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Divergent requirement of a DNA repair enzyme during picornavirus infections

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Divergent requirement for a DNA repair enzyme during picornavirus infections

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Sonia Monica Maciejewski

Dissertation Committee:
Professor Bert L. Semler
Professor Rozanne M. Sandri-Goldin
Professor Klemens J. Hertel
Professor Paul D. Gershon

2016

DEDICATION

To

my mother Leticia Jaramillo,

who has always valued a good education and a strong work ethic.

Without her I would not be the person I am today.

To

my husband Nicholas Flores,

who has taught me to be patient, ambitious, and most importantly, a kind person.

You have kept me grounded throughout this whole experience.

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ACKNOWLEDGMENTS

Graduate school is a journey that requires a lot of persistence, hard work, and dedication. These are all values that have been instilled into me by my mother. She has also taught me to value a good education, which is why I probably wanted to go to graduate school immediately after graduating from college. However, getting into graduate school was not done with my efforts alone. Getting to this point in my academic career was a group effort that needs to be acknowledged.

First and foremost, my mother is the first person that should be acknowledged for helping me get to where I am today. She came from Mexico to the United States to obtain the "American Dream." Her idea of this dream was to raise children who were well educated and career-driven. I remember her working two jobs, even three at one point, so that my brothers and I could all attend private school and get the best education possible. On top of this, she always encouraged us to participate in extracurricular activities, even if it meant at an extra financial cost. These activities ranged from sports programs to science clubs. I look back and realize how important it was to her that we succeeded both academically and personally. This continued effort and sacrifice on her behalf has only motivated me to be my best in school. In the end, everything that I do is to make her proud.

As an undergraduate at San Diego State University I did not know what I wanted to major in. I started off as a business major only to find myself interested by my general science courses. Growing up as a first generation American college student, I felt lost in what career paths I could take with a degree in science. One day I decided to approach my general biology teacher, Dr. Robert Pozos, after class to ask him for career advice. I remember talking with him for hours in his office. He then referred me to a woman named Veronica Bejar, the administrator for an

undergraduate summer research program known as the McNair Scholars Program. I joined this program and began researching in Dr. Diane Smith's laboratory, she was my first mentor in the science field. Following this summer, I applied to the Minority Biomedical Research Support Program (MBRS). After a year I switch to Dr. Ralph Feuer's laboratory, where my interest in virology became very apparent to me. The MBRS program also provided workshops for their participants on how to apply to graduate school and networking events to meet current graduate students. At one of these networking events is where I met Richard Virgen-Slane. I approached him since he was the only virologist at the event. After talking with him, he invited me to visit Dr. Bert Semler's laboratory at the University of California, Irvine. I took him up on the offer and got to meet with Bert and his team. This encounter is what motivated me to apply to the Ph.D. program at the University of California, Irvine. To this day I still consider all of these people and programs as key components in helping me get to where I am today. I value all of these individuals' continued mentorship and friendship.

Next I would like to acknowledge Dr. Bert Semler and both past and current members of his laboratory. Bert has had the greatest impact throughout my graduate career serving as my mentor. He has not only taught me about virology but has pushed me to become a better speaker, writer, thinker, and team player. These are all extremely important skills that I will need to carry on in my future employments and for this I am deeply thankful to him. I am also thankful to Richard for mentoring me throughout my summer rotation and for being my first great friend upon joining the graduate program. I am thankful to the former laboratory members Andrea Cathcart, Amanda Chase, and Kerry Fitzgerald for always being available to talk in person or e-mail about how to do certain experiments, even after graduating. I am also thankful for the current lab members Eric Baggs, Dylan Flather, Autumn Holmes, Alexis Bouin, and especially Wendy Ullmer

for their suggestions throughout all of my laboratory meetings and manuscript preparations. All of their comments were greatly appreciated.

