). Mature miRNAs are bound by a member of the Argonaute protein family within the RNA-induced silencing complex (RISC), which together act as the effector complex that targets complementary mRNAs for RNA interference (156).

miRNA binding can lead directly to mRNA degradation, or indirectly through degradation of repressed mRNAs in PBs. Direct degradation of a miRNA target in animal cells is most often initiated through recruitment of deadenylases by the RISC, and on rare occasions, by endonucleolytic cleavage of the mRNA by Argonaute 2 (Ago2), the only catalytically active member of the Argonaute family (153, 157). Until a few years ago, it was assumed that the antiviral potential of the miRNA pathway is not utilized during picornavirus infections, since the cytoplasmic replication cycle of these viruses does not encounter nuclear miRNA biogenesis pathways. However, regions of the viral genome, such as the IRES, contain hairpin structures that resemble structured miRNA transcripts.

These structured regions in the viral RNA provide an opportunity for the cytoplasmic miRNA machinery to generate miRNA-like interfering RNAs.

Recent studies using deep sequencing techniques have revealed that viral small RNAs (vsRNAs) are produced from HAV, EMCV and EV71 RNA in a Dicer-dependent manner (158-161). While the roles of the HAV and EMCV vsRNAs during infection have not been elucidated, the study of EV71 vsRNA has revealed interesting new ways in which the virus both inhibits and re-purposes yet another potentially antiviral cellular pathway. The EV71 3A protein has been shown to inhibit antiviral RNA interference by binding and sequestering double stranded viral RNA from Dicer nuclease activity. Furthermore, infection by EV71 encoding a mutant 3A resulted in the Dicer-dependent production of virus-derived small interfering RNAs (siRNAs) that are loaded into RISC and result in the cleavage and degradation of cognate viral RNA (161). An additional study of a small RNA produced from the second stem loop (SL-II) of the EV71 5' NCR, vsRNA-1, demonstrates that this small RNA negatively regulates viral translation and replication without resulting in viral RNA degradation (160). Instead of inhibiting virus replication via canonical miRNA mechanisms, it was discovered that vsRNA-1 may regulate EV71 IRES-driven translation by promoting the binding of both positive and negative ITAFs to SL-II of the 5' NCR. Surprisingly, vsRNA-1 recruits Ago2 to the viral RNA, but instead of acting as a negative regulator through translational repression or cleavage of viral RNA, Ago2 acts a positive regulator of translation (27). These data reveal the possibility for a new and interesting regulator in the picornavirus life cycle. Whether these small, virus-derived RNAs represent a bona fide antiviral defense mechanism or act in regulating replication for other picornaviruses has yet to be determined.

Conclusion

Picornavirus disruption of the cellular RNA decay machinery generally involves a broad approach of cleaving, degrading, inhibiting, disassembling, or re-purposing components of these processes. However, several pathways that may contribute to viral RNA instability have received relatively little attention to date, and the focus has been on only a few members of the *Picornaviridae* family. For instance, 5’→3’ RNA degradation is inhibited during infection through cleavage of decapping enzymes and Xrn1, but it is unclear whether exosomes, which participate in 3’→5’ RNA degradation, remain intact or functional. There has also been little investigation into the cellular proteins that bind viral RNA and promote either viral RNA stability or degradation. Insight into these issues can be provided by approaches like TUX-MS, which may reveal novel RNA binding proteins that contribute to viral RNA stability (29). Additional studies like these will reveal not only new viral mechanisms to preserve the stability of their RNA, but also host defense mechanisms that target viral RNA for degradation.

III. Host restriction of picornavirus infection

Introduction

The cytoplasmic life cycle of picornaviruses is dependent upon modification of many cellular processes and the re-purposing of host proteins for the generation of progeny virions. As part of the alteration of the host cell landscape to promote virus replication, picornaviruses modify lipid metabolism and reorganize membrane architecture to form replication complexes, down regulate host cell transcription and translation to redirect cellular resources to favor viral replication, and disrupt nucleocytoplasmic trafficking to

relocate nuclear proteins required for replication into the cytoplasm (1, 162-164) (Figure 1.1). Along with modification of cellular pathways to favor virus replication, picornaviruses must also disrupt processes that restrict virus replication. Triggered by double-stranded RNAs that form during viral RNA synthesis, the innate immune system is the best described process that restricts virus replication and, as a result, picornaviruses have evolved numerous strategies to counteract this response (165, 166). Outside of the antiviral activity of the innate immune system, few host factors have been identified that restrict picornavirus replication. However, the dependence on cellular proteins to promote each step of the virus replication cycle provides opportunities for viral interactions with cellular restriction factors.

Antiviral interferon stimulated genes

The innate immune response to infection serves as the first line of defense against replication and spread of a viral pathogen. The detection of viral RNA (discussed in more detail in section II of the Introduction) ultimately leads to the expression of hundreds of interferon-stimulated genes (ISGs) that both generally restrict virus replication and lead to the development of a virus-specific adaptive immune response. Strategies employed by ISGs to restrict virus replication involve inhibition of translation, viral RNA degradation, enhancement of pathogen sensing, and blockage of viral entry and release (167, 168). While several ISGs have been characterized for their roles as antiviral effector molecules, the functions of most remain poorly understood. The involvement of antiviral ISGs in the picornavirus life cycle has been investigated for only a few of these factors, with most studies focused on the role of PKR.

PKR, also discussed previously in the context of viral RNA decay, is an ISG that restricts viral replication through inhibition of cellular translation. The expression and activation of PKR during infection results in phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) leading to the accumulation of stalled cellular and viral mRNAs in the 48S pre-initiation complex (169). PKR has been shown to be activated during poliovirus and EV71 infection but is subsequently degraded or cleaved (170, 171). PKR is degraded through an unknown mechanism during poliovirus infection but was determined to be cleaved by the 3C proteinase during EV71 infection. The cleavage of PKR likely serves as a viral defense mechanism but may also have additional pro-viral consequences. It was demonstrated that a PKR cleavage product that formed during EV71 infection was able to enhance virus replication (171). PKR expression is also induced during FMDV infection, but instead of using cleavage as a defense mechanism, the 3C proteinase inhibits its activation and indirectly promotes PKR degradation through the lysosomal pathway (172).

In addition to PKR, ISGs with possible roles in inhibition of picornavirus entry and modification of viral proteins have also been investigated. Myxovirus resistance (Mx) proteins belong to a family of guanosine triphosphatases (GTPases) and have been shown to act as antiviral effector molecules at an early stage of infection, immediately post-entry. It has been proposed that Mx traps or sequesters incoming viral proteins due to its tendency to oligomerize into ring-like structures, which may form around viral components (173-176). It is unclear whether Mx proteins act against picornaviruses in this manner; however, knockdown of Mx1 in bovine cells was shown to increase FMDV replication, indicating that Mx1 acts as an antiviral against at least one picornavirus (177).

In addition to Mx, interferon-induced transmembrane (IFITM) family members are also thought to inhibit virus entry. Enriched on endosome and lysosome membranes, IFITM may inhibit entry of viruses that require pH- or cathepsin-dependent fusion with endosomes (178). FMDV uncoating has been shown to be dependent on acidification of early endosomes and overexpression of IFITM in cells has been shown to inhibit FMDV replication (179). IFITM may restrict FMDV entry in cell culture, but its overexpression in transgenic pigs did not protect the animals from FMDV infection (180). ISGs may also restrict infection by modifying the activity of viral proteins. Tripartite motif (TRIM) is a large family of interferon-stimulated proteins that includes E3 ubiquitin ligases. Expression of TRIM22 prior to infection by EMCV conferred resistance to the virus, possibly through TRIM22 ubiquitination of the viral 3C proteinase (181). Overall, the effect of antiviral ISGs on the picornavirus life cycle is an area that requires further exploration. The identification of ISGs that restrict picornavirus infection could provide new insight into host strategies used to defend against these viruses.

Host restriction factors

Few host proteins have been identified that are able to restrict picornavirus infection which are not involved in the innate immune response. For picornaviruses, three proteins that are normally expressed in cells have been reported to restrict infection. DRBP76, KHSRP, and AUF1 were all identified in RNA affinity screens of the 5' NCRs of different members of the Enterovirus genus of the *Picornaviridae* family. DRBP76, an isoform of interleukin enhancer binding factor 3 (ILF3), was found to bind the 5' NCR of human rhinovirus 2 (HRV2) RNA and restrict infection in a cell-type specific manner by negative regulation of the viral IRES (182, 183). KHSRP has been characterized as a

negative ITAF for enterovirus 71 (EV71) following binding to multiple sites within its 5' NCR (145). The ability of KHSRP to act as a negative ITAF is regulated by ubiquitination, which appears to enhance its ability to compete with a positive regulator of viral translation, far upstream element binding protein 1 (FUBP1), for binding to the EV71 IRES (184). Of the identified restriction factors, AUF1 is the only protein shown to negatively regulate replication of several picornaviruses. Using knockdown or knockout mouse cell or human cell models, AUF1 has been shown to negatively regulate infection by poliovirus, coxsackievirus B3 (CVB3), HRV, and EV71 (136-139).

AUF1 has been primarily described as that of a negative regulator of picornavirus translation. AUF1 has been shown to bind poliovirus, CVB3, and EV71 RNA during infection, suggesting that restriction occurs through interaction with viral RNA (29, 138, 139). For poliovirus, HRV, and EV71, binding has been shown to occur in the 5' NCR, specifically within stem loops that make up the poliovirus and EV71 IRES (136, 138, 139). Consistent with these results, AUF1 has been shown to reduce *in vitro* translation of poliovirus RNA (136). Using bicistronic reporter assays, AUF1 was shown to negatively regulate EV71 IRES-driven translation, likely through competition with the positive ITAF, hnRNP A1 (139, 150, 185). Given its role in mRNA decay, AUF1 may also restrict picornavirus infection through degradation of viral RNA. It was shown that AUF1 can bind a CVB3 3' NCR reporter RNA and that knockdown of AUF1 lead to increased stability of this RNA (138). Whether AUF1 promotes the decay of CVB3 genomic RNA during infection has not yet been determined. Like many host restriction factors, AUF1 is cleaved during infection by poliovirus, HRV, and CVB3 which may serve as a virus defense against its

activity (138, 140). The work presented in this dissertation will further dissect the role of AUF1 in the restriction of poliovirus and CVB3 infection.

Conclusion

Viral infection leads to a large-scale activation of antiviral processes; however, little is known of many individual players in host restriction of infection. At the cellular level, research aimed at studying the control of infection has focused on the innate immune system with little emphasis on normally-expressed cellular proteins capable of restricting virus replication. The three described picornavirus restriction factors (DRBP76, KHSRP, and AUF1) were all identified though RNA affinity screens, which is a useful approach to identify host proteins that may restrict infection through direct binding of viral RNA. Many proteins have been identified that bind poliovirus RNA but whose functions remain unknown and may represent novel inhibitors of infection by poliovirus and other picornaviruses (29). Genome-wide screens using CRISPR or RNAi may also identify proteins whose knockout or knockdown result in increased virus replication and could include factors that restrict infection through indirect mechanisms. The discovery of additional host mechanisms for restriction of virus infection could provide new directions for the development of novel antiviral therapies.

IV. Overall summary

Picornaviruses must modify many cellular processes and counteract host defense mechanisms to successfully complete their life cycle. Most investigations have focused on the innate immune system as the first critical line of defense against infection with little attention paid to identifying non-canonical host restriction factors. This dissertation

describes work aimed at determining the mechanism of restriction of one of these non-canonical factors, AUF1, during picornavirus infection. Taken together, previous studies of AUF1 do not paint a clear picture of its role during picornavirus infections, with evidence pointing at a role in inhibition of viral translation or in promoting viral RNA decay. Results presented in **Chapter 2** describe the effect of AUF1 on replication of poliovirus and CVB3. Distinct from its role as an mRNA decay protein in uninfected cells, AUF1 appears to restrict infection of poliovirus and CVB3 by negatively regulating viral translation. Furthermore, the disruption of nucleocytoplasmic trafficking, resulting in the unintended relocalization of AUF1 from the nucleus to the cytoplasm, is required for AUF1 inhibition of infection. **Chapter 3** explores the mechanism by which AUF1 restricts viral translation, focusing on identifying competitive interactions of AUF1 with positive translation factors for binding to the poliovirus 5' NCR. The work presented in this chapter will also address whether there is an isoform-specific effect of AUF1 on virus replication and if cleavage of AUF1 during infection ameliorates its negative effect. Overall, the studies described in this dissertation seek to better characterize a restriction factor of picornavirus infection and illuminate a host mechanism for limiting virus replication separate from the innate immune response.

CHAPTER 2

Direct and indirect effects on viral translation and RNA replication are required for AUF1 restriction of enterovirus infections in human cells

I. Introduction

Picornaviruses induce extensive modification of cellular processes to complete their cytoplasmic replication cycle. As part of the alteration of the host cell landscape to promote virus replication, viral proteins induce membrane rearrangements to form replication complexes, down regulate host translation, and disrupt nucleocytoplasmic trafficking to relocate nuclear proteins required for replication into the cytoplasm (1, 162-164). In addition to modifications that promote their replication, picornaviruses must also inhibit cellular processes that restrict infection. The innate immune system is the first line of defense against virus infection and as a result, picornaviruses have evolved numerous strategies to counteract this response (165, 166). Few host factors have been identified that restrict picornavirus replication at the cellular level outside of interferon-induced antiviral effector proteins. This chapter focuses on determining the effect of one non-canonical restriction factor, AUF1, on replication of poliovirus and CVB3.

Upon release of a single-stranded, positive sense RNA genome into infected cells, picornavirus proteins are translated by a cap-independent mechanism using an internal ribosome entry site (IRES) encoded within the 5’ non-coding region (NCR) of viral RNA. This structured region of RNA interacts with both canonical translation initiation factors

and other host proteins known as IRES transacting factors (ITAFs) to recruit ribosomes to viral RNA (186-188). To date, a number of host proteins have been proposed to act as ITAFs for picornavirus translation, including the established ITAFs polypyrimidine tract-binding protein (PTB), poly(rC)-binding protein 1 and 2 (PCBP1/2), upstream of N-ras (Unr), Lupus La protein (La), nucleolin, and serine and arginine rich splicing factor 3 (SRSF3 or SRp20) (186, 188). In addition, three host proteins have been identified that restrict picornavirus translation: double-stranded RNA binding protein 76 (DRBP76), K homology-type RNA splicing regulatory protein (KHSRP, KSRP, or FBP2), and AU-rich element degradation factor 1 (AUF1 or hnRNP D).

DRBP76, KHSRP, and AUF1 were all identified in RNA affinity screens of the 5' NCRs of different members of the enterovirus genus of the *Picornaviridae* family. DRBP76, an isoform of interleukin enhancer binding factor 3 (ILF3), was found to bind the 5' NCR of human rhinovirus 2 (HRV2) RNA and restrict infection in a cell-type specific manner by negative regulation of the viral IRES (182, 183). KHSRP has been characterized as a negative ITAF for enterovirus 71 (EV71) following binding to multiple sites within its 5' NCR (145). The ability of KHSRP to act as a negative ITAF is regulated by ubiquitination, which appears to enhance its ability to compete for binding to the EV71 IRES with a positive regulator of viral translation, far upstream element binding protein 1 (FUBP1) (184). Of the identified restriction factors, AUF1 is the only protein shown to negatively regulate replication of several picornaviruses. Using knockdown or knockout mouse or human cell models, AUF1 has been shown to negatively regulate infection by poliovirus, coxsackievirus B3 (CVB3), HRV, and EV71 (136-139).

AUF1 is most often described as an mRNA decay protein that regulates the stability and translation of mRNAs following binding to sites within the 3' UTR or introns of target transcripts. Four isoforms of AUF1 are generated through alternative pre-mRNA splicing and are named based on their apparent molecular weights: p37, p40, p42, and p45 (124). All four isoforms of AUF1 are predominantly nuclear proteins, but the smaller isoforms, p37 and p40, shuttle between the nucleus and cytoplasm (127). During infection by poliovirus, CVB3, HRV, or EV71, AUF1 was shown to relocalize to the cytoplasm following disruption of nucleocytoplasmic trafficking by viral proteinases (136-141). Additionally, AUF1 relocalizes to the cytoplasm during infection by encephalomyocarditis virus (EMCV), a non-human pathogen belonging to the Cardiovirus genus of *Picornaviridae*. However, AUF1 does not negatively regulate infection of this virus in a mouse cell model (137).

The role of AUF1 as a restriction factor during picornavirus infection has been primarily described as that of a negative regulator of viral translation. AUF1 has been shown to bind poliovirus, CVB3, and EV71 RNA during infection, suggesting that restriction occurs through interaction with viral RNA (29, 138, 139). For poliovirus, HRV, and EV71, binding has been shown to occur in the 5' NCR, specifically within stem loops that make up the poliovirus and EV71 IRES (136, 138, 139). Consistent with these results, AUF1 has been shown to reduce *in vitro* translation of poliovirus RNA (136). Using bicistronic reporter assays, AUF1 was shown to negatively regulate EV71 IRES-driven translation, likely through competition with the positive ITAF, hnRNP A1 (139, 150, 185). Given its role in mRNA decay, AUF1 may also restrict picornavirus infection through degradation of viral RNA. AUF1 was found to bind to a reporter RNA harboring a CVB3 3' NCR, and knockdown

of AUF1 was shown to stabilize that RNA (138). These data suggest that AUF1 may regulate the stability of CVB3 RNA through binding of its 3' NCR.