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Last, and most importantly, I would like to thank my husband Nicholas Flores for his continued support throughout graduate school. He has been the most patient, loving, and kind person throughout this whole experience. I know it has not been easy with all of the late nights in the laboratory or listening to me do my practice talks for my seminars, but in the end it has shown me how much he cares about me. It is extremely comforting to know that he is so selfless and would sacrifice so much for my own success. I hope that we can continue supporting each other for the rest of our lives.

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PUBLICATIONS

1. **Maciejewski S**, Nguyen JHC, Gomez-Hereros F, Cortes-Ledesma F, Caldecott KW, and Semler BL. 2016. A divergent requirement of a DNA repair enzyme during enterovirus infections. mBio 7(1):e01931-15.
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March 2014	Europic 2014 Blankenberge, Belgium Poster Presentation: Defining the functional role of 5' tyrosyl-DNA phosphodiesterase 2 during picornavirus infections
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ABSTRACT OF THE DISSERTATION

Divergent requirement for a DNA repair enzyme during picornavirus infections

By

Sonia Monica Maciejewski

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2016

Professor Bert L. Semler, Chair

Viruses of the *Picornaviridae* family, including poliovirus, coxsackievirus B3 (CVB3), human rhinovirus, and encephalomyocarditis virus (EMCV), commandeer the functions of host cell proteins to aid in the replication of their small viral RNA genomes during infection. One of these host proteins is a cellular DNA repair enzyme known as 5' tyrosyl-DNA phosphodiesterase 2 (TDP2). TDP2 was previously demonstrated to mediate the cleavage of a unique covalent linkage between a viral protein (VPg) and the 5' end of picornavirus RNAs. Although VPg is absent from actively translating poliovirus mRNAs, the removal of VPg is not required for the *in vitro* translation and replication of the RNA. However, TDP2 appears to be excluded from replication and encapsidation sites during peak times of poliovirus infection of HeLa cells, suggesting a role for TDP2 during the viral replication cycle. Using a mouse embryonic fibroblast cell line lacking TDP2, we found that TDP2 is differentially required among picornaviruses. Our single-cycle viral growth analysis shows that CVB3 replication has a greater dependency on TDP2 compared to poliovirus, human rhinovirus, or EMCV replication in murine cells. During infection CVB3 protein accumulation is undetectable by Western blot analysis in the absence of TDP2, while poliovirus and EMCV protein accumulation is still detectable at reduced levels. Following

transfection of an infectious CVB3 RNA with a reporter, CVB3 RNA replication could still be detected in the absence of TDP2, albeit at reduced levels. EMCV showed the least dependence on TDP2 of the picornaviruses we studied and a possible alternative mechanism for modulating TDP2 activity during infection. TDP2 was shown to relocalize from the nucleus to the cytoplasm during EMCV infection. Unlike during poliovirus infection, TDP2 was not excluded from putative replication sites at peak times of EMCV infection. TDP2 was also cleaved during peak times of EMCV infection, but not during other picornavirus infections, suggesting that EMCV regulates TDP2 activity differently than enteroviruses. Despite these differences, these findings collectively show that TDP2 potentiates viral replication during picornavirus infections of cultured mouse cells, making TDP2 a putative target for antiviral therapeutic development for picornavirus infections.

CHAPTER 1

Introduction

Summary

Picornaviruses are responsible for many viral illnesses worldwide. These viruses include the human pathogens poliovirus, coxsackievirus, and rhinovirus, as well as the livestock pathogens encephalomyocarditis virus and foot and mouth disease virus. Despite their small RNA genomes, this family of viruses can efficiently replicate in the host by using their viral proteins to usurp cellular proteins to facilitate their replication cycle. This is done by virus-mediated modifications of cellular proteins via their viral proteinases or other nonstructural proteins. Additionally, these viral proteins inhibit host antiviral pathways to create an environment that favors viral replication. Although picornaviruses have a highly conserved genome, subtle differences in the genomic sequences can lead to differential usage of host proteins and mechanisms for viral replication. These differences can make it difficult to develop broad-spectrum antiviral therapeutics targeting host proteins. Thus it is imperative that these virus-host protein interactions are identified and their function in the viral replication cycle is elucidated for the development of effective antiviral therapeutics.