In the study described in this chapter, the effect of AUF1 on replication of poliovirus or CVB3 in human cells was investigated. Following AUF1 knockdown, infection by poliovirus or CVB3 resulted in increased viral translation, RNA synthesis, and progeny virion production. Although AUF1 targets many cellular mRNAs by binding within the 3' NCR (189), it was found that AUF1 was able to restrict the replication of a mutant poliovirus lacking its 3' NCR, demonstrating that restriction of poliovirus infection does not occur through binding to its 3' NCR. Importantly, these data show that AUF1 has no detectable effect on the stability of poliovirus or CVB3 RNA during infection. Using poliovirus and CVB3 5' NCR reporter RNAs, it was demonstrated that AUF1 negatively regulates both poliovirus and CVB3 IRES-driven translation during infection. These findings reveal that the effect of AUF1 on enterovirus RNA synthesis is, in part, indirect due to a reduction in the levels of viral proteins required for replication. Finally, it was revealed that AUF1 had no effect on viral IRES-driven translation in uninfected cells, suggesting that relocalization of AUF1 from the nucleus to the cytoplasm during infection is a crucial requirement for its negative effects.

II. Materials and Methods

Cell culture and viruses

HEK-293 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells were cultured in DMEM supplemented with 8% newborn calf serum (NCS). Both cell lines were maintained at 37°C,

5% CO_2. Virus stocks were produced in HeLa cells transfected with *in vitro* transcribed RNA generated from infectious cDNA clones. The following clones were used to generate virus stocks: pT7PV1 for Mahoney strain poliovirus (190), pCVB3-0 for CVB3 Nancy strain (191), and pT7PV1(Δ3'NCR) for poliovirus Δ3' NCR (192).

AUF1 knockdown

HEK-293 cells were transfected with linearized pSilencer/U6/tetO/shCtrl or pSilencer/U6/tetO/shAUF1 using jetPRIME reagent (Polyplus-transfection) and selected for stable expression using hygromycin B (Calbiochem) as previously described (193).

Virus infections

293-shCtrl or -shAUF1 cells were infected with wild type or Δ3' NCR poliovirus at a multiplicity of infection of 1 (MOI 1) or CVB3 at MOI 20. Virus was diluted in serum-free DMEM and adsorbed for 30 (poliovirus) or 40 (CVB3) min at room temperature. Following adsorption, cells were overlaid with DMEM supplemented with 10% FBS and incubated at 37°C, 5% CO_2. Cells and supernatant were harvested at specified time points post-infection and four freeze-thaw cycles were performed prior to titration by plaque assay on HeLa cells. Virus titers were normalized to cell count and represented as plaque forming units per cell (PFU/cell). Values represent the means of triplicate experiments ± standard error of the mean (SEM). Statistical significance was measured by unpaired Student's t-test.

Reverse transcription and quantitative PCR

293-shCtrl or -shAUF1 cells were infected with poliovirus (MOI 1) or CVB3 (MOI 20) as described above. At specified times after infection, cells were harvested in 1 ml TRIzol (Invitrogen) and RNA was extracted following the manufacturer's protocol. Complementary DNA (cDNA) was generated from 1 μg total RNA using either oligo $d(T)_{18}$

or virus-specific reverse primers (listed below) and AMV reverse transcriptase (Life Sciences Advanced Technologies). cDNA was analyzed for viral RNA and GAPDH expression using PowerUp SYBR Green Master Mix (Applied Biosystems) and the 7900HT Fast Real-Time PCR System (Applied Biosystems). Fold change in viral RNA relative to the 0 h time point for each cell type was calculated using the ΔΔCt method. Virus- or gene-specific primer pairs were as follows; poliovirus forward 5'-GTCAATGATCACAACCCGAC-3' and reverse 5'-AAGAGGTCTCTATTCCACAT-3', CVB3 forward 5'-ACTCTGCAGCGGAACCGACTA-3' and reverse 5'-GCTGTATTCAACTTAACAATG-3', and GAPDH forward 5'-GTCCACTGGCGTCTTCAC-3' and reverse 5'-CTTGAGGCTGTTGTCATACTTC-3'.

Viral RNA stability measurement

Cells were infected as above and incubated for 4 h (poliovirus) or 6 h (CVB3) before vehicle (DMSO) or 300 μM cordycepin (Tocris Bioscience) was added to the culture media. Cells were harvested in TRIzol (Invitrogen) at 0, 2, 4, and 6 h post-cordycepin addition and RNA was extracted. RT-qPCR was performed as above.

Western blot

Proteins were extracted from HEK-293 cells using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail), and Bio-Rad Protein Assay Dye Reagent was used to determine concentration. Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% resolving gel, 5% stacking gel) and transferred to an Immobilon-P Membrane (Millipore). Membranes were blocked with 5% non-fat milk in phosphate buffered saline with Tween-20 (PBST) followed by incubation with rabbit polyclonal anti-AUF1 (1:2,000; Millipore) or

rabbit polyclonal anti-nucleolin (1:1,000; Abcam) antibodies diluted in PBST with 5% bovine serum albumin (BSA). Membranes were washed 3 times with PBST followed by incubation with a 1:4,000 dilution of goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG-heavy and light chain secondary antibody (Bethyl) diluted in PBST with 5% BSA. Membranes were washed 3 times with PBST followed by exposure to ECL Western Blotting Reagent (Pierce) for chemiluminescent detection of HRP.

Reporter virus infection and luciferase assay

Renilla luciferase-expressing reporter virus stocks were generated by transfecting RNA transcribed from linearized pT7-R-Luc-PPP (PV-PPP) or p53CB3/T7-RLuc (RLuc-CVB3) plasmids into HeLa cells (194, 195). 293-shCtrl or -shAUF1 cells were plated in 12-well plates (2 x 10^5 cells/well) and infected with 100 µl of undiluted virus (MOI <1) as described above. To inhibit viral RNA synthesis, 5 mM guanidine hydrochloride (GuHCl; MP Biomedicals) or 5 mM GuHCl and 5 µg/ml enviroxime (kind gift from Beverly Heinz of Lilly Research Laboratories, Indianapolis, IN) was added during and after adsorption for PV-PPP or RLuc-CVB3, respectively. At 4 h and 8 h post-infection, cells were lysed in Renilla Luciferase Assay Lysis Buffer and luciferase activity was measured using the Renilla Luciferase Assay System (Promega) and a SIRIUS luminometer (Berthold Detection System). Luminescence was measured as relative light units per second (RLU/s) normalized to cell count. Data represent the means of triplicate experiments ± standard error of the mean (SEM). Statistical significance was calculated by unpaired Student's t-test.

Immunofluorescence

HEK-293 cells were plated on glass coverslips and infected with poliovirus as described above. For guanidine hydrochloride (GuHCl; MP Biomedicals) treatment, 5 mM GuHCl was

added to both the virus inoculum and growth media added post-adsorption. At specified times after infection, cells were fixed in 3.7% formaldehyde in phosphate buffered saline (PBS). Fixed cells were washed with PBS and permeabilized in PBS with 0.25% Triton X-100. Cells were washed 3 times with PBS and blocked in 1% BSA in PBS prior to incubation with primary antibody. Cells were incubated with rabbit polyclonal anti-AUF1 (1:100; Millipore) and mouse monoclonal anti-enterovirus capsid (1:500; Dako) antibodies diluted in PBS with 1% BSA followed by 3 washes with PBS. Cells were incubated with goat anti-mouse IgG-heavy and light chain DyLight 488- and goat anti-rabbit IgG-heavy and light chain DyLight 650-conjugated secondary antibodies (1:400; Bethyl) diluted in PBS with 1% BSA. Following 3 washes with PBS, cells were counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) and coverslips were mounted on slides with Fluoro-Gel (Electron Microscopy Sciences). Cells were imaged using a LSM700 laser scanning confocal microscope and ZEN software (Zeiss).

***In vitro* transcription, RNA co-transfection, and luciferase assays**

Capped *Renilla* luciferase (RLuc) control RNA was *in vitro* transcribed from the CVB3 bicistronic reporter construct, pRstCVB3F (196). pRstCVB3F was linearized with BlpI (New England BioLabs) immediately following the RLuc cistron to prevent transcription of the CVB3 5' NCR and Firefly luciferase cistron. Linearized pRstCVB3F was transcribed in the presence of $m^7G(5')ppp(5')G$ cap analog using the mMESSAGE mMACHINE T7 Transcription Kit (Ambion). Uncapped poliovirus or CVB3 5' NCR-Firefly luciferase (FLuc) reporter RNAs were transcribed using MEGAscript T7 Transcription Kit (Ambion) from the p5'PVLuc or p5'CVBLuc plasmid linearized with XbaI (26). Cells were seeded in 12-well plates overnight and co-transfected with 0.85 μg 5'NCR-Fluc and 0.15 μg capped RLuc

RNAs using TransIT-mRNA Transfection Kit (Mirus). To measure the effect of AUF1 on translation of the reporter RNAs during infection, cells were infected with either poliovirus (MOI 1) or CVB3 (MOI 20) as described above. Immediately following adsorption, the transfection mixture was added to the culture media applied to the infected cells. At 4 h and 8 h post-transfection, cells were harvested in passive lysis buffer for 15 min and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and SIRIUS luminometer (Berthold Detection System). Luminescence was measured as relative light units per second (RLU/s) normalized to cell count. Data represent the means of triplicate experiments ± standard error of the mean (SEM). Statistical significance was calculated by unpaired Student's t-test.

III. Results

AUF1 negatively regulates replication of poliovirus and CVB3 in human cells

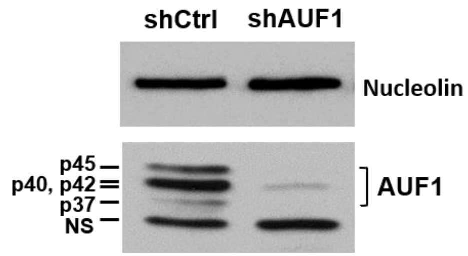
AUF1 has previously been shown to negatively regulate replication of several picornaviruses. Poliovirus was found to replicate to higher titers in mouse embryonic fibroblasts (MEFs) genetically ablated for AUF1 (136). However, since mouse cells do not express the human poliovirus receptor (PVR), the effect of AUF1 on virus replication was measured following transfection of *in vitro* transcribed or virion RNA into cells. The impact of AUF1 on poliovirus replication in human cells, the natural host for this virus, has not been measured. To assess the effect of AUF1 on replication of poliovirus in a human cell model, HEK-293 cells stably expressing an shRNA targeting all four isoforms of AUF1 (293-shAUF1) were generated. Expression of shAUF1 resulted in over a 90% reduction of AUF1 protein expression relative to control (293-shCtrl) cells (Figure 2.1A). Following infection

by poliovirus at a multiplicity of infection of 1 (MOI 1), an approximately 10-fold increase in poliovirus titer was observed by 6 h post-infection of 293-shAUF1 cells (Figure 2.1B). These results demonstrate that AUF1 restricts poliovirus replication during infection of a human cell line, similar to the observations made in mouse cells.

Knockout or knockdown of AUF1 in MEFs or HeLa cells, respectively, has been shown to increase replication of CVB3 (136, 138). To confirm that AUF1 also negatively regulates replication of CVB3 in HEK-293 cells, 293-shCtrl or -shAUF1 cells were infected with CVB3 at an MOI of 20. Consistent with previous results, an approximately a 10-fold increase in CVB3 titer was observed in 293-shAUF1 cells by 8 h post-infection (Figure 2.1C). These data demonstrate that AUF1 negatively regulates infection by the enteroviruses poliovirus and CVB3 in multiple cell types.

Figure 2.1. AUF1 knockdown enhances replication of poliovirus and CVB3 in HEK-293 cells. (A) Cell lysates were prepared from HEK-293 cells stably expressing control (shCtrl) or AUF1 (shAUF1) targeting shRNAs and analyzed for AUF1 protein expression by Western blot. The four isoforms of AUF1 (p37, p40, p42, and p45) are labeled, and nucleolin was used as a loading control. NS = non-specific band. 293-shCtrl or -shAUF1 cells were infected with poliovirus at an MOI of 1 (B) or CVB3 at an MOI of 20 (C). Cells and supernatant were harvested at specified time points and virus titer was determined by plaque assay on HeLa cells. Data represent the mean of three individual experiments ± standard error of the mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$

A



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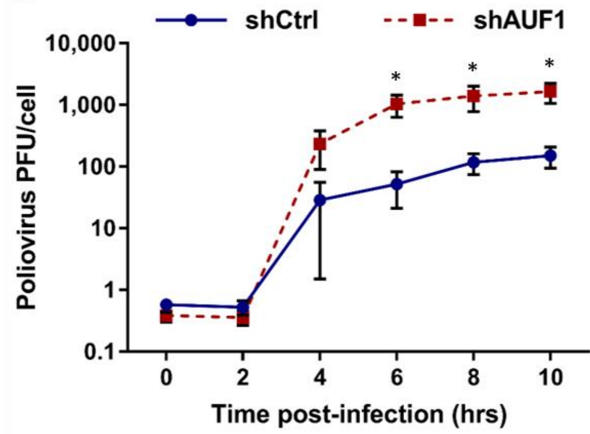

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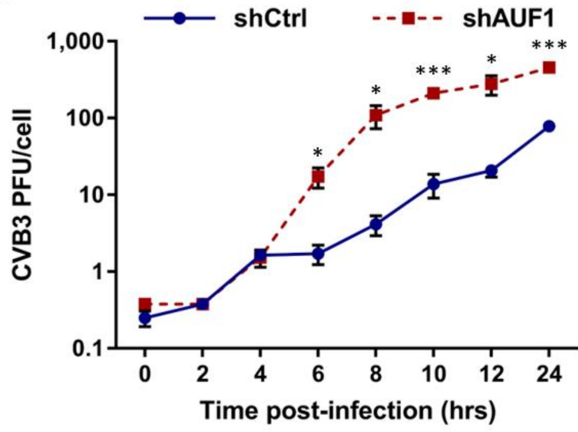


Poliovirus and CVB3 RNA synthesis is negatively regulated by AUF1

The effect of AUF1 on picornavirus RNA synthesis has been measured for CVB3 and EV71. AUF1 knockdown was shown to increase synthesis of CVB3 RNA in HeLa cells, while there was no effect on EV71 RNA synthesis in the human glioblastoma cell line, SF268 (138, 139). These apparently discordant results suggest that the negative impact of AUF1 on enterovirus replication may differentially affect viral RNA synthesis. To determine the effect of AUF1 on poliovirus and CVB3 RNA replication in HEK-293 cells, viral RNA was measured using quantitative reverse transcription PCR (RT-qPCR). Following infection by either poliovirus or CVB3, a significant increase in viral RNA synthesis was observed in 293-shAUF1 cells (Figure 2.2). The increase in viral RNA synthesis was detected by 4 h and 8 h post-infection for poliovirus and CVB3, respectively. These results demonstrate that AUF1 negative regulation of poliovirus and CVB3 infection results in decreased viral RNA synthesis. However, these results do not distinguish between a direct or indirect effect on viral RNA synthesis since restriction of other steps in the replication cycle or destabilization of viral RNA may contribute to the decrease in viral RNA.

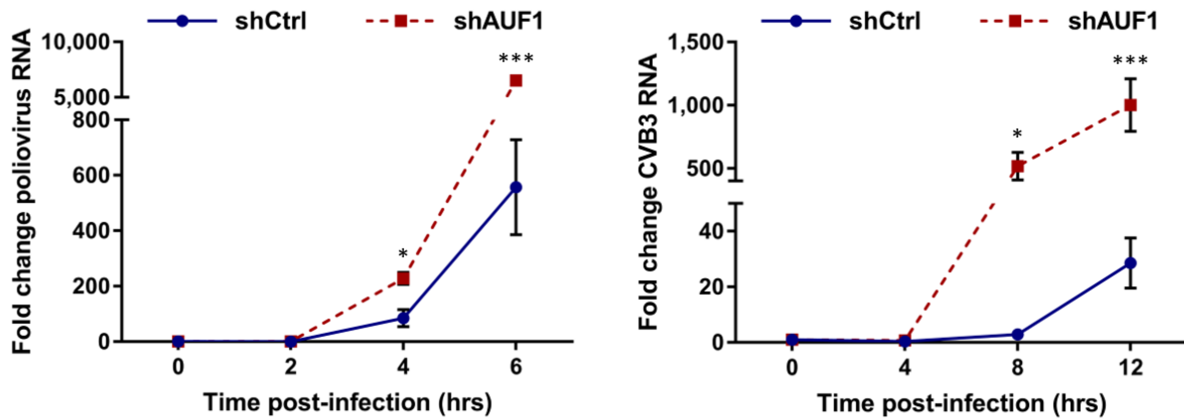


Figure 2.2. AUF1 inhibits poliovirus and CVB3 RNA synthesis. 293-shCtrl or -shAUF1 cells were infected with poliovirus (MOI 1) or CVB3 (MOI 20) and RNA was extracted at specified time points. Viral RNA synthesis relative to the 0 h time point for each cell line was analyzed by RT-qPCR using the ΔΔCt method. Data represent the mean of three individual experiments ± standard error of the mean (SEM). *, $P < 0.05$; *** $P, < 0.005$

AUF1 does not restrict poliovirus replication through its 3' non-coding region or promote viral RNA decay

AUF1 has been shown to bind the CVB3 3' NCR using a 3' NCR reporter construct expressed in HeLa cells. Based upon siRNA-mediated knockdown of AUF1 in HeLa cells and an indirect measurement of RNA stability, Wong and colleagues concluded that AUF1 may restrict CVB3 replication by promoting viral RNA degradation analogous to its role in mRNA decay (138). To determine whether AUF1 negatively regulates poliovirus replication through binding of the 3' NCR, a mutant poliovirus lacking the 3' NCR (Δ3' NCR) of its genomic RNA was utilized. Poliovirus Δ3' NCR is an infectious virus but exhibits a replication impairment due to a reduction in positive strand RNA synthesis (192, 197). Following infection by the Δ3' NCR virus, an approximately 10-fold increase in virus titer was measured in 293-shAUF1 cells, similar to the effect of AUF1 knockdown on wild type poliovirus (Figure 2.3). As previously reported, there were overall reduced virus yields and a delay in replication of the Δ3' NCR virus compared to wild type; however, the effect of AUF1 knockdown on replication of the Δ3' NCR virus demonstrates that AUF1 does not negatively regulate poliovirus through its 3' NCR. The effect of AUF1 on replication of CVB3 lacking its genomic 3' NCR could not be assessed due to lack of viability of the CVB3 Δ3' NCR virus (data not shown).