Importance

The *Picornaviridae* family contains 29 genera, including the two that will be discussed throughout this dissertation: enterovirus and cardiovirus (ictvonline.org/virusTaxonomy.asp). While cardioviruses are divided into three species, including encephalomyocarditis virus (EMCV) and theilovirus, the enterovirus genus is more diverse. The enterovirus genus encompasses 12 species, including poliovirus, coxsackievirus, rhinovirus, enterovirus, and echovirus serotypes. These viruses are responsible for the most prevalent human diseases worldwide (Khetsuriani et al., 2006). Diseases caused by picornaviruses pose a major public health problem and have significant economic impact. Poliovirus can cause paralytic poliomyelitis. Coxsackievirus can cause hand, foot, and mouth disease and myocarditis. Human rhinovirus is the causative agent of the common cold. Although the symptoms of respiratory illnesses caused by picornaviruses are almost never fatal, these viral infections have a negative economic impact due to lost work time and can severely affect individuals with respiratory dysfunction, such as asthma (Gavala et al., 2011). Such respiratory infections are commonly caused by human rhinovirus, coxsackievirus, and enterovirus D68. Enterovirus D68 was first identified in California in 1962, but has had sporadic outbreaks throughout the years in North America, Europe, and Asia (Tokarz et al., 2012). Enterovirus D68 outbreaks have been associated with severe respiratory illnesses and are quickly spreading throughout the United States (Midgley et al., 2014). Another enterovirus with recurring outbreaks is enterovirus 71. Enterovirus 71 is a potentially neurotropic virus with the majority of symptoms similar to hand, foot, and mouth disease and remains endemic in the Asia-Pacific region. Neurological diseases caused by enterovirus 71 infection can cause aseptic meningitis and brainstem encephalitis, which can lead to mortality [reviewed in (Shih et al., 2011)]. Although a vaccine against poliovirus is available, no effective antivirals for treating picornavirus infections

currently exist. Since symptoms caused by such infections can lead to severe complications in certain individuals, including children and immunocompromised individuals, it is necessary to develop antiviral therapeutics against picornaviruses, especially enteroviruses. Antivirals can target a host protein required for picornavirus replication, a viral protein, or the viral RNAs. Antivirals solely targeting host factors used during the viral replication cycle can lead to cell toxicity, while antivirals against a viral protein can lead to antiviral-resistant mutants. Although several of the host proteins involved in picornavirus replication have been identified, the specific roles these proteins play during picornavirus translation and RNA synthesis remain largely unknown. Therefore, to develop an effective broad-spectrum antiviral, the steps of the picornavirus replication mechanism and the roles of key molecular players, both host and viral, must be elucidated.

Picornavirus genome

While infection may result in diverse diseases, all picornaviruses have a small (~7-8 kb) positive-sense, single-stranded RNA genome that is replicated in the cytoplasm of infected cells. This genome is packaged into an icosahedral shell made up of four capsid proteins known as VP1, VP2, VP3, and VP4. The genome contains a highly structured 5' noncoding region (NCR) that is necessary for viral translation and RNA replication. Downstream of the 5' NCR is a single open reading frame that encodes both the structural and nonstructural proteins necessary for viral replication. Following the coding region is a 3' NCR and a short genetically encoded poly(A) tract necessary for viral replication (Kitamura et al., 1981; Wimmer et al., 1993; Yogo and Wimmer, 1972). Picornaviruses lack a 7-methylguanosine (7mG) cap at the 5' end of their RNA and instead contain a small viral protein known as VPg covalently linked to the 5' end by a phosphotyrosyl bond (Ambros and Baltimore, 1978; Flanegan et al., 1977; Lee et al., 1977; Rothberg et al., 1978).