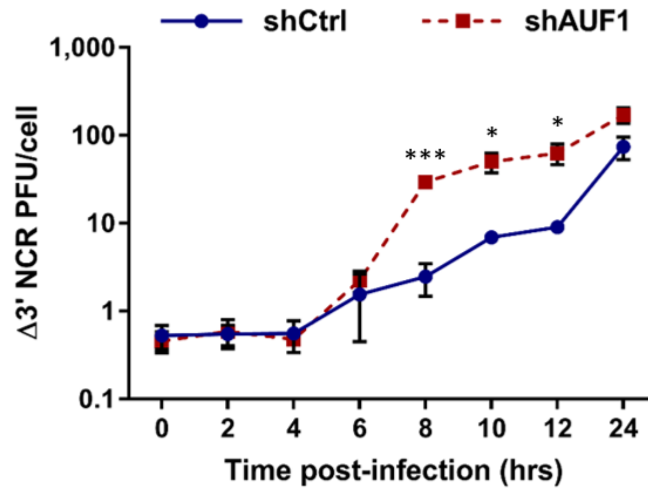


Figure 2.3. AUF1 does not negatively regulate poliovirus through its 3’ NCR. 293-shCtrl or -shAUF1 cells were infected with poliovirus lacking its 3’ NCR (Δ3’ NCR) at an MOI of 1. Cells and supernatant were harvested at specified time points and virus titer was determined by plaque assay on HeLa cells. Data represent the mean of three individual experiments ± standard error of the mean (SEM). *, $P < 0.05$; ***, $P < 0.005$

Although the 3' NCR of poliovirus genomic RNA is not involved in negative regulation of infection by AUF1, it is possible that AUF1 destabilizes viral RNA through interactions with RNA sequences outside of the 3' NCR. To directly measure the stability of poliovirus and CVB3 RNA during infection, viral RNA synthesis was inhibited using the adenosine analog, cordycepin (3'-deoxyadenosine), which when taken up by cells and phosphorylated to produce cordecypin triphosphate, acts as a chain terminator for RNA synthesis (198). Cordycepin has been previously shown to inhibit poliovirus and HRV-14 RNA synthesis and is more effective at inhibiting RNA synthesis at later times of infection compared to the commonly used inhibitor, guanidine hydrochloride (GuHCl) . Cells were infected with poliovirus (Figure 2.4A) or CVB3 (Figure 2.4B) and cordycepin was added to the culture media at 4 h or 6 h post-infection, respectively. Viral RNA synthesis was inhibited at a time during infection when there is both a detectable increase in viral RNA (refer to Figure 2.2) and AUF1 has relocalized to the cytoplasm (138, 140). Upon inhibition of viral RNA synthesis, the stability of viral RNA was analyzed for the next 6 h by RT-qPCR. Consistent with our previous results, in both the untreated poliovirus- and CVB3-infected 293-shAUF1 cells, viral RNA was synthesized at higher levels than in 293-shCtrl cells. However, when viral RNA synthesis was inhibited by cordycepin, there was no measurable difference between viral RNA stability in the 293-shCtrl or -shAUF1 cells for either poliovirus or CVB3. These data demonstrate that AUF1 does not restrict infection by these viruses through destabilization of viral RNA.

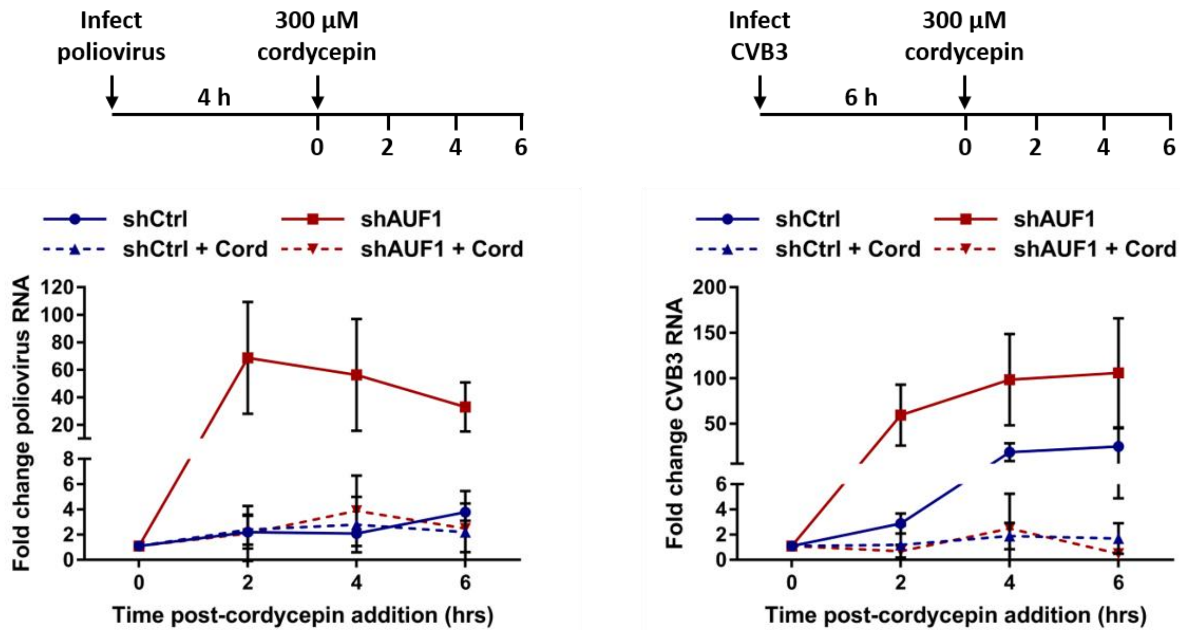


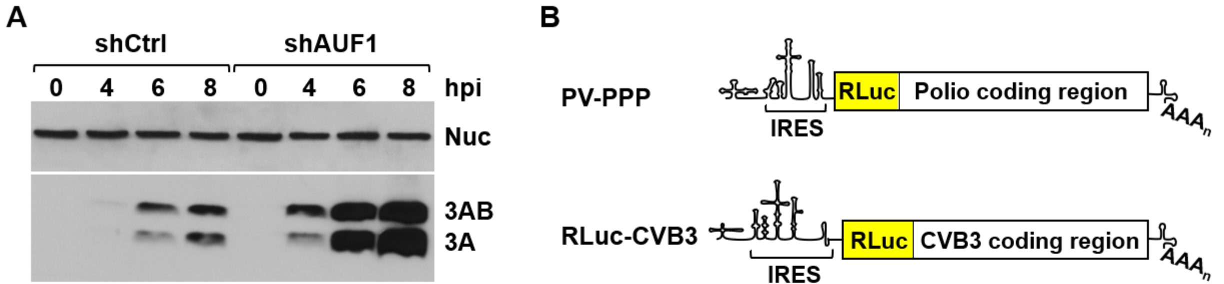
Figure 2.4. AUF1 does not promote poliovirus or CVB3 RNA decay. 293-shCtrl or -shAUF1 cells were infected with poliovirus (MOI 1) or CVB3 (MOI 20) and treated with vehicle (DMSO) or 300 µM cordycepin (cord) at the indicated times post-infection. RNA was extracted at 0, 2, 4, or 6 h post-cordycepin addition and the fold change in viral RNA relative to the 0 h time point for each cell line was determined by RT-qPCR using the ΔΔCt method. Data represent the mean of three or four individual experiments ± standard error of the mean (SEM).

AUF1 inhibits poliovirus and CVB3 translation during infection

The inhibitory effect of AUF1 on poliovirus translation was previously measured using *in vitro* translation assays (136). Given that cell-free assays are unable to recapitulate the nuclear-cytoplasmic partitioning of a host protein like AUF1, we turned to cell culture assays in the context of viral infection. Following infection of HEK-293 cells, viral protein accumulation was analyzed by Western blot. Production of viral protein 3A, and its precursor 3AB, revealed a clear increase in poliovirus translation in 293-shAUF1 compared to 293-shCtrl cells by 4 h post-infection (Figure 2.5A). Since the observed increase in viral translation may have been the result of increased viral RNA synthesis and subsequent translation of these progeny RNAs, *Renilla* luciferase-expressing poliovirus (PV-PPP) and CVB3 (RLuc-CVB3) were used to separate the effect of AUF1 on viral translation or RNA synthesis. (Figure 2.5B). In untreated cells, quantification of luminescence represented translation of both input and newly synthesized viral RNAs. To separate the two processes, viral RNA synthesis was inhibited using either GuHCl for poliovirus or GuHCl combined with enviroxime (Env) for CVB3 (199-201). Inhibition of viral RNA synthesis allowed for the measurement of translation from input viral RNA only, without the contribution of newly synthesized RNAs. Cells treated with RNA synthesis inhibitors were treated both during and after adsorption to ensure complete inhibition of viral RNA synthesis. Following infection by PV-PPP or RLuc-CVB3, luciferase activity was measured at 4 h and 8 h post-infection. In untreated cells, luciferase activity was higher in 293-shAUF1 cells following PV-PPP or RLuc-CVB3 infection (Figure 2.5C and D). Significantly, when viral RNA synthesis was inhibited following either PV-PPP or RLuc-CVB3 infection, there was no difference in luciferase activity measured from the 293-shCtrl or -shAUF1 cells (Figure 2.5C and D).

These data demonstrate that AUF1 negatively regulates poliovirus and CVB3 translation when infection is allowed to proceed normally but has no effect on translation of input viral RNA.

Figure 2.5. AUF1 restricts poliovirus and CVB3 translation during infection of HEK-293 cells. (A) Protein lysates were generated from 293-shCtrl or -shAUF1 cells infected with poliovirus (MOI 1) at 0, 4, 6, and 8 h post-infection (hpi) and analyzed for viral 3A/3AB expression by Western blot. Nucleolin was used as a loading control. (B) Schematic diagrams of PV-PPP (top) and RLuc-CVB3 (bottom) RNA. Both RNAs contain a full-length viral genome with the *Renilla* luciferase (RLuc) gene immediately following the 5' NCR and a 3C cleavage site to liberate RLuc from the viral polyprotein. (C) 293-shCtrl or -shAUF1 cells were infected with PV-PPP with or without 5 mM guanidine hydrochloride (GuHCl) treatment during and after adsorption. Luciferase activity was measured at 4 and 8 hpi and represented as relative light units per second (RLU/s) normalized to cell count. The mean of three individual experiments ± SEM is represented. (D) 293-shCtrl or -shAUF1 cells were infected with RLuc-CVB3 and treated with 5 mM GuHCl and 5 μg/ml enviroxime (Env) during and after adsorption. Luciferase activity was measured at 4 and 8 hpi and represented as relative light units per second (RLU/s) normalized to cell count. The mean of three individual experiments ± SEM is represented. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$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shCtrl
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0 4 6 8 0 4 6 8 hpi
Nuc
3AB
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PV-PPP
IRES
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Polio coding region
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CVB3 coding region
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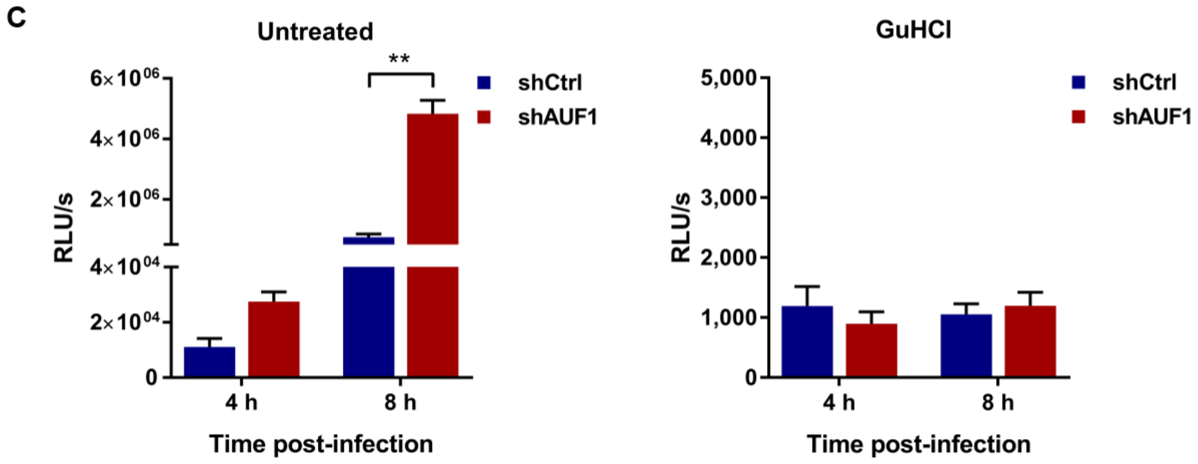
C
Untreated
GuHCl
shCtrl
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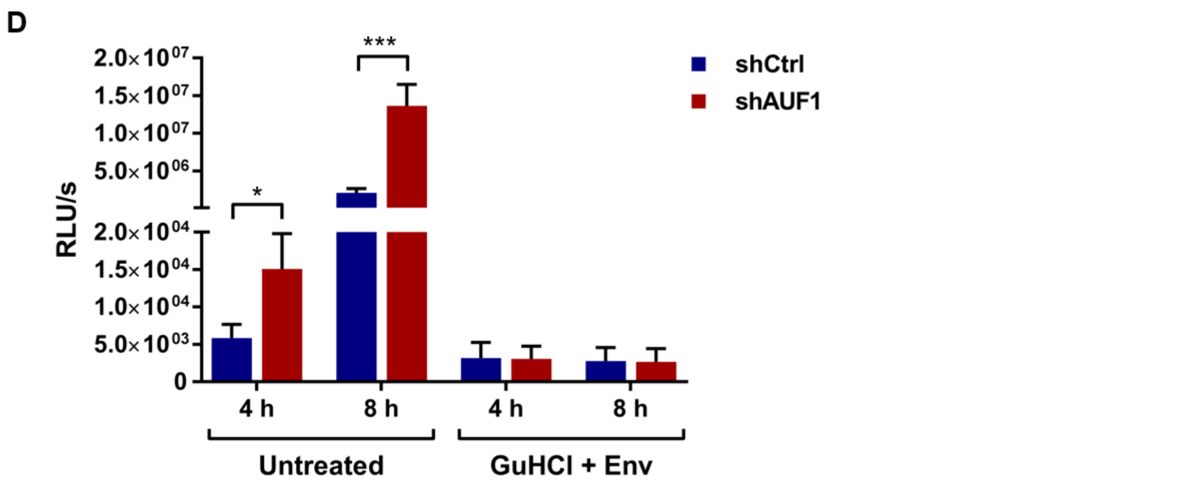
RLU/s
4 h
8 h
Time post-infection


D
shCtrl
shAUF1
RLU/s
4 h
8 h
Untreated
GuHCl + Env

Inhibition of viral RNA synthesis during infection leads to reduced accumulation of viral proteins in cells. Viral proteinase activity is required for the disruption of nucleocytoplasmic trafficking in infected cells, so low viral protein expression may prevent relocalization of nuclear proteins into the cytoplasm. Cytoplasmic relocalization of AUF1 occurs during poliovirus, CVB3, HRV, EV71, and EMCV infection and may contribute to its negative effect on virus replication (136, 137, 139-141). To determine whether inhibition of viral RNA synthesis prevents relocalization of AUF1, HEK-293 cells were infected with poliovirus with or without GuHCl treatment and AUF1 localization was analyzed by immunofluorescence assay (IFA) (Figure 2.6). Following poliovirus infection of untreated cells, AUF1 clearly translocated from the nucleus to the cytoplasm of infected cells at 4 h and 8 h post-infection. However, in cells where viral RNA synthesis was inhibited, viral protein expression was not detected by IFA and AUF1 remained predominantly in the nucleus. Taken together, these results suggest that AUF1 does not negatively regulate translation of input poliovirus or CVB3 RNA but impacts viral translation at later stages of infection. Furthermore, the relocalization of AUF1 as an unintended consequence of the disruption of nucleocytoplasmic trafficking appears to be required for its negative effect on virus replication.