This VPg-RNA linkage is highly conserved among picornaviruses. Picornaviruses have evolved to use VPg as a protein-primer for RNA synthesis, since their RNA-dependent RNA polymerase (RdRP) 3D ($3D^{pol}$) cannot initiate viral RNA replication *de novo* (Flanegan and Baltimore, 1977; Paul et al., 1998). **Figure 1.1** depicts an overview of the poliovirus genome and the structural and nonstructural viral proteins it encodes.
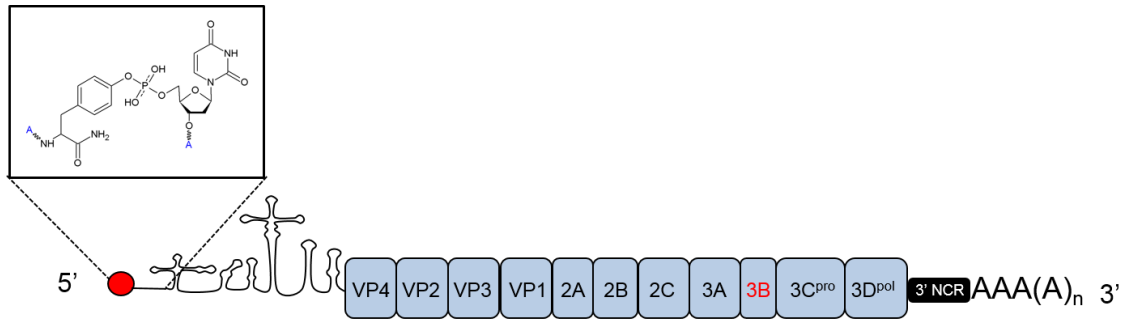
Figure 1.1 Overview of the poliovirus genome.

Illustration depicting the poliovirus genome. The phosphotyrosyl bond between the viral RNA and the third tyrosine in the VPg (red) amino acid sequence is magnified in the box. Following this linkage is the 5' NCR made up of RNA secondary structures known as stem-loops I-VI that are required for viral replication and internal ribosome entry site (IRES)-mediated translation of the viral polyprotein. The structural or capsid proteins include VP1-VP4. The nonstructural viral proteins include 2A, 2B, 2C, 3A, 3B (VPg), 3C, and 3D^{pol}. The schematic of the phosphotyrosyl bond depicted in the box was made by Eric Baggs.

Viral translation and RNA replication

Following uncoating, picornavirus genomic RNA is released into the cell cytoplasm where it is then translated into a single viral polyprotein. Since picornavirus genomic RNAs lack a 7mG cap at the 5' end, the viral polyprotein is translated in a cap-independent manner via an internal ribosome entry site (IRES). The IRES is located in the 5' NCR of the viral RNA genome and is composed of a number of stem-loop secondary structures depending on the picornavirus. EMCV has a more structured type II IRES in comparison to the enterovirus type I IRES [reviewed in (Fitzgerald and Semler, 2009)]. Due to their limited coding capacity, picornaviruses have evolved to use and modify host cellular functions (in addition to their viral proteins) to carry out the translation and replication of their genomes. Viral translation is mediated by cellular IRES trans-acting factors (ITAFs). After translation of the single open reading frame of viral genomic RNA, the viral polyprotein is proteolytically processed by the newly synthesized viral proteinases. The viral polyprotein is initially processed into three precursor molecules referred to as P1, P2, and P3. P1 is further proteolytically processed into the mature capsid proteins (VP4, VP2, VP3, VP1), while P2 and P3 are further processed into the mature nonstructural viral proteins (2A, 2B, 2C, 3A, 3B, 3C, 3CD) as shown in **Figure 1.1**. Some viral proteins may also function in carrying out the viral replication cycle in a precursor form (i.e. 3AB and 3CD). In addition to the autocatalytic nature of viral proteinases, they can also cleave host proteins. These cellular protein modifications result in events such as the shut down of cellular translation and transcription, allowing host proteins normally involved in these cellular functions to become hijacked to function in the viral replication cycle, and the alteration of nucleo-cytoplasmic trafficking. The alteration of nucleo-cytoplasmic trafficking is advantageous to the virus since the predominantly nuclear host proteins involved in viral replication relocalize to the cytoplasm, where picornavirus replication occurs.