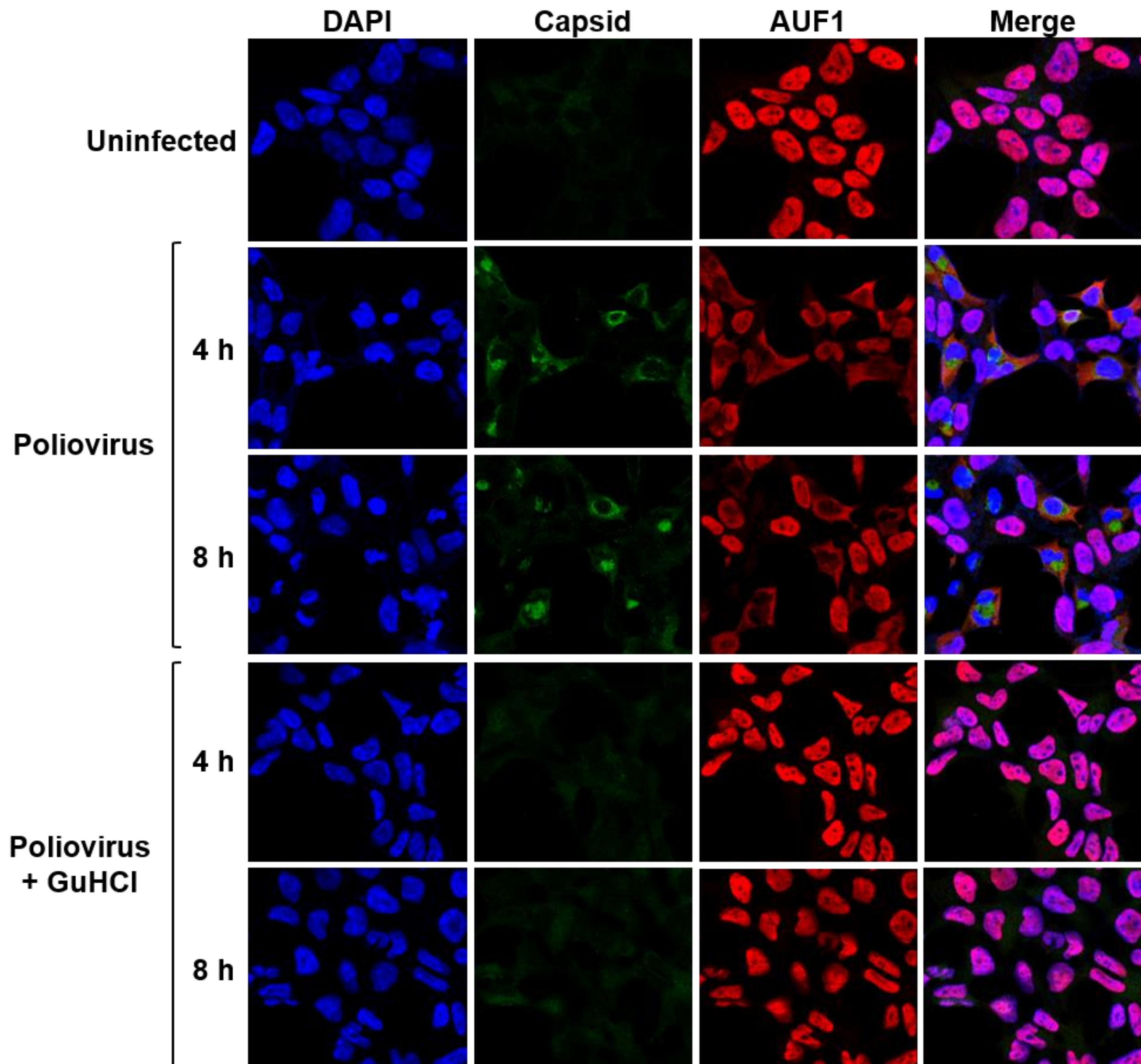


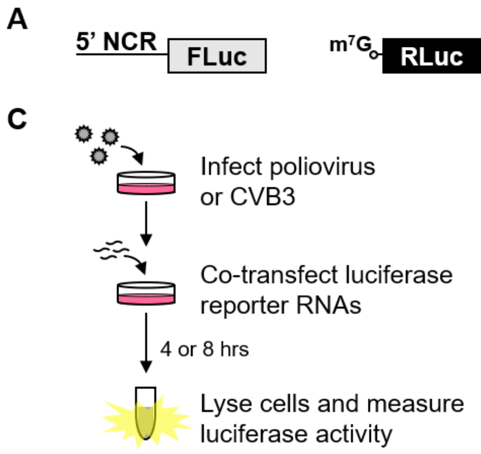
Figure 2.6. Inhibition of poliovirus RNA synthesis blocks nuclear-cytoplasmic relocalization of AUF1 during infection. HEK-293 cells were infected with poliovirus (MOI 1) with or without 5 mM GuHCl treatment during and after adsorption. Cells were fixed at 4 and 8 h post-infection and analyzed for AUF1 (red) and poliovirus capsid (green) localization by immunofluorescence assay. Nuclei were counterstained with DAPI (blue). Cells were imaged using confocal microscopy.

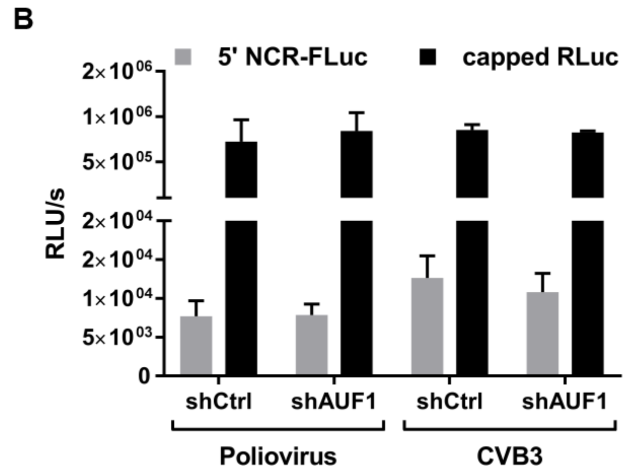
AUF1 negatively regulates poliovirus and CVB3 IRES-driven translation

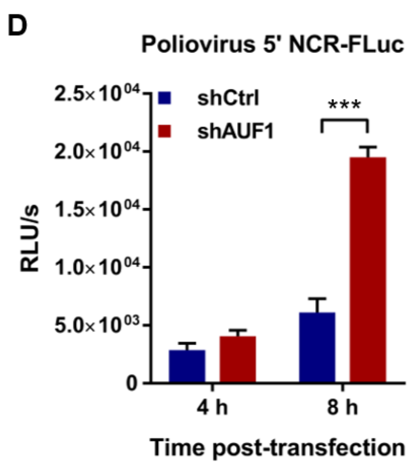
AUF1 has been previously shown to bind the 5' NCR of poliovirus, HRV, and EV71 RNA (136, 139, 140). Evidence that AUF1 binds to stem loop structures within the IRES of poliovirus and EV71 suggest that it may negatively regulate viral IRES-driven translation by acting as a negative ITAF, perhaps by competing with *bona fide* ITAFs for binding sites in the viral IRES (136, 139). The effect of AUF1 on poliovirus or CVB3 IRES-driven translation has not been measured in cells. To determine whether AUF1 negatively regulates the poliovirus or CVB3 IRES during infection, two different reporter RNAs were used. The first RNA harbors the 5' NCR of poliovirus or CVB3 upstream of sequences encoding firefly luciferase (5' NCR-FLuc), and the second encodes a control *Renilla* luciferase (RLuc) (Figure 2.7A). The 5' NCR-FLuc RNAs were *in vitro* transcribed in the absence of mRNA cap analog so that FLuc activity represented viral IRES-driven translation. The RLuc control RNA was *in vitro* transcribed in the presence of cap analog so that RLuc activity represented cellular cap-dependent translation. When uninfected cells were co-transfected with the 5' NCR-FLuc and RLuc RNAs, there were no differences in either viral IRES- or cap-driven translation between the 293-shCtrl and -shAUF1 cells (Figure 2.7B). To measure the effect of AUF1 on translation of the reporter RNAs during infection, cells were infected with poliovirus or CVB3 prior to co-transfection of the respective reporter RNAs (Figure 2.7C). Following infection, an increase in both poliovirus and CVB3 IRES-driven translation was observed in the 293-shAUF1 cells (Figure 2.7D and E). These results demonstrate that AUF1 negatively regulates poliovirus and CVB3 IRES-driven translation during infection, but not in uninfected cells. The inability of AUF1 to negatively regulate viral IRES-driven

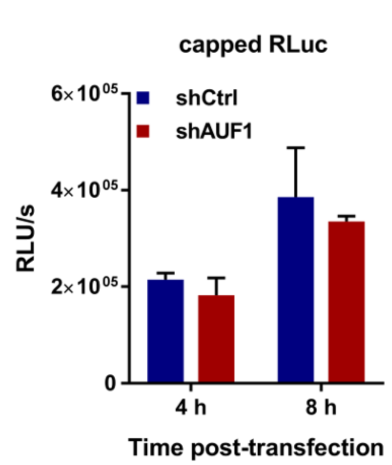
translation in uninfected cells provides strong evidence that relocalization of AUF1 is required for its activity as a restriction factor during picornavirus infection.

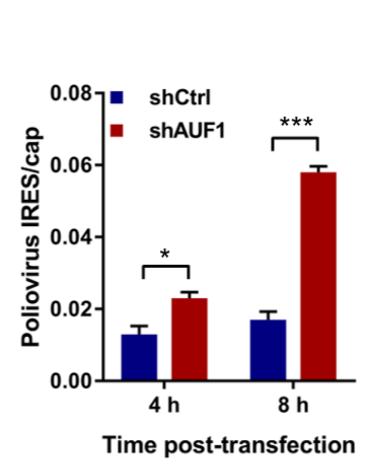
Figure 2.7. AUF1 inhibits poliovirus and CVB3 IRES-driven translation during infection. (A) Schematic of luciferase reporter RNAs. Viral IRES-driven translation was measured using *in vitro* transcribed RNA encoding the poliovirus or CVB3 5' non-coding region (5' NCR) upstream of firefly luciferase (FLuc). Cap-dependent translation was measured using a *Renilla* luciferase (RLuc) construct *in vitro* transcribed in the presence of $m^7G(5')ppp(5')G$ cap analog. (B) Viral IRES- and cap-dependent translation measured in uninfected cells 8 h after co-transfection of 293-shCtrl or -shAUF1 cells. FLuc and RLuc activities were measured using a dual luciferase assay and represented as relative light units per second (RLU/s) normalized to cell count. The mean of three individual experiments ± SEM is represented. (C) Schematic of infection followed by co-transfection experiment. 293-shCtrl or -shAUF1 cells were infected with poliovirus (MOI 1) or CVB3 (MOI 20). Immediately following virus adsorption, cells were co-transfected with the 5'NCR-FLuc and capped RLuc RNAs. At 4 h and 8 h post-transfection, cells were lysed and dual luciferase activity was measured. (D) 293-shCtrl or -shAUF1 were infected with poliovirus (MOI 1) prior to co-transfection with luciferase reporter RNAs. FLuc and RLuc activities were measured at 4 h and 8 h post-transfection and represented as relative light units per second (RLU/s) normalized to cell count. Data from co-transfections were graphed separately due to differences in scale and represent the mean of three individual experiments ± SEM. The ratios of IRES- to cap-dependent translation from these experiments are also presented. (E) 293-shCtrl or -shAUF1 cells were infected with CVB3 (MOI 20) prior to co-transfection with luciferase reporter RNAs. Results are presented the same as in *(D)*. *, $P < 0.05$; ***, $P < 0.001$


A
5' NCR
FLuc
m^7G
RLuc
C
Infect poliovirus or CVB3
Co-transfect luciferase reporter RNAs
4 or 8 hrs
Lyse cells and measure luciferase activity


B
5' NCR-FLuc
capped RLuc
RLU/s
shCtrl
shAUF1
shCtrl
shAUF1
Poliovirus
CVB3


D
Poliovirus 5' NCR-FLuc
shCtrl
shAUF1
***
RLU/s
4 h
8 h
Time post-transfection


capped RLuc
shCtrl
shAUF1
RLU/s
4 h
8 h
Time post-transfection


shCtrl
shAUF1
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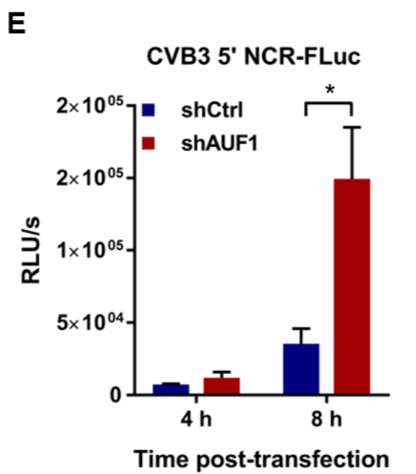
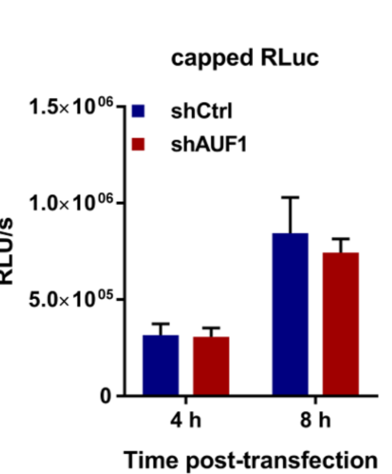
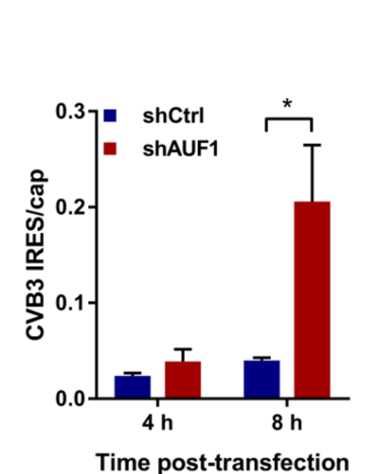
*
Poliovirus IRES/cap
4 h
8 h
Time post-transfection


E
CVB3 5' NCR-FLuc
shCtrl
shAUF1
*
RLU/s
4 h
8 h
Time post-transfection


capped RLuc
shCtrl
shAUF1
RLU/s
4 h
8 h
Time post-transfection


shCtrl
shAUF1
*
CVB3 IRES/cap
4 h
8 h
Time post-transfection

IV. Conclusion

The work presented in this chapter provides new mechanistic insights into how AUF1 acts as a host restriction factor during picornavirus infections of human cells. AUF1 normally participates in mRNA stability, translation, and telomere maintenance in the host cell; however, AUF1 has been primarily described as an mRNA decay protein (128, 202, 203). Following binding to AU-, U-, or GU-rich regions of RNA in the 3' NCR or introns of target transcripts, AUF1 promotes rapid deadenylation followed by decapping and degradation of the body of the mRNA (111-114, 128). It has been previously reported that AUF1 could bind to and promote the decay of a CVB3 3' NCR reporter RNA in HeLa cells (138). These results, although indirect, were interpreted to show that AUF1 destabilizes viral RNA through binding to the 3' NCR. Here, a mutant poliovirus lacking a 3' NCR (Δ3' NCR) in its genomic RNA was used to determine whether this region of RNA is required for AUF1 negative regulation of poliovirus infection. Knockdown of AUF1 resulted in increased replication of the Δ3' NCR virus similar to wild type poliovirus. These results demonstrate that the 3' NCR is not involved in AUF1 negative regulation of poliovirus infection. The contribution of the CVB3 3' NCR to AUF1 restriction of an authentic viral infection could not be analyzed due to the lack of viability of the CVB3 Δ3' NCR virus (data not shown). Since AUF1 may regulate viral RNA stability by binding to regions of RNA outside of the 3' NCR, a direct measurement of viral RNA stability was performed. Use of an RNA chain terminator to inhibit viral RNA synthesis at mid times of infection revealed that poliovirus and CVB3 RNAs were equally stable in 293-shCtrl or -shAUF1 cells. These data demonstrate that AUF1 negative regulation of infection occurs by a mechanism distinct from its role in mRNA decay.

The results presented here suggest that AUF1 negatively regulates poliovirus and CVB3 translation by acting as a negative ITAF during infection or by sequestering factors normally required for viral IRES-dependent translation. Viral IRES-driven translation was not affected by AUF1 in uninfected cells. Since AUF1 is predominantly a nuclear-resident protein in uninfected cells, or in infected cells treated with viral RNA synthesis inhibitors, low levels of AUF1 in the cytoplasm appear to be insufficient to negatively regulate translation initiated from the viral IRES. The disruption of nucleocytoplasmic trafficking during picornavirus infection results in a major relocalization of AUF1 into the cytoplasm, which has been demonstrated during poliovirus, CVB3, HRV, EV71, and EMCV infection. Once relocalized to the cytoplasm, AUF1 is present at sufficient levels to act as an inhibitor of viral translation (refer to the model shown in Figure 2.8). It has been previously reported that AUF1 knockdown does not affect translation from the EMCV IRES in uninfected HeLa cells, but translation was not measured during infection, when AUF1 has been shown to relocalize in these cells (132, 137). It has also been reported that AUF1 knockdown in the human glioblastoma cell line, SF268, resulted in increased EV71 IRES-driven translation following reporter RNA transfection. In contrast to the work presented here, these findings were determined in uninfected cells (139). The differences in the results presented here and by Lin and colleagues may be attributed to variations in AUF1 subcellular localization between cell types or differences in experimental design. Luciferase activity was measured at 4 h and 8 h post-transfection in the work presented here and at 2 days post-transfection by Lin and colleagues. Given a longer incubation time, it may be possible that low levels of cytoplasmic AUF1 have a cumulative effect on viral IRES-driven translation.