Following viral protein synthesis, specific viral proteins alter cytoplasmic membranes to form replication complexes, where negative- and positive-sense viral RNA is synthesized. These newly synthesized positive-sense RNAs can either undergo further rounds of translation and replication or become encapsidated into virions that go on to infect neighboring cells. In addition to the modifications of host proteins for viral replication, viral proteinases can also alter cellular proteins to suppress antiviral response pathways, including the type I interferon (IFN) response, generation of stress granules (SGs) [reviewed in (Feng et al., 2014)], and processing body (P body) formation (Dougherty et al., 2011). Suppression of the cellular antiviral signaling pathways leads to a favorable cellular environment for viral replication. Collectively, viral-mediated modifications of cellular proteins aid in the viral replication cycle.

Viral proteinase disruption of host functions

Picornaviruses can disrupt host cell translation and transcription machinery to benefit viral replication by using viral-encoded proteinases to cleave host cell proteins. To carry out these modifications, enteroviruses utilize the virus-encoded proteinases 2A and 3C (and the precursor protein, 3CD), while cardioviruses use 3C only, since the 2A protein they encode lacks enzymatic activity. In addition to recognizing multiple cleavage sites in host cellular proteins, these proteins are responsible for proteolytically processing the picornavirus polyprotein. Poliovirus proteinase 2A cleaves between phenylalanine-glycine or tyrosine-glycine residues in the viral polyprotein (Toyoda et al., 1986), while 3C/3CD cleaves primarily at glutamine-glycine sites but can cleave at additional sites as well. 3C/3CD cleavage activity is dependent on surrounding sequences, specifically an amino acid with a small aliphatic side chain in the amino acid located four positions (P4) proximal to the cleavage site (Blair and Semler, 1991; Nicklin et al., 1986). The somewhat divergent recognition sites for these proteinases allow for cleavage of host proteins, which disrupts

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cellular functions and alters protein activities. This section will focus on how picornavirus proteinases cleave host proteins to shut down the cellular translation and transcription machinery, subverting host functions to augment viral translation and RNA synthesis. These cleavage events and cellular function alterations are outlined in **Table 1.1**.

Host protein	Viral proteinase	Host function disrupted	References
eIF4GI/II	PV and CVB3 2A	Cap-dependent translation	Devaney et al. 1988; Etchison et al., 1982; Krausslich et al., 1987; Bovee et al., 1998; Lamphear et al., 1995
PABP	PV and CVB3 2A; PV 3C	Cap-dependent translation	Joachims et al., 1999; Kuyumcu et al., 2002; Kerekatte et al., 1999
UBF	PV 3C	RNA pol I transcription	Banerjee et al., 2005
TAF$_{110}$	PV 3C	RNA pol I transcription	Banerjee et al., 2005
TBP	PV 2A; PV 3C	PIC formation for RNA pol II transcription	Yalamanchili et al., 1997a; Das and Dasgupta, 1993; Yalamanchili et al., 1996
CREB-P	PV 3C	RNA pol II transcription	Yalamanchili et al., 1997b
p53	PV 3C	Transcription	Weidman et al., 2001
TFIIIC	PV 3C	RNA pol III transcription	Clark et al., 1991; Shen et al., 1996

Table 1.1. Targets of picornavirus-mediated cleavage to disrupt the host cell translation and transcription machinery. Viral-encoded proteinases mediate cleavage of cellular proteins to shut down host cell functions, including cap-dependent translation and cellular transcription. Viral disruption allows host functions to become available for viral translation and RNA synthesis activities. Table 1.1 outlines the cellular proteins involved in cellular translation and transcription targeted by poliovirus (PV) or coxsackievirus B3 (CVB3) proteinases.

During cap-dependent translation of cellular mRNAs, eukaryotic initiation factors are recruited to the 7mG cap structure at the 5' ends of mRNA. These factors form a complex that interacts with the 43S pre-initiation complex (PIC) to recruit ribosomes for translation initiation. Eukaryotic initiation factor 4G (eIF4G) serves as a scaffold protein that aids in the recruitment of eIF4E and eIF4A, to form a ribonucleoprotein (RNP) complex termed eIF4F, as well as additional proteins such as poly(A) binding protein (PABP), for initiation of cap-dependent translation (Jackson et al., 2005; Wells et al., 1998). Poliovirus and coxsackievirus 2A proteinases have been shown to cleave both eIF4G isoforms, eIF4GI and eIF4GII, early in viral infection, resulting in the loss of the N-terminal domain required for both eIF4E and PABP interaction [reviewed in (Daijogo and Semler, 2011)] (Devaney et al., 1988; Etchison et al., 1982; Krausslich et al., 1987). 2A proteinase preferentially cleaves eIF4G when the cellular protein is bound to cap-binding protein eIF4E, thus leading to rapid shut down of cap-dependent cellular translation (Bovee et al., 1998). Unlike enteroviruses, cardioviruses inhibit cellular translation by altering ribosomes in a 2A-dependent manner (Aminev et al., 2003; Groppo and Palmenberg, 2007). This shut down of host machinery allows for resource allocation to cap-independent translation, benefiting viral protein synthesis. Additionally, evidence suggests that the cleaved form of eIF4G is required to stimulate IRES-dependent enterovirus translation (Lamphear et al., 1995; Liebig et al., 1993). eIF4G interacts with stem-loop V of the poliovirus and coxsackievirus IRES (de Breyne et al., 2009). The central domain of eIF4G interacts with eIF3, a component of the 43S PIC, *in vitro* (Sweeney et al., 2014). This direct interaction between eIF4G and eIF3 at stem-loop V suggests that this interaction may be required in recruiting the 43S PIC to the proximal stem-loop IV, an essential step in 48S complex formation for viral translation initiation to occur.

In addition to viral-mediated cleavage of the cap-binding complex scaffold protein eIF4G, infection also results in the cleavage of host protein PABP causing disruption of cap-dependent translation. PABP is a cellular protein that binds to the 3' poly(A) tract of mRNAs and interacts with eIF4G to functionally circularize the mRNA for efficient translation and mRNA stability in the uninfected cell [reviewed in (Fitzgerald and Semler, 2009; Smith et al., 2014)]. During infection, PABP is cleaved by poliovirus and coxsackievirus 2A proteinases (Joachims et al., 1999; Kerekatte et al., 1999). In addition, poliovirus and human rhinovirus 3C proteinases preferentially cleave ribosome-associated PABP (Kuyumcu-Martinez et al., 2002). PABP has three conserved putative cleavage sites in the flexible linker domain between its RNA recognition motifs (RRMs) and C-terminal domain, and two additional putative cleavage sites in the RRMs (Kozlov et al., 2004; Kozlov et al., 2001; Lloyd, 2006). Cleavage of PABP at these different sites by either 2A or 3C results in cellular translation inhibition by disrupting mRNA circularization.

Enterovirus proteinases 2A and 3C play roles in shutting down host cellular transcription during infection by disrupting RNA polymerases (pol) I, II, and III. Poliovirus proteinase 3C is responsible for inhibiting RNA pol I transcription activity approximately 90 to 180 minutes post-infection by targeting the pol I transcription factor upstream binding factor (UBF). UBF is a sequence-specific DNA-binding protein that stabilizes the selectivity factor (SL-1) protein complex on the rRNA promoter for pol I transcription. Poliovirus 3C also inhibits the SL-1 protein complex subunit, TATA-binding protein (TBP)- associated factor (TAF) (TAF$_{110}$) (Banerjee et al., 2005). RNA pol II is responsible for transcribing host cellular mRNAs and is targeted by both poliovirus proteinases 2A and 3C. These enzymes cleave TBP, which is involved in forming a PIC that contains transcription factor II D (TFIID) for pol II binding to transcription start sites (Das and Dasgupta, 1993; Yalamanchili et al., 1997a). However, it is 3C-mediated cleavage of TBP and