Figure 2.8. Proposed model for AUF1 negative regulation of poliovirus and CVB3 infection. Following poliovirus and CVB3 infection, disruption of the nuclear pore complex (NPC) by the viral 2A proteinase leads to relocalization of AUF1 (yellow circles) from the nucleus to the cytoplasm. Once in the cytoplasm, AUF1 restricts infection through inhibition of viral translation by acting as a negative ITAF. Inhibition of viral translation leads to decreased viral RNA synthesis and generation of progeny virions. A direct inhibition of viral RNA synthesis may also contribute to the negative effect of AUF1 on infection, although the involvement of AUF1 in RNA replication complexes has yet to be analyzed. Poliovirus and CVB3 may defend against restriction by AUF1 through proteolytic cleavage of AUF1 by viral proteinases. AUF1 is cleaved (yellow half circles) by the 3C proteinase during infection, which may ameliorate its negative effect on viral translation and enhance virus replication.

A possible mechanism by which AUF1 negatively regulates picornavirus IRES-driven translation is through competitive binding to the IRES with positive ITAFs. Evidence for this mechanism was previously demonstrated using the EV71 5' NCR, where AUF1 was shown to compete with another AU-rich element binding protein, hnRNP A1, for association with the viral IRES (139). This has not yet been shown for other picornaviruses negatively regulated by AUF1. AUF1 may also restrict picornavirus IRES-driven translation through indirect mechanisms. Following AUF1 knockdown, dysregulation of AUF1 target transcripts may contribute to increased IRES translation during infection. Additionally, the enhanced infection observed in AUF1 knockdown cells may drive IRES translation in a non-specific manner. While indirect mechanisms may be possible, multiple reports of direct interactions between AUF1 and picornavirus RNA, specifically the 5' NCR, support a direct mechanism for negative regulation.

Picornaviruses have evolved to extensively modify the host cell landscape to promote their replication and defend against restrictive cellular processes. Proteinases encoded by these viruses are employed to cleave cellular proteins, resulting in inhibition or modification of their activity. For example, the poliovirus, CVB3, and EV71 2A proteinases cleave MDA5 and MAVS, inhibiting viral RNA sensing and activation of the innate immune response (43). Picornaviruses may also inhibit restriction by AUF1 through proteolytic cleavage. AUF1 has been shown to be cleaved during poliovirus, CVB3, and HRV infection by the viral 3C proteinase and pre-incubation of recombinant AUF1 with 3C proteinase reduced its inhibitory effect on *in vitro* translation of poliovirus RNA (136, 138, 140). However, it is not yet clear whether the cleavage of AUF1 serves as a viral defense mechanism during infection (Figure 2.8).

In this study, it was shown that knockdown of AUF1 resulted in increased replication of poliovirus and CVB3 in a human cell model. Enhanced infection of poliovirus and CVB3 resulted in increased viral RNA synthesis and translation in AUF1 knockdown cells. These data demonstrated that the effect on viral RNA synthesis is largely indirect, due to the increased production of viral proteins required for replication in cells knocked down for AUF1 expression. In addition, direct evidence was provided to indicate that AUF1 does not exert its restrictive effects via alterations in RNA stability for either poliovirus or CVB3. Using 5' NCR reporter RNAs, it was shown that AUF1 negatively regulates poliovirus and CVB3 IRES-driven translation during infection but not in uninfected cells. These results suggest that the relocalization of AUF1 as an unintended consequence of the disruption of nucleocytoplasmic trafficking is required for its negative effect. Taken together, these findings reveal that AUF1 restricts viral translation subsequent to protein synthesis directed by input viral RNAs released from virions and that poliovirus and CVB3 encoded proteinases may serve as both the initiators of AUF1 antiviral defense activity and, ultimately, the destroyers of such activity.

CHAPTER 3

Mechanism of restriction by AUF1 and viral defense against restriction during poliovirus infection

I. Introduction

Picornaviruses are positive sense, single-stranded RNA viruses that replicate in the cytoplasm of infected cells through the actions of both viral and host proteins. Nuclear proteins required for replication are relocalized to the cytoplasm of infected cells following cleavage of nucleoporins by viral proteinases. As an unintended consequence of the disruption of nucleocytoplasmic trafficking, host proteins that have a negative impact on virus replication are also mislocalized. AUF1 is one such example of a negative regulator of picornavirus infection. Identified from an RNA affinity screen as a poliovirus 5' NCR-binding protein, it was later discovered that AUF1 relocalizes from the nucleus to the cytoplasm during infection by multiple picornaviruses including poliovirus, CVB3, HRV, and EMCV (136-141). Studies presented in **Chapter 2** and by others have demonstrated that knockout or knockdown of AUF1 results in increased replication of the enteroviruses poliovirus, CVB3, HRV1a, and EV71, but not the cardiovirus EMCV (136-139). In **Chapter 2**, it was shown that the mechanism of restriction of AUF1 during poliovirus and CVB3 infection involves negative regulation of viral IRES-driven translation and that the relocalization of AUF1 to the cytoplasm is required for its negative effect. Consistent with these results, AUF1 has also been shown to negatively regulate translation driven by the

EV71 IRES (139). The work presented in this chapter seeks to further probe the mechanism by which AUF1 negatively regulates poliovirus translation and whether the activity of AUF1 is inhibited during infection.

AUF1 is a member of a family of proteins that bind to AU-rich elements within RNA that are referred to as AU-rich element binding proteins (AUBPs). Once bound to an mRNA, the mechanism by which AUF1 regulates the stability or translation of that transcript remains largely unknown, but it has been proposed that AUF1 may act, in part, through competitive or cooperative binding for the target RNA with other AUBPs (123, 204). This mechanism may also be used by AUF1 to negatively regulate picornavirus translation. It has been shown that AUF1 competes for binding to the EV71 IRES with another AUBP and positive regulator of viral translation, hnRNP A1 (139, 150, 185, 205). Competitive binding for the viral IRES may be a mechanism shared by other picornavirus restriction factors. KHSRP, also an AUBP, may inhibit EV71 translation by competing for binding to the viral IRES with the positive regulator, FUBP1 (145, 184). Given these results, it is plausible that AUF1 restricts replication of other picornaviruses by competitive binding to the viral IRES with hnRNP A1 or other AUBPs that positively regulate viral translation. Using thiouracil crosslinking mass spectrometry, numerous AUBPs have been identified as binding to poliovirus genomic RNA (29). Several of the identified AUBPS have been shown to have positive roles in picornavirus replication, making them good candidates for a possible competitive interaction with AUF1 for binding to the 5’ NCR (Table 3.1).

Table 3.1. List of AU-rich element binding proteins (AUBPs) that bind to poliovirus genomic RNA. Proteins were identified by thiouracil crosslinking mass spectrometry (adapted from (29)). Previously described roles in enterovirus replication are listed for each protein.

AUBP	Role in enterovirus replication	Virus	Reference
AUF1/hnRNP D	Negative regulator of viral translation	Poliovirus, CVB3, HRV, EV71	(42, 136, 138)
hnRNP C	Positive regulator of viral RNA synthesis	Poliovirus	(206)
hnRNP K	Positive regulator of viral RNA synthesis or translation	EV71	(25)
hnRNP Q/SYNCRIP	Unknown		
HuR/ELAVL1	Positive regulator of viral IRES translation	EV71	(27)
Nucleolin	Positive regulator of viral IRES translation	Poliovirus, HRV	(207)
YB-1/YBX1	Unknown		
hnRNP A1	Positive regulator of viral IRES translation	EV71	(150, 185, 205)
KSRP	Negative regulator of viral translation	EV71	(145, 146)
TIA-1	Positive regulator of viral RNA synthesis	EV71	(208)

There are four isoforms of AUF1 that are expressed through alternative pre-mRNA splicing and are named based on their apparent molecular weights: p37, p40, p42, and p45 (124). All isoforms are composed of the same two non-identical RNA recognition motifs, glutamine-rich domain, and a dimerization domain. Variation between isoforms is generated by alternative inclusion or exclusion of exon 2 or 7 (Figure 3.1). Most studies of AUF1 have focused on its primary role in promoting mRNA decay. Each of the four isoforms of AUF1 has been reported to be involved in regulating the stability of specific transcripts, with the smallest isoform, p37, being the most studied (209). A large-scale investigation into the RNAs bound by AUF1 revealed that many targeted transcripts are bound by all four isoforms. Taken together, these data suggest that both overlapping and separate regulatory roles exist for each isoform (128). In addition, the two larger isoforms of AUF1, p42 and p45, have been shown to be involved in telomere maintenance both by binding to telomeric transcripts and by activating transcription of the catalytic subunit of telomerase (203, 210, 211). The contribution of each isoform to the negative impact of AUF1 on picornavirus replication has not been measured in cells; however, the isoform-specific effect of AUF1 on *in vitro* poliovirus translation has been measured. The addition of recombinant AUF1 to *in vitro* translation assays revealed that all four isoforms were able to inhibit translation of poliovirus RNA, with the p45 isoform having the greatest inhibitory effect and p42 the least (136).

Figure 3.1. Schematic of AUF1 isoforms. Isoforms of AUF1 are generated by alternative pre-mRNA splicing and named based on their apparent molecular weights (p37, p40, p42, p45). Each isoform contains the same two non-identical RNA recognition motifs (RRM), glutamine-rich domain (Q), and dimerization domain. Differences between isoforms result from alternative inclusion or exclusion of exons 2 or 7. Adapted from (209).

The cellular landscape is heavily modified during picornavirus infection, in large part due to the activity of viral proteinases. Proteolytic cleavage of host proteins is a strategy employed by picornaviruses to concomitantly direct cellular resources to favor virus replication and inhibit antiviral processes. The innate immune response to infection is the first line of defense against virus replication and as such, picornaviruses have evolved to inhibit this process at multiple stages. Viral proteinases cleave many proteins involved in this response including proteins involved in pathogen sensing (MDA5), signal transduction (MAVS), interferon expression (IRF-3/7), and proinflammatory cytokine production (IKKγ, p65/RelA) (39, 43, 44, 46-49, 52-54, 59-61). Picornavirus proteinases may also target host restriction factors for proteolysis. AUF1 has been shown to be cleaved by the 3C proteinase, or its precursor 3CD, during infection by poliovirus, CVB3, or HRV (138, 140). Pre-incubation of recombinant AUF1 with 3CD proteinase reduced its ability to bind the poliovirus IRES *in vitro*, suggesting that cleavage of AUF1 may ameliorate the negative effect on viral translation by disrupting its ability to bind viral RNA (140). Interestingly, AUF1 remains intact during infection by EMCV, a picornavirus belonging to a different genus that is not negatively regulated by AUF1 (137). While the cleavage of AUF1 is suggestive of a viral defense mechanism, it has not yet been determined whether its cleavage impacts virus replication.

This chapter seeks to explore both the mechanism by which AUF1 restricts viral translation and whether the cleavage of AUF1 reduces its ability to act as a restriction factor. To gain insight into the mechanism used by AUF1 to negatively regulate infection by the prototypic picornavirus, poliovirus, the isoform-specific effect of AUF1 on virus replication was determined, along with possible competitive interactions of AUF1 with

other AUBPs for binding to the viral 5' NCR. When expressed individually, all four isoforms of AUF1 were able to rescue inhibition of poliovirus replication, suggesting that the effect of AUF1 on picornavirus infection is not isoform-specific. Unlike the observations reported with EV71, AUF1 was not found to compete for binding to the poliovirus 5' NCR with hnRNP A1, suggesting that the mechanism used by AUF1 to inhibit translation may differ between viruses. Since the cleavage of AUF1 by viral proteinases may be a defense strategy used to limit the negative effect of AUF1 on infection, uncleavable AUF1 isoform constructs were used to measure the impact of cleavage on poliovirus replication. It was determined that the expression of uncleavable AUF1 did not enhance the inhibitory effect of each isoform on poliovirus infection. Finally, it was found that EMCV, a picornavirus previously thought to be unaffected by AUF1 during infection of mouse cells, can be negatively regulated by AUF1 in a cell type- or species-specific manner. Taken together, these results suggest that AUF1 cleavage may not serve as a defense mechanism used by all AUF1-sensitive picornaviruses.

II. Materials and Methods

Cell culture and viruses

HEK-293 and mouse embryonic fibroblast (MEF) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells were cultured in DMEM supplemented with 8% newborn calf serum (NCS). Both cell lines were maintained at 37°C, 5% CO_2. Virus stocks were produced in HeLa cells transfected with *in vitro* transcribed RNA generated from infectious cDNA clones. The

following clones were used to generate virus stocks: pT7PV1 for Mahoney strain poliovirus (190), pCVB3-0 for CVB3 Nancy strain (191), and pEC9 for EMCV (212).

AUF1 knockdown

HEK-293 cells were transfected with linearized pSilencer/U6/tetO/shCtrl or pSilencer/U6/tetO/shAUF1 using jetPRIME reagent (Polyplus-transfection) and selected for stable expression using hygromycin B (Calbiochem) as previously described (193).

Virus infections

HEK-293 or MEF cells were infected with poliovirus at a multiplicity of infection (MOI) of 1 or EMCV at an MOI of 5. Virus was diluted in serum-free DMEM and adsorbed for 30 (poliovirus) or 40 (EMCV) min at room temperature. Following adsorption, cells were overlaid with DMEM supplemented with 10% FBS and incubated at 37°C, 5% CO_2. Cells and supernatant were harvested at specified time points post-infection and four freeze-thaw cycles were performed prior to titration by plaque assay on HeLa cells. Virus titers were normalized to cell count and represented as plaque forming units per cell (PFU/cell). Values represent the means of triplicate experiments ± standard error of the mean (SEM). Statistical significance was measured by unpaired Student's t-test.

Western blot

Proteins were extracted from HEK-293 or MEF cells using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail), and Bio-Rad Protein Assay Dye Reagent was used to determine concentration. Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% resolving gel, 5% stacking gel) and transferred to an Immobilon-P Membrane (Millipore).

Membranes were blocked with 5% non-fat milk in phosphate buffered saline with Tween-20 (PBST) followed by incubation with rabbit polyclonal anti-AUF1 (1:1,000; Millipore), rabbit polyclonal anti-nucleolin (1:1,000; Abcam), mouse monoclonal anti-Flag (1:1,000; Sigma), mouse monoclonal pan-enterovirus anti-capsid (1:1,000; Dako), or rabbit polyclonal anti-EMCV 3C (1:2,000; gift from T. Glen Lawson) antibodies diluted in PBST with 5% bovine serum albumin (BSA) (213). Membranes were washed 3 times with PBST followed by incubation with a 1:4,000 dilution of goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG-heavy and light chain secondary antibody (Bethyl) diluted in PBST with 5% BSA. Membranes were washed 3 times with PBST followed by exposure to ECL Western Blotting Reagent (Pierce) for chemiluminescent detection of HRP.

shRNA-resistant and uncleavable AUF1 isoform construct generation

AUF1 isoform constructs were provided by Robert Schneider (New York University School of Medicine) and cloned into a pFLAG-CMV2 mammalian expression vector for N-terminal flag-tagging of each isoform. Mutagenesis of each construct was performed using the Q5 Site-Directed Mutagenesis Kit (New England BioLabs) and primers were designed using the NEBaseChanger tool (New England BioLabs). Primers were designed to render each isoform resistant to silencing by shAUF1 based on a previous description (214) and were as follows: forward 5’-ATTGTACCCTAAAGTTAGATCCTATC-3’ and reverse 5’-CTACCACTTCACCAAATTTGGAAAAG-3’. shRNA-resistant constructs were then used to generate “uncleavable” constructs that were resistant to cleavage by the 3C/3CD proteinase. The glutamine-glycine cleavage pair in the dimerization domain of each construct was mutated to an isoleucine-aspartic acid pair based on previously reported results (140). Primers designed for this mutagenesis were as follows:

5'-GGCGGCGACAattgatGCAGCGGCGGC-3' and reverse 5'-ACCATGGCTCCCTCCTGC-3'.

AUF1 isoform expression and infection

To analyze wild type or uncleavable AUF1 isoform expression in uninfected cells, 293-shCtrl and -shAUF1 cells were plated 3 x 10^5 cells/well of 6-well plates in DMEM + 10% FBS. The next day, cells were transfected with 2 µg/well of plasmid DNA using Lipofectamine 3000 Transfection Reagent (Invitrogen) and 1 µg/ml doxycycline (Sigma) was added to the growth media to knockdown endogenous AUF1 expression. Vector (pFlag-CMV2)-transfected 293-shCtrl or -shAUF1 cells were used as controls. To measure the effect of each AUF1 isoform on poliovirus replication, 293-shCtrl or -shAUF1 cells were plated 2 x 10^5 cells/well of 6-well plates in DMEM containing 10% FBS and 1 µg/ml doxycycline. 24 hours after plating, cells were transfected with 2 µg/well of plasmid DNA using Lipofectamine 3000 and incubated for 36 h prior to infection. Cells were infected with poliovirus at an MOI of 1 or 10 as described above. After virus adsorption, cells were overlaid with DMEM containing 10% FBS and 1 µg/ml doxycycline. Six hours post-infection, cells and supernatant were harvested to determine virus titer by plaque assay on HeLa cells. Cell lysates were also prepared for Western blot analysis as described above.

Biotinylated RNA pulldowns

The poliovirus 5' NCR firefly luciferase reporter construct (p5'PVLuc) used in Chapter 2 was mutated to insert a ClaI restriction enzyme site immediately following the 5' NCR. Mutagenesis was performed using the Q5® Site-Directed Mutagenesis Kit (New England BioLabs) and primers were designed using the NEBaseChanger™ tool (New England BioLabs) as follows: forward 5'-ATCATACCATCGATGACGCCAAAAAC-3' and reverse 5'-ACAATTGTCTGATTGAAATAAC-3'. Linearization by ClaI digestion resulted in removal of

the firefly luciferase gene and allowed for *in vitro* transcription of only the 5' NCR from the T7 promoter. RNA was generated from ClaI-linearized p5'PVLuc using the MEGAscript T7 Transcription Kit (Ambion). To generate biotinylated RNA, a ratio of 1 biotin-14-CTP (Invitrogen) to 4 CTP was used in the *in vitro* transcription reaction. RNA affinity assays were performed by incubating 3 μg RNA with 200 μg cell lysate prepared using polysome extraction buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$, 0.5% NP-40, Pierce™ Protease Inhibitor Tablet EDTA-free [ThermoFisher Scientific]), RNasin (Promega) RNase inhibitor, and RNA mobility shift buffer (5 mM HEPES [pH 7.1], 40 mM KCl, 2 mM MgCl2, 0.1 M EDTA, 2 mM dithiothreitol) for 15 min at 30°C followed by 15 min at room temperature. RNA-protein complexes were isolated using 100 μl of pre-washed Hydrophilic Streptavidin Magnetic Beads (New England BioLabs) incubated for 10 min at room temperature and washed 5 times using RNA mobility shift buffer. Proteins samples were prepared by heating beads to 95°C for 5 min in 1X Laemmli sample buffer (63 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, 0.0005% bromophenol blue, 10% glycerol). Proteins were resolved by SDS-PAGE and Western blot analysis performed as described above. For detection of AUBPs, membranes were incubated with 1:1,000 dilutions of anti-hnRNP A1 (1:1,000; Abcam), anti-hnRNP Q (1:1,000; Invitrogen), or anti-hnRNP K (1:1,000; Abcam) antibodies diluted in PBS + 5% BSA.

III. Results

All isoforms of AUF1 negatively regulate poliovirus infection

The individual contribution of each isoform of AUF1 to its negative impact on picornavirus infection has not yet been determined. Addition of recombinant AUF1 isoforms to *in vitro* translation assays revealed that each isoform of AUF1 was able to inhibit translation of poliovirus RNA (140). These data suggest that all isoforms may participate in restriction of infection. To measure the isoform-specific effect of AUF1 on replication of poliovirus, individual flag-tagged AUF1 isoform constructs were transiently expressed in HEK-293 cells stably expressing a tetracycline-inducible shRNA that targets all four isoforms of AUF1 (shAUF1). To ensure that the exogenously expressed isoforms were not targeted by shAUF1, silent mutations were generated in each construct to render them resistant to knockdown. Over a 90% knockdown of endogenous AUF1 was achieved by tetracycline-induced expression of shAUF1 (fFigure 3.2A). The high degree of endogenous AUF1 knockdown ensured that the flag-tagged isoforms were the dominant form of AUF1 expressed in the cells. Individual AUF1 isoform constructs were expressed in 293-shAUF1 cells for 36 h prior to infection by poliovirus (Figure 3.2B). Virus from cells and supernatant was harvested 6 h post-infection (hpi) and virus titer was determined by plaque assay (Figure 3.2C). As expected, poliovirus titer increased in 293-shAUF1 cells expressing vector only (V) relative to the 293-shCtrl cells. Expression of each AUF1 isoform individually in 293-shAUF1 cells resulted in virus titers similar to 293-shCtrl cells, demonstrating a near complete rescue of AUF1 inhibition of poliovirus replication. These results indicate that all four isoforms may contribute to the negative effect of AUF1 on poliovirus infection.

Figure 3.2. All four isoforms of AUF1 inhibit poliovirus replication. (A) Western blot analysis of AUF1 protein expression in HEK-293 cells stably expressing tetracycline-inducible control (shCtrl) or AUF1 (shAUF1) targeting shRNAs. Cells were treated with 1 μg/ml doxycycline (a tetracycline derivative) for 48 h prior to harvesting cell lysates. The four isoforms of AUF1 (p37, p40, p42, and p45) are labeled and nucleolin was used as a loading control. NS, non-specific band. (B) 293-shAUF1 cells were treated with 1 μg/ml doxycycline and transfected with individual flag-tagged AUF1 isoform constructs for 36 h before analyzing isoform expression by Western blot. Nucleolin was used as a loading control. (C) 293-shCtrl and -shAUF1 cells were treated with 1 μg/ml doxycycline for 24 h prior to transfection with vector or individual flag-tagged AUF1 isoform constructs. 293-shCtrl cells were transfected with vector only. Constructs were expressed for 36 h prior to infection by poliovirus at a multiplicity of infection of 1 (MOI 1). Cells and supernatant were harvested 6 h post-infection (hpi) and virus titer was determined by plaque assay on HeLa cells. Virus titer was normalized to cell count and referred to as plaque forming units per cell (PFU/cell).

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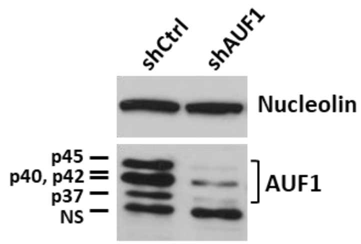

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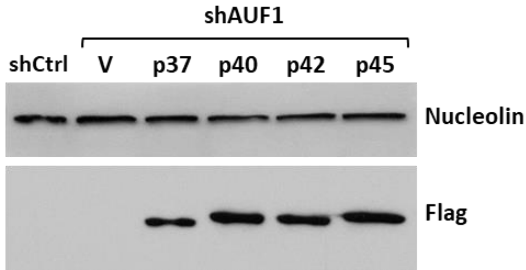

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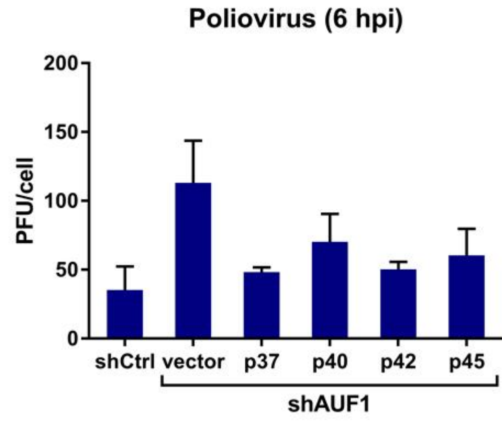


AUF1 does not compete for binding to the poliovirus 5' NCR with multiple AUBPs

AUF1 belongs to a family of RNA binding proteins called AU-rich element binding proteins (AUBPs) that bind to similar sites in host RNA. AUF1 is known to cooperatively or competitively bind to RNA with other AUBPs and competition for binding to the EV71 5' NCR has already been demonstrated for the AUBP, hnRNP A1 (139). Since hnRNP A1 is a positive regulator of EV71 translation, it was proposed that AUF1 negatively regulates EV71 infection by competing with hnRNP A1 for binding to the viral IRES. In a subsequent study, it was shown that AUF1 does not compete for binding to the EV71 5' NCR with another AUBP, HuR, which is also a positive regulator of viral translation and binds to the same RNA stem loop within the viral IRES (27). Since numerous AUBPs have been identified as binding to poliovirus genomic RNA, it is possible that AUF1 negatively regulates poliovirus translation by competing with one or more of these AUBPs for binding to the 5' NCR (Table 3.1) (29).

To determine if AUF1 competes for binding to the poliovirus 5' NCR with other AUBPs, RNA affinity pulldowns were performed. A poliovirus 5' NCR construct was *in vitro* transcribed in the presence or absence of biotinylated CTP and the resulting RNA was incubated with cell lysates prepared from mock- or poliovirus-infected 293-shCtrl or -shAUF1 cells. Ribonucleoprotein complexes were captured using magnetic streptavidin beads and bound proteins were analyzed by Western blot. Possible candidates for competitive binding with AUF1 include the known positive regulators hnRNP A1 and hnRNP K, along with an AUBP having an unknown function, hnRNP Q. From uninfected 293-shCtrl lysate, biotinylated poliovirus 5' NCR pulled down all 4 isoforms of AUF1 when compared to background (Figure 3.3, compare lanes 5 and 9). Background binding was determined by incubating

non-biotinylated poliovirus 5' NCR with cell lysates, followed by the same pulldown procedure used for biotinylated RNA. Using lysate prepared from 293-shCtrl cells infected with poliovirus for 4 hours, the predominant isoform bound to the poliovirus 5' NCR appeared to be the smallest isoform, p37 (Figure 3.3, lanes 6 and 10). Binding of the AUF1 cleavage products to the 5' NCR could not be detected. HnRNP A1 was only pulled down by the poliovirus 5' NCR from 293-shCtrl lysates, and binding appeared to increase following infection (Figure 3.3, lanes 5 and 7). These results differ from the EV71 5' NCR study, which showed that addition of recombinant AUF1 (p40) resulted in decreased hnRNP A1 binding to the EV71 5' NCR (139). Based on these results, it could be expected that hnRNP A1 binding to the poliovirus 5' NCR would increase in lysates prepared from AUF1 knockdown cells. Instead, little to no hnRNP A1 was pulled down from mock or infected 293-shAUF1 lysates (Figure 3.3, lanes 6 and 8). These results demonstrate that AUF1 may cooperatively, or at least non-competitively, bind with hnRNP A1 to the poliovirus 5' NCR. In contrast to EV71, hnRNP A1 is unlikely to contribute to the increase in poliovirus translation following AUF1 knockdown.

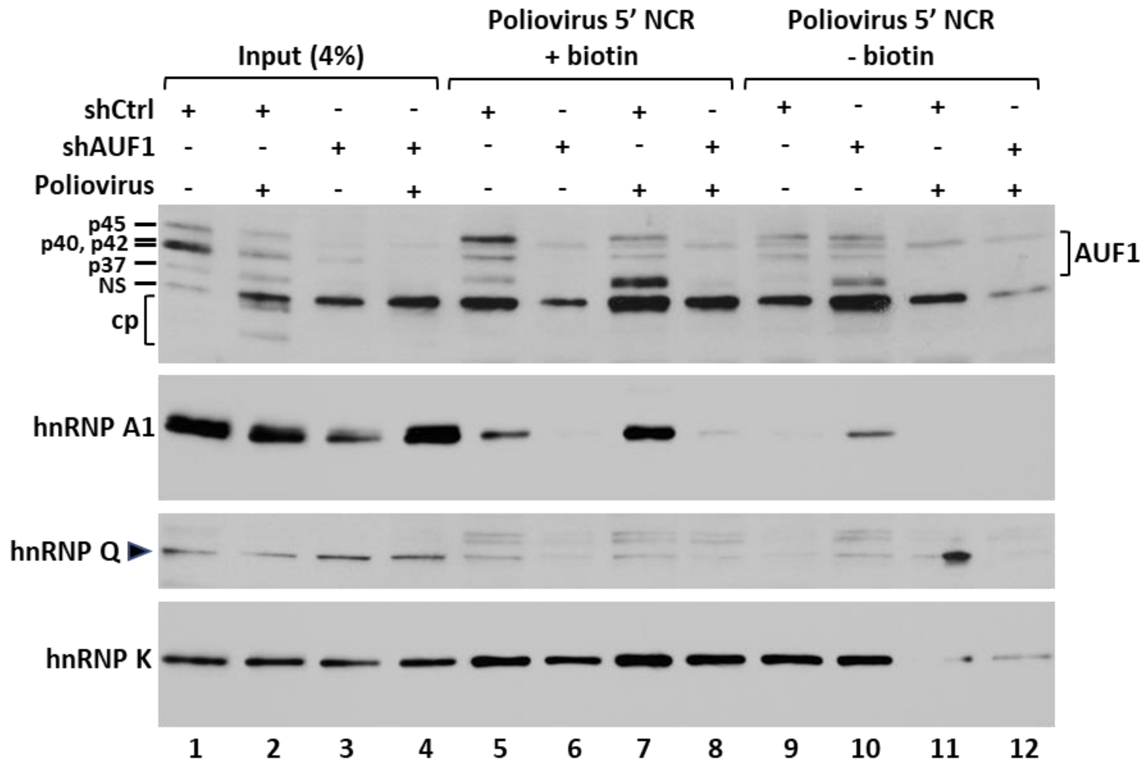


Figure 3.3. AUBP interactions with the poliovirus 5' NCR. Cell lysates were prepared from 293-shCtrl and -shAUF1 cells either mock- or poliovirus-infected (MOI 10) for 4 hours. RNA affinity pulldowns were performed by incubating 200 μg cell lysate with 3 μg poliovirus 5' NCR RNA which was *in vitro* transcribed in the presence or absence of biotin-14-CTP (+/- biotin). Protein-RNA complexes were isolated using magnetic streptavidin beads and bound proteins were analyzed by Western blot. The four isoforms of AUF1 (p37, p40, p42, and p45) are indicated as well as AUF1 cleavage products (cp) formed during infection. NS, non-specific band.

Results aimed at determining whether hnRNP Q or hnRNP K bind to the poliovirus 5' NCR were less clear using this technique. HnRNP Q was weakly detected in the input samples (Figure 3.3, lanes 1-4) and was not pulled down by the poliovirus 5' NCR above background (Figure 3.3, compare lanes 5-8 to 9-12). This result may be due to the low expression level of this protein, poor antibody affinity leading to weak detection, or that hnRNP Q does not bind the poliovirus 5' NCR. HnRNP K was pulled down from all cell lysates incubated with biotinylated 5' NCR and shows no indication of a competitive interaction with AUF1 (Figure 3.3, lanes 5-8); however, it is unclear whether these results reflect true binding since similar levels of hnRNP K were also detected in the 293-shCtrl background samples (Figure 3.3, lanes 9 and 10). The low background levels of hnRNP K detected using either the 293-shCtrl or -shAUF1 infected lysates suggest that the poliovirus 5' NCR may bind hnRNP K during infection; however, the high background from samples from uninfected cells limits the confidence with which this assessment can be made.

AUF1 is not cleaved during infection by all AUF1-sensitive picornaviruses

AUF1 has been shown to be cleaved during infection of the human cell line, HeLa, by poliovirus, CVB3, and several human rhinoviruses (HRV1a, HRV14, and HRV16) (136, 138, 140). Since these viruses are sensitive to inhibition by AUF1, it has been proposed that proteolytic cleavage by viral proteinases may serve as a viral defense mechanism to ameliorate its negative effect. Supporting this hypothesis, it has also been shown that AUF1 is not cleaved during EMCV infection of HeLa cells and that AUF1 does not restrict replication of this virus in mouse embryonic fibroblasts (MEFs) (137). Presumably, cleavage of AUF1 by EMCV would not be required since it does not appear to impact virus replication in mouse cells. Since cleavage of AUF1 has not been demonstrated in human cell

lines other than HeLa cells, HEK-293 cells were infected with poliovirus or CVB3 and the appearance of cleavage products was analyzed by Western blot. Consistent with results observed in HeLa cells, AUF1 cleavage products were clearly visible by 6 h post-poliovirus infection (Figure 3.4A). By 8 h post-infection, much of the full length AUF1, particularly the larger isoforms, had disappeared. Since signal from the cleavage products does not appear to increase as full-length proteins decrease, it appears that cleavage of AUF1 may also lead to its degradation during poliovirus infection. AUF1 cleavage products do not form until later times during CVB3 infection since the life cycle of this virus in HEK-293 cells is protracted relative to poliovirus. Low levels of cleavage products can be seen at 8 h post-infection, with obvious cleavage and loss of full-length protein at 24 h post-infection (Figure 3.4B). These data show that cleavage of AUF1 by poliovirus and CVB3 is not limited to one human cell line and may be a generalized phenomenon for these viruses.