phosphorylated CREB, both upstream cellular transcription factors, that is required for pol II transcription inhibition (Das and Dasgupta, 1993; Yalamanchili et al., 1997b; Yalamanchili et al., 1996). Poliovirus 3C activity can also lead to the degradation of transcription activator p53, in a non-ubiquitin mediated pathway (Weidman et al., 2001). RNA pol III is responsible for transcribing ribosomal RNA genes, tRNA genes, and genes encoding other small RNAs. Pol III activity is inhibited during poliovirus infection by 3C-mediated cleavage of TFIIIC (Clark et al., 1991; Shen et al., 2004). TFIIIC binds to the promoter element, B box, downstream of the transcription start site to recruit TFIIIB, which recruits pol III to the transcription start site. Once TFIIIB recruits pol III, TFIIIC dissociates, allowing pol III-mediated transcription to occur. 3C cleavage of TFIIIC inhibits recruitment of TFIIIB, thus indirectly inhibiting pol III transcription (Clark et al., 1991; Kassavetis et al., 1990). These proteinase-mediated cleavages of host proteins all work together to inhibit cellular transcription.

The cleavage of host proteins can directly or indirectly lead to the disruption of cellular translation and transcription. Such cleavage events are summarized in **Table 1.1**. Viral modifications of cellular proteins are not restricted to down regulation of host cell machinery but can also extend to the enhancement of viral IRES-mediated translation and viral RNA synthesis, which will be further discussed in the following sections of this chapter, as well as evasion of the host antiviral and stress response mechanisms.

Use and abuse of host cell functions for viral translation and RNA replication

Use of host factors for IRES-dependent translation and viral RNA synthesis

Since picornaviruses replicate in the host cytoplasm, and a number of cellular proteins involved in viral replication are predominantly nuclear, picornaviruses modify the cellular nucleo-cytoplasmic trafficking mechanism to accumulate proteins used for viral replication in the cell

cytoplasm, including La autoantigen, polypyrimidine tract-binding protein (PTB), poly(rC)-binding protein 2 (PCBP2), and serine/arginine-rich splicing factor 3 (SRSF3), also known as serine/arginine-rich protein (SRp20) (**Table 1.2**). These nucleo-cytoplasmic shuttling proteins contain an amino acid sequence known as a nuclear localization signal (NLS) that is recognized by a specific import receptor complex (Gorlich and Kutay, 1999). Protein-receptor complexes relocalize from the cytoplasm to the nucleus through nuclear pore complexes (NPCs) embedded in the nuclear envelope of the host cell. The NPC is made up of nucleoporins (Nups) that contain phenylalanine-glycine repeats necessary for shuttling the protein-receptor complex through the nuclear membrane. During enterovirus infection, the NPC becomes modified when poliovirus or human rhinovirus proteinase 2A cleaves Nup62, Nup98, and Nup153. These cleavage events correlate with proteins accumulating in the cytoplasm and inhibition of nuclear import pathways (Belov et al., 2000; Castello et al., 2009; Fitzgerald et al., 2013; Gustin and Sarnow, 2001; Park et al., 2008; Park et al., 2010; Watters and Palmenberg, 2011). Cleavage of Nups results in loss of the phenylalanine-glycine repeats necessary for protein-receptor complex docking in the NPC domain during shuttling through the nuclear membrane (Bayliss et al., 2000; Stewart et al., 2001). Pathways that are inhibited during enterovirus infections include the transportin import pathway and K nuclear shuttling (KNS) import pathway. The KNS import pathway mediates the import of RNA-binding proteins required for enterovirus replication known as heterogeneous nuclear ribonucleoproteins (hnRNPs) (Gustin, 2003; Gustin and Sarnow, 2001; Michael et al., 1997; Pollard et al., 1996). Unlike enteroviruses, cardioviruses disrupt the nucleo-cytoplasmic trafficking through its Leader (L) protein. The L protein is a viral protein encoded only by the cardioviruses. The L protein disrupts the nucleo-cytoplasmic trafficking by binding tightly to Ran-GTPase, a regulator of the import/export pathways, and mediating hyperphosphorylation of Nup62, Nup153,