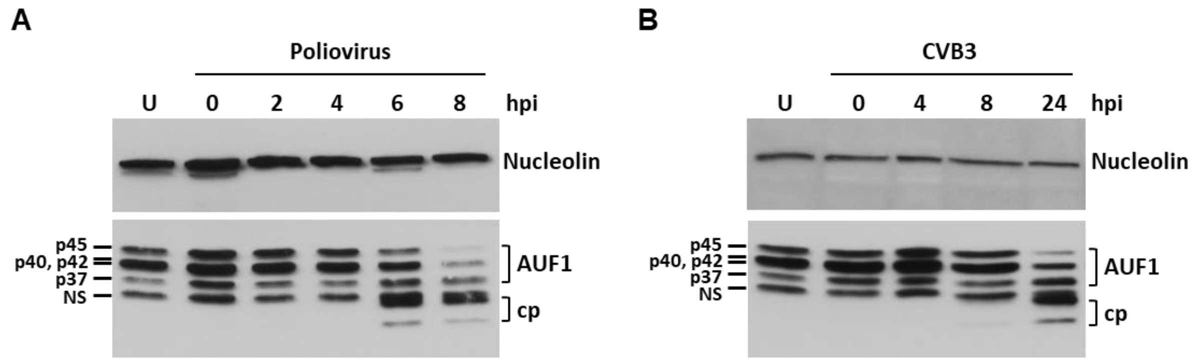
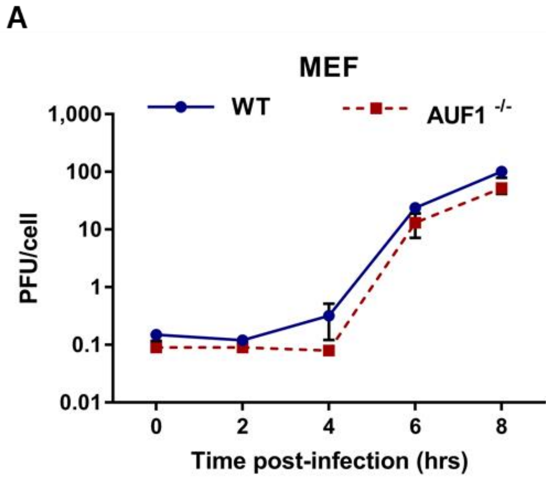


Figure 3.4. AUF1 is cleaved during infection of HEK-293 cells by poliovirus or CVB3. (A and B) HEK-293 cells were infected with poliovirus at an MOI of 10 (A) or CVB3 at an MOI of 20 (B), cell lysates were harvested indicated hours post-infection (hpi), and proteins were analyzed by Western blot. Nucleolin was used as a loading control and both full length isoforms (p37, p40, p42, and p45) and AUF1 cleavage products (cp) are indicated.

The effect of AUF1 on replication of EMCV in human cells has not been measured. EMCV, a picornavirus belonging to a different genus than poliovirus or CVB3, is an animal pathogen but can infect humans, typically asymptomatically. For comparison, 293-shCtrl and -shAUF1 cells were infected with EMCV alongside wild type (WT) or AUF1 knockout ($AUF1^{-/-}$) MEFs at identical MOIs. Surprisingly, while AUF1 did not negatively regulate EMCV infection in MEFs (Figure 3.5A), consistent with the report from Cathcart and Semler (137), AUF1 did inhibit infection by EMCV in HEK-293 cells (Figure 3.5C). AUF1 is not cleaved during EMCV infection of either MEFs or HEK-293 cells, despite its inhibitory effect on EMCV replication in HEK-293 (Figure 3.5B and D). These results demonstrate that AUF1 differentially regulates EMCV infection in a cell type- or host species-specific manner. Furthermore, cleavage of AUF1 does not occur during infection by all AUF1-sensitive picornaviruses.

Figure 3.5. Differential regulation of EMCV infection by AUF1 in mouse and human cells. (A) Wild type (WT) or AUF1 knockout (AUF1$^{-/-}$) mouse embryonic fibroblasts (MEFs) were infected with EMCV at an MOI of 5. Cells and supernatant were harvested at indicated time points and virus titer was determined by plaque assay on HeLa cells and normalized to cell count as plaque forming units per cell (PFU/cell). (B) WT MEFs were infected with EMCV at an MOI of 5, cell lysates were harvested at indicated hours post-infection (hpi), and proteins were analyzed by Western blot. Nucleolin was used as a loading control and EMCV infection was confirmed by detection of the viral 3C protein. U, uninfected (C) 293-shCtrl or -shAUF1 cells were infected with EMCV at an MOI of 5. Cells and supernatant were harvested at indicated time points and virus titer was determined by plaque assay on HeLa cells and normalized to cell count. (D) Parental HEK-293 cells were infected with EMCV at an MOI of 5, cell lysates were harvested at indicated hours post-infection (hpi), and proteins were analyzed by Western blot. Nucleolin was used as a loading control and EMCV infection was confirmed by detection of the viral 3C protein. Virus growth curves represent the mean of three individual experiments ± standard errors of the means (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$


A
MEF
WT
AUF1 -/-
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1
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PFU/cell
0
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6
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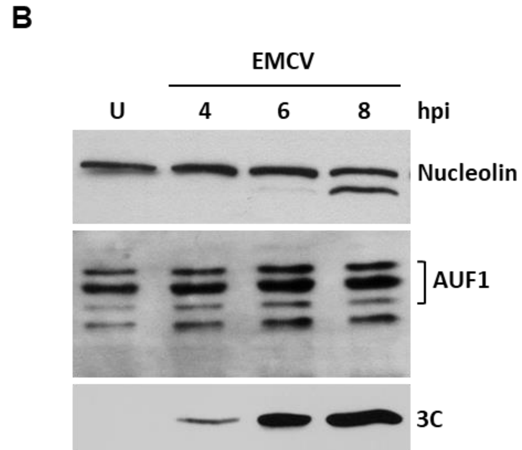
Time post-infection (hrs)


B
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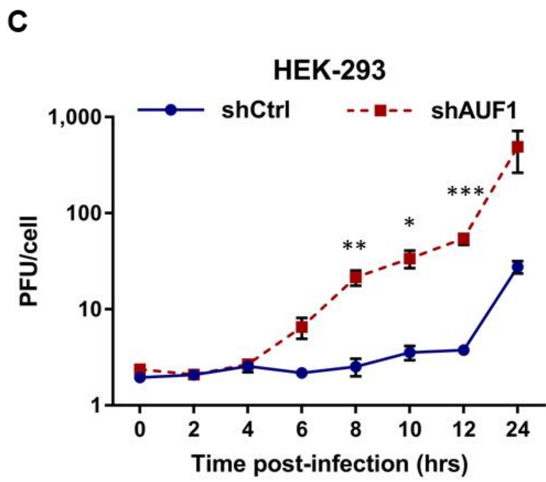
C
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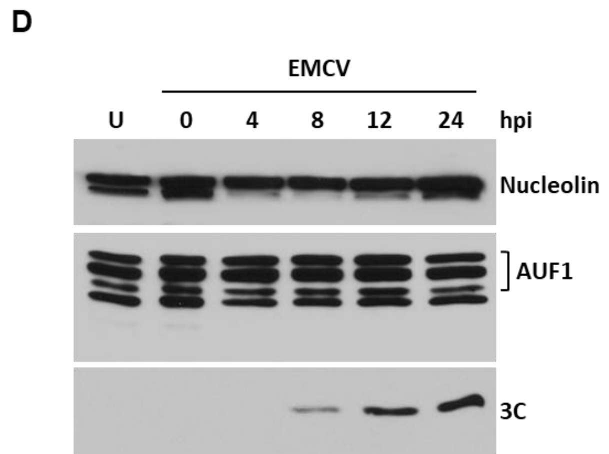
D
EMCV
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0
4
8
12
24
hpi
Nucleolin
AUF1
3C

Expression of uncleavable AUF1 does not enhance restriction of poliovirus infection

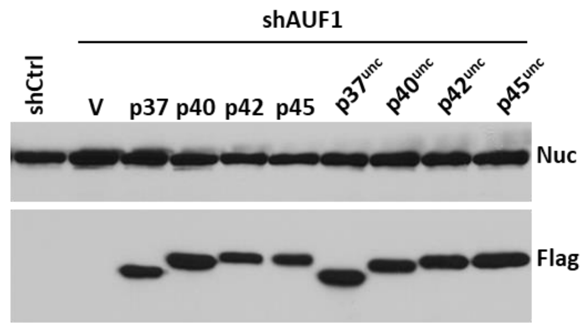
It has been proposed that the cleavage of AUF1 by the viral 3C/3CD proteinase may serve as a defense mechanism used by AUF1-sensitive picornaviruses to ameliorate its negative effect on virus replication. To measure the impact of AUF1 cleavage on poliovirus replication, flag-tagged, shRNA resistant AUF1 isoform constructs were mutated to render their 3C/3CD cleavage site uncleavable. The poliovirus 3C/3CD cleavage site in AUF1 has been previously determined and is located near the N-terminal end of all isoforms in the dimerization domain (Figure 3.6A). Mutation of the glutamine-glycine (Q-G) amino acid pair at the scissile bond site to isoleucine-aspartic acid (I-D) rendered AUF1 uncleavable by recombinant poliovirus 3CD (140). Cleavable and uncleavable isoform constructs were expressed in 293-shAUF1 cells to limit the impact of endogenous AUF1 on subsequent analyses. Mutation of the 3C/3CD cleavage site had no impact on expression of any of the isoform constructs (Figure 3.6B). Cleavable or uncleavable AUF1 isoform constructs were expressed in 293-shAUF1 cells for 36 h prior to infection by poliovirus. Cell lysates were harvested 8 hpi and isoform cleavage was analyzed by Western blot (3.6C). An MOI of 10 was used to ensure that all cells were infected. In infected cells expressing cleavable AUF1 isoforms, cleavage products could not be detected by Western blot using an antibody to detect the N-terminal flag tag. This was not an unexpected result since 3C/3CD cleavage of the N-terminal portion of AUF1 would result in loss of the flag tag and the generation of a low molecular weight peptide. However, loss of full-length protein, particularly the p42 and p45 isoforms, can be seen relative to the uncleavable protein levels. The loss of full length protein is likely the result of viral 3C/3CD activity which may lead to protein degradation. When compared to the Western blot analysis of endogenous AUF1 8 hpi (Figure 3.4A), it

was clear that cleavage of the exogenously expressed isoforms is not as extensive. This may be due to the overexpression of exogenous AUF1 relative to endogenous levels, which leaves a higher proportion of AUF1 uncleaved due to incomplete processing by the viral proteinase. Cells and supernatant were also harvested 6 hpi and virus titer was determined by plaque assay (Figure 3.6D). Expression of either cleavable or uncleavable AUF1 isoforms in 293-shAUF1 cells prior to infection resulted in relatively equivalent rescue of AUF1 inhibition of poliovirus replication by all isoforms. Expression of uncleavable AUF1 did not enhance inhibition of poliovirus replication. These results could be due to inefficient cleavage of exogenous AUF1, which leaves enough intact AUF1 to fully restrict infection or that higher levels of inhibition by AUF1 cannot be achieved. It is also possible that cleavage of AUF1 may be a consequence of viral proteinase activity without having any impact on virus replication.

Figure 3.6. Expression of uncleavable AUF1 does not enhance restriction of poliovirus infection. (A) Schematic of N-terminal flag-tagged AUF1 and its 3C/3CD cleavage site. The poliovirus 3C/3CD cleavage site is a glutamine-glycine (Q-G) pair in the dimerization domain of all four isoforms of AUF1 with an alanine (A) at position P4 upstream of the scissile bond. Uncleavable constructs were generated by mutation of the Q-G pair to isoleucine-aspartic acid (I-D) as determined by Rozovics and colleagues. (B) 293-shAUF1 cells were treated with 1 μg/ml doxycycline to induce AUF1 knockdown and transfected with vector (V), individual flag-tagged AUF1 isoform constructs (p37, p40, p42, or p45), or individual uncleavable flag-tagged AUF1 isoform constructs ($p37^{unc}$, $p40^{unc}$, $p42^{unc}$, or $p45^{unc}$) for 36 h prior to cell lysis and protein analysis by Western blot. 293-shCtrl cells were transfected with vector only. Nucleolin (nuc) was used as a loading control. (C) 293-shAUF1 cells were treated with 1 μg/ml doxycycline and transfected with vector, cleavable, or uncleavable individual flag-tagged AUF1 isoform constructs prior to infection with poliovirus at an MOI of 10. 293-shCtrl cells were transfected with vector only. Cell lysates were prepared at 8 h post-infection and protein expression analyzed by Western blot. AUF1 isoforms were detected using an anti-flag antibody, poliovirus infection was confirmed by detection of viral capsid protein, and nucleolin (nuc) was used as a loading control. (D) 293-shAUF1 cells were treated with 1 μg/ml doxycycline and transfected with vector, cleavable, or uncleavable individual flag-tagged AUF1 isoform constructs prior to infection with poliovirus at an MOI of 1. Virus from cells and supernatant at 6 h post-infection was titered by plaque assay and normalized to cell count (PFU/cell).

A

B



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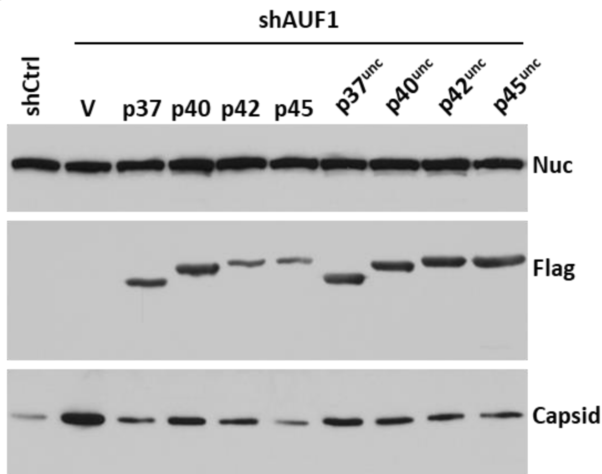

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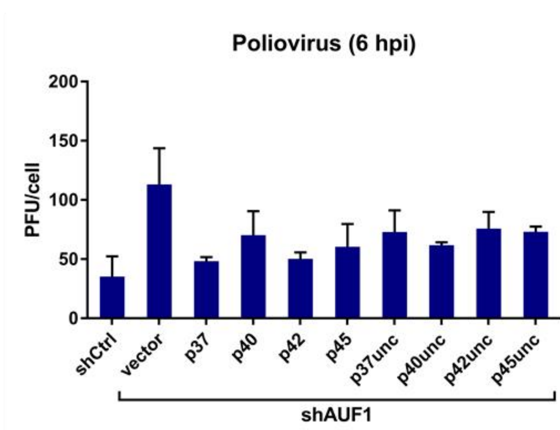

IV. Conclusions

The results presented in this chapter explore different aspects of the mechanism of AUF1 negative regulation of poliovirus infection and the possibility of a viral defense against its activity. In **Chapter 2**, it was shown that AUF1 restricts infection by poliovirus and CVB3 by negatively regulating viral IRES-driven translation. It was not clear whether all, or specific, isoforms of AUF1 contributed to the inhibition of viral translation. It was demonstrated in this chapter that all isoforms of AUF1 were able to rescue the inhibitory effect on poliovirus replication. Since AUF1 isoforms have been shown to bind many of the same mRNAs (128), the site on poliovirus RNA which is bound by AUF1 may also be shared by all isoforms. Consistent with these results, it was also shown that all isoforms of AUF1 can bind the poliovirus 5' NCR using an RNA affinity pulldown assay of lysate from uninfected cells. However, the ability of AUF1 isoforms to bind the 5' NCR may change during infection. Using lysate from infected cells, the predominant isoform of AUF1 bound to the 5' NCR was the smallest isoform, p37. These results suggest that there may be isoform-specific effects that occur during infection and that the overexpression of individual isoforms prior to infection may have overwhelmed this effect. Further studies of the specific effects of AUF1 will require reducing expression levels of each isoform and measuring their effect on replication of other AUF1-sensitive picornaviruses.

RNA affinity pulldowns were used to determine whether inhibition of poliovirus translation by AUF1 could be achieved through competition for binding to the 5' NCR with other AUBPs. Specifically, interactions by hnRNP A1, hnRNP Q, and hnRNP K were investigated. HnRNP A1 is a positive regulator of EV71 translation, and addition of recombinant p40 isoform to RNA affinity assays has been shown to reduce binding of

hnRNP A1 to the EV71 5' NCR (139). It was shown here that AUF1 knockdown reduces hnRNP A1 binding to the poliovirus 5' NCR, contrary to what might be expected based on the previously published EV71 results. It is possible that AUF1 exerts a negative effect on poliovirus and EV71 translation using different mechanisms or that these assays are not an accurate measure of protein binding. Addition of recombinant AUF1 to an RNA affinity assay may compete with hnRNP A1 in a way that does not represent true competition during infection. The lysates used in these assays were prepared from different cell lines and may also reflect cell type-specific variations in RNA-protein complexes that form on viral RNA.

Binding of two additional AUBPs, hnRNP Q and hnRNP K, did not yield clear results. Both of these proteins were identified as binding to poliovirus genomic RNA, but it is unclear where that binding occurs (29). hnRNP Q was detected at low levels in cell lysates and was faintly detected in the pulldown samples, but not above background. hnRNP K was clearly detected in both pulldown and background samples, thereby making it impossible to determine whether it is a true 5' NCR binding protein. On-going and future RNA affinity screens will probe for competitive interactions with other AUBPs such as HuR, nucleolin, and YB-1 along with other known IRES transacting factors (ITAFs). Instead of competing with AUBPs for binding to the 5' NCR, AUF1 may compete with established picornavirus ITAFs like PCBP2, PTB, or La, among others.

The cleavage of AUF1 that has been observed during infection by several picornaviruses may serve as a defense mechanism used to inhibit its negative effect on virus replication. Previously, proteolytic cleavage has been shown to occur during infection by viruses which are restricted by AUF1, but not by EMCV, which was thought to be unaffected by this

protein. In this chapter, it was shown that EMCV can be negatively regulated by AUF1, but in a cell type- or species-specific manner. Furthermore, while AUF1 inhibits replication of EMCV in human cells, it is not cleaved. These data demonstrate that cleavage of AUF1 does not occur during infection by all AUF1-sensitive picornaviruses. It is possible that AUF1 may be inhibited during infection by EMCV using means other than proteolytic cleavage, such as protein modification, protein-protein interactions, or sequestration away from sites of viral translation or RNA synthesis. To directly study the impact of AUF1 cleavage on virus replication, uncleavable AUF1 isoform constructs were expressed in cells prior to infection by poliovirus. When compared to expression of their cleavable counterparts, all forms of AUF1 were able rescue inhibition of poliovirus replication. These results may indicate that cleavage of AUF1 does not impact virus replication; however, sufficient amounts of full-length AUF1 may remain during expression of the cleavable isoforms to fully inhibit virus replication. An alternative approach to be used in future experiments will involve expression of only the cleaved portion of each isoform prior to infection to eliminate the interference of full-length protein.

Results presented in this chapter demonstrate that AUF1 inhibition of picornavirus replication is complex and may use different mechanisms depending on the cell type, host species, or virus. All isoforms of AUF1 were able to inhibit poliovirus replication, but RNA affinity assays revealed that each isoform may not bind equally to the 5’ NCR during infection. Furthermore, AUF1 may compete for binding to the 5’ NCR with different positive regulators of translation, depending on the virus. It is not clear whether cleavage of AUF1 inhibits its negative effect on virus replication. However, AUF1 cleavage products were not detected as binding to the poliovirus 5’ NCR, which suggests that cleavage of AUF1 disrupts

its ability to bind viral RNA. Future studies will aim to identify alterations in the composition of proteins bound to the poliovirus and CVB3 5' NCRs that occur following AUF1 knockdown. Identification of host factors with altered binding to the 5' NCRs of different viruses will reveal whether AUF1 inhibits viral translation through a common mechanism, or if different mechanisms are used to achieve a similar outcome.

CHAPTER 4

Discussion

The work presented in this dissertation further characterizes the function of AUF1 as a host restriction factor for picornavirus infections. Distinct from its role as an mRNA decay protein, AUF1 does not restrict poliovirus or CVB3 infection through destabilization of viral RNA, but by inhibiting viral translation. As an unintended consequence of the disruption of nucleocytoplasmic trafficking by viral proteinases, AUF1 is relocalized from the nucleus to the cytoplasm where it inhibits rounds of viral translation that occur subsequent to translation of input viral RNA (Figure 4.1). Inhibition of viral translation indirectly leads to decreased viral RNA synthesis and progeny virion production. All four isoforms of AUF1 were shown to inhibit poliovirus replication when exogenously expressed, but endogenous AUF1 may bind the viral IRES in an isoform-specific manner during infection. These results suggest that even though all isoforms of AUF1 are be able to inhibit virus replication, not all isoforms may be involved during infection. It was further demonstrated that AUF1 may act as a broad-spectrum picornavirus restriction factor, inhibiting infection of picornaviruses from different genera in a cell type- or species-specific manner.

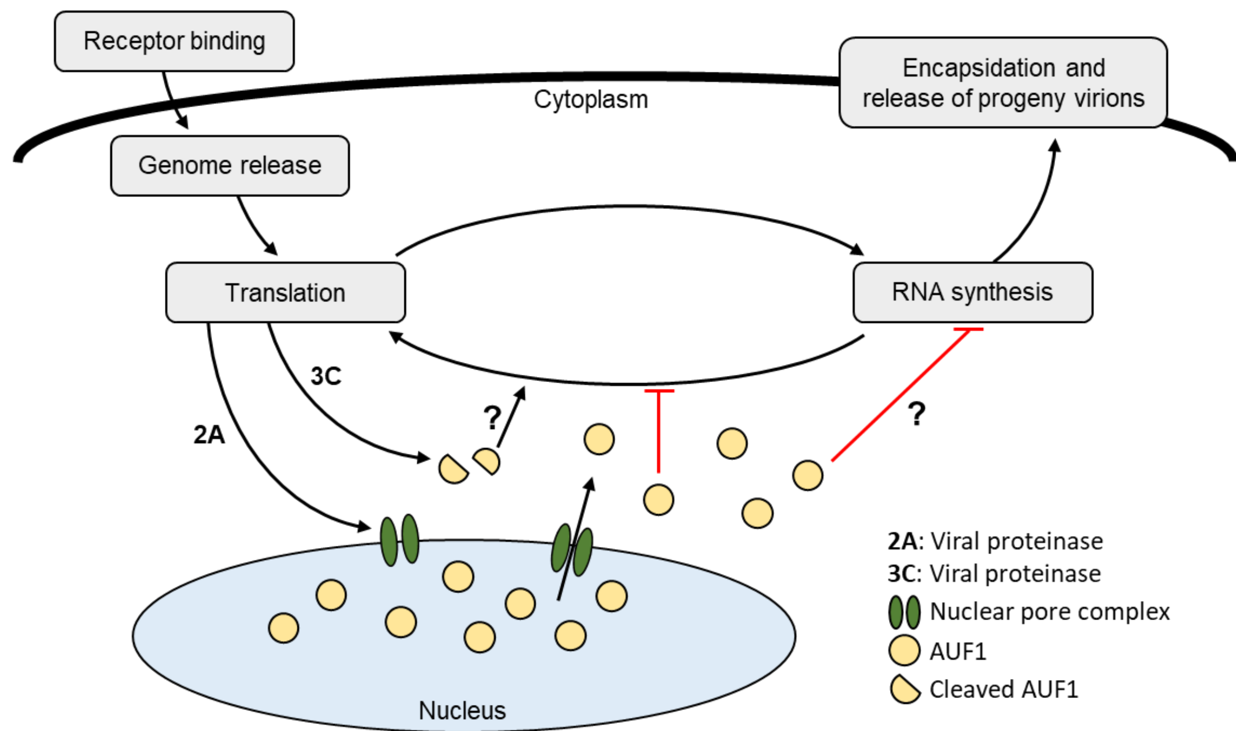

Figure 4.1. Proposed model for restriction of poliovirus and CVB3 infection by AUF1.

During poliovirus and CVB3 infection, the disruption of the nuclear pore complex (NPC) by the viral 2A proteinase results in the relocalization of AUF1 from the nucleus to the cytoplasm. Once in the cytoplasm, AUF1 restricts virus replication by inhibiting rounds of viral translation that occur after translation of input viral RNA has occurred. Inhibition of viral translation leads indirectly to decreased viral RNA accumulation and reduced progeny virion production. A direct inhibition of viral RNA synthesis may also contribute to the negative effect of AUF1 on infection but has not been measured. Poliovirus and CVB3 may defend against restriction by AUF1 through proteolytic cleavage of AUF1 by viral proteinases. AUF1 is cleaved by the 3C proteinase during infection, which could ameliorate its negative effect on viral translation and enhance virus replication.

Although AUF1 may act as a broad inhibitor of picornavirus infection, it is not a general restriction factor for viral infection. AUF1 has been shown to act as a positive regulator of infection by several members the *Flaviviridae* family of positive-sense, single-stranded RNA viruses. Flaviviruses include the mosquito-borne viruses dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), and Zika virus (ZIKV) as well as the major human pathogen, hepatitis C virus (HCV). The p45 isoform of AUF1 has been shown to bind and destabilize stem loop structures at the 5' and 3' ends of WNV, DENV, and ZIKV, facilitating cyclization of the genome and viral RNA replication (133-135). AUF1 has also been shown to positively regulate replication of HIV-1. Similar to picornaviruses, AUF1 is relocalized from the nucleus to the cytoplasm during HIV-1 infection and promotes expression of the Gag and Env proteins (131). AUF1 p42 and p45 have been shown to regulate splicing of HIV-1 mRNA through association with the HIV exonic splicing silencer complex (215). AUF1 also regulates the splicing and expression of human papillomavirus type 16 (HPV-16) genes, a double-stranded DNA (dsDNA) virus. In this case, the smaller p37 and p40 isoforms are responsible for HPV-16 gene regulation (216). In addition to these viruses, AUF1 is involved in gene expression for Epstein-Barr virus, another dsDNA virus (129). AUF1 was found associated with viral and host proteins that transactivate viral latency genes. Interestingly, knockdown of AUF1 resulted in increased replication of another dsDNA virus, human cytomegalovirus (217). How AUF1 may restrict replication of this virus remains unknown.

Even though AUF1 restricts infection of several picornaviruses by acting as a negative viral IRES transacting factor (ITAF), it does not act as a negative ITAF for all viruses having an IRES. HCV contains an IRES in its 5' NCR which is composed of fewer

domains and utilizes a reduced number of host factors for translation initiation compared to a picornavirus IRES (218). AUF1 was found to bind to an RNA stem loop structure in the HCV IRES and promote viral translation (132). Based on these published studies, the role of AUF1 as a viral restriction factor may be limited to picornaviruses. However, since the *Picornaviridae* family of viruses is responsible for more viral infections than any other virus family, this makes AUF1 a significant player in non-innate immune-mediated restriction of viral infections.

In addition to AUF1, two other host restriction factors have been identified for picornaviruses: KHSRP and DRBP76. KHSRP appears to act on viral translation in a manner similar to AUF1 involving competitive binding to the viral IRES with positive regulators of translation (145, 184). While cell type- or species-specific regulation of infection for one picornavirus, EMCV, has been demonstrated, it is generally not clear whether AUF1 or KHSRP regulate tissue-specific permissiveness to infection. DRBP76 has been shown to play a role in the pathogenicity of a recombinant poliovirus currently in clinical trials for the treatment of recurrent glioblastoma (219). A chimeric poliovirus, called PV-RIPO, was generated that replaces the 5' NCR of poliovirus with the HRV 5' NCR. PV-RIPO replicates efficiently in cancer cells, but not in cells of neuronal lineage even though poliovirus is a neurotropic virus (182). The reduced neuropathogenicity of PV-RIPO makes it a promising treatment for glioblastoma since it replicates in tumor cells and not surrounding brain tissue. DRBP76 was identified as an HRV 5' NCR binding protein that inhibits HRV IRES-driven translation. Since neuronal cells express relatively high levels of DRBP76, it is thought that this restriction factor is responsible for eliminating the neuropathogenicity of PV-RIPO (182, 183). Whether AUF1 acts like DRBP76 and contributes to the tissue tropism

of specific picornaviruses is yet to be determined. Results presented in **Chapter 3** suggest that AUF1 may regulate infection of EMCV in a species-specific manner. AUF1 did not negatively regulate EMCV infection in mouse cells, a host which is susceptible to pathogenic infection by EMCV. In contrast, AUF1 was able to negatively regulate infection of EMCV in a human cell line. EMCV is an animal pathogen but can cause asymptomatic infections in humans. Given these results, AUF1 may act as a restriction factor to infection in host cells where EMCV replicates less efficiently or with reduced pathogenic effects. In cells where EMCV replicates efficiently, the virus may be able to overwhelm the negative effects of AUF1.

The identification of host restriction factors for picornavirus infection is an important aspect of the study of virus-host interactions. Since the three restriction factors that have been identified thus far all appear to act as negative ITAFs, inhibition of viral translation may be an important restriction imposed by host cells to limit virus replication. However, the importance of this type of restriction may also be a consequence of the screens used to identify those factors. All three proteins were identified in RNA affinity screens of the 5' NCRs of different picornaviruses. Identification by these means would bias discovery toward IRES-binding proteins. Analysis of proteins which bind full-length genomic RNA may lead to the identification of factors that restrict virus replication using means other than inhibition of translation. A study aimed at identifying poliovirus RNA binding proteins identified 66 host proteins that bind viral RNA but have no described function in virus replication (29). Among these proteins may be novel picornavirus restriction factors, leasing to new insights into host mechanisms used to limit virus replication.

The identification of picornavirus RNA binding proteins may reveal novel restriction factors that directly inhibit virus replication through binding of viral RNA; however, many host restriction factors may use a less direct mechanism. The cleavage of host proteins by viral proteinases results in the modification or disruption of many cellular processes, including those having a negative impact on virus replication. Identification of host proteins that are targeted by viral proteinases may reveal novel negative regulators of infection. Using an *in vitro* proteomics approach, targets of the poliovirus and CVB3 3C proteinase were recently identified by terminal amine isotopic labeling of substrates (TAILS) followed by tandem mass spectrometry (MS/MS) (220). Interestingly, there were only 3 high confidence 3C targets that overlapped between the two viruses, with 69 substrates identified for poliovirus 3C and 31 for the 3C proteinase of CVB3. One substrate targeted by both poliovirus and CVB3 3C, phosphoribosylformylglycinamidine synthase (PFAS), acted as a negative regulator of poliovirus infection and can represent a novel picornavirus restriction factor. Since the majority of 3C substrates are not shared between poliovirus and CVB3, this presents the possibility that more restriction factors are virus-specific than broadly active like AUF1. While the specific players may differ between viruses, the overall mechanisms used by these restriction factors may be similar (e.g. inhibition of viral IRES-driven translation). Additional candidates will almost certainly be identified as substrates of the 2A or L (for FMDV) proteinases. Not all host restriction factors may be substrates for viral proteinases. Several proteins in the innate immune response pathway have been shown to be inhibited by viral proteins, independent of proteolysis. Additional restriction factors may be identified through screens of viral-host protein interactions.

Identification of host proteins that are cleaved by viral proteinases is likely to reveal novel restriction factors whose function is disrupted during picornavirus infection; however, results presented in **Chapter 3** do not clearly reveal whether cleavage of AUF1 impacts its inhibitory function. Expression of uncleavable AUF1 isoforms during poliovirus infection did not enhance inhibition of virus replication relative to "cleavable" AUF1 isoforms. These results may indicate that cleavage of AUF1 is a byproduct of 3C proteinase activity and does not affect its function or that cleavage may occur too late during infection, after negative regulation by intact AUF1 is already established. Complicating the interpretation of these results, it was determined that exogenously expressed AUF1 is only partially cleaved during infection and enough intact AUF1 may remain to completely rescue inhibition of virus replication. A better measure of the effect of AUF1 cleavage will be achieved through the expression of AUF1 cleavage products during infection, thereby eliminating interference by the full-length protein. Results from this type of experiment will reveal whether the cleavage products of AUF1 have the same function of full-length protein or whether such cleavage products are unable to inhibit virus replication.

Results presented in **Chapters 2** and **3** show that AUF1 inhibits poliovirus and CVB3 IRES-driven translation, likely by direct binding to the viral IRES. These data are the first report of AUF1 negative regulation of CVB3 translation. Previous studies using RNA affinity assays suggest that AUF1 binds to different RNA stem loop structures within the EV71 and poliovirus IRES; however, unlike the EV71 study, a complete comparison of all poliovirus stem loops was not performed and the stem loop with which AUF1 binds with the highest affinity may not have been identified (136, 139). It has yet to be determined where AUF1 binds on the CVB3 5' NCR or whether AUF1 negatively regulates EMCV infection of human

cells by inhibiting viral translation. Differences in 5' NCR binding sites for AUF1 between these viruses could underscore the possibility that AUF1 inhibits translation using different mechanisms for each virus. Evidence presented in **Chapter 3** suggests that this is a possibility for poliovirus and EV71. AUF1 was shown to compete for binding to the EV71 5' NCR with hnRNP A1, a positive regulator of EV71 translation (139). These results lead the authors to speculate that AUF1 negatively regulates EV71 translation by interfering with hnRNP A1 binding to the IRES. In contrast to EV71, the opposite result was found for the poliovirus 5' NCR. AUF1 knockdown resulted in reduced binding of hnRNP A1 to the poliovirus 5' NCR, suggesting a possible cooperative, instead of competitive, interaction between these proteins for poliovirus RNA. The opposing results from these studies could be due to differences in the assays used to identify competitive interactions or could reflect virus-specific variation in the mechanism used by AUF1 to inhibit viral translation. It is possible that AUF1 inhibits translation by competing with positive ITAFs for the viral IRES, but the nature of the positive ITAF may differ between viruses. A more complete analysis of competitive interactions for the poliovirus 5' NCR is required to determine which positive ITAF(s) is involved for regulation by AUF1. AUF1 interactions with the CVB3 5' NCR have not been studied and may reveal additional insights into the complexity of the mechanism used by AUF1 to negatively regulate viral translation.

Picornaviruses represent a family of RNA viruses composed of many human and animal pathogens of historic, current, and emerging significance. There are no specific treatments for picornavirus infections, and vaccines exist for only two viruses: poliovirus and hepatitis A virus. Given the worldwide distribution and prevalence of picornaviruses, it is important to gain insight into the host mechanisms used to restrict infection. Other than

proteins involved in the innate immune response, few host factors have been identified that restrict picornavirus replication. The work presented in this dissertation sought to further characterize and define the mechanism of action of the host restriction factor AUF1 during infection by poliovirus and CVB3. AUF1 becomes a restriction factor during infection as an unintended consequence of the disruption of nucleocytoplasmic trafficking by viral proteinases. Normally a predominantly nuclear-resident protein, AUF1 moves into the cytoplasm during infection along with many host factors that promote virus infection. Once in the cytoplasm, AUF1 inhibits poliovirus and CVB3 IRES-driven translation, resulting in reduced viral RNA synthesis and virion production. The study of AUF1, and other restriction factors like it, illuminates non-canonical pathways used by host cells to limit virus infection. Discovery of new inhibitory mechanisms used by cells to restrict virus replication may help direct development of effective antivirals against this large family of viruses.

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