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and Nup214 via the effector kinases ERK and p38 (Porter et al., 2006; Porter et al., 2010; Porter and Palmenberg, 2009). Regardless of their mechanism, all picornavirus-mediated disruption of nucleo-cytoplasmic trafficking results in an accumulation of nuclear proteins necessary to enhance viral translation and RNA synthesis in the cytoplasm.

Host protein La was initially described as an autoantigen found in sera from patients with systemic lupus erythematosus and Sjögren syndrome (Tan, 1989). La is predominantly a nuclear protein in the uninfected cell and plays a role in the maturation of RNA pol III transcripts, due to its ability to bind various RNA structures via its RNA binding domain (Gottlieb and Steitz, 1989; Kenan et al., 1991). During poliovirus or coxsackievirus serotype B3 (CVB3) infection, La becomes relocalized to the cell cytoplasm and interacts with the 5' NCR of the viral RNA to enhance IRES-mediated viral translation (Meerovitch et al., 1993; Ray and Das, 2002). During the course of poliovirus infection, La is cleaved by viral proteinase 3C but is still able to bind the viral IRES and mediate translation of the viral genome (Shiroki et al., 1999). Previous studies showed that the addition of purified La protein to rabbit reticulocyte lysate, a lysate that contains the minimal requirements for viral translation, enhances viral protein synthesis (Meerovitch et al., 1993; Svitkin et al., 1994). La is only one of the several known IRES trans-acting factors (ITAFs) that interact with the viral IRES to enhance translation of the enterovirus genome. Another nuclear RNA binding protein, nucleolin, has also been shown to interact with both the 5' and 3' NCR of poliovirus RNA to stimulate viral translation and replication, although the exact mechanism remains unclear (Izumi et al., 2001; Waggoner and Sarnow, 1998).

Host cell shuttling protein PTB is a member of the hnRNP complex and also functions as a cellular ITAF. In the uninfected cell, PTB functions as a repressive regulator of alternative splicing (Mulligan et al., 1992). During poliovirus infection, PTB, like La, relocalizes to the cell

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cytoplasm and becomes redistributed (Back et al., 2002). Full length PTB has been implicated in the enhancement of viral translation (Florez et al., 2005) by binding to stem-loop V of the poliovirus IRES and modulating adjacent eIF4G binding (Kafasla et al., 2010). Like poliovirus, EMCV also requires PTB for IRES-mediated viral translation (Kaminski et al., 1995). Full-length PTB also interacts with PCBP2 when bound to stem-loop IV of the poliovirus 5' NCR to stimulate translation (Kim et al., 2000). However, the multiple isoforms of PTB are cleaved between the RRM domains by poliovirus 3C/3CD proteinase late during viral infection (Back et al., 2002). Cleavage of the N-terminal domain of PTB could result in loss of interaction with PCBP2 and the hnRNP complex. Alternatively, cleavage of the C-terminal domain could result in loss of interaction with the IRES element (Back et al., 2002). The accumulation of cleaved PTB corresponds with a decrease in viral translation levels *in vitro*, suggesting a role in mediating the switch from viral translation to negative-strand RNA synthesis during the replication cycle (Back et al., 2002). A switch in viral translation to RNA synthesis is required during viral replication since the positive-strand viral RNA is translated in a 5' to 3' direction by the translation machinery, while the negative-strand viral RNA is synthesized using the same template but in the opposite direction by the viral-encoded RdRP 3D^{pol} [reviewed in (Daijogo and Semler, 2011)]. Interestingly, cleavage of PTB seems to be specific to poliovirus- or human rhinovirus-infected HeLa cells. A recent study shows that PTB is not efficiently cleaved in human rhinovirus-infected WisL cells, a human lung fibroblast cell line, suggesting that host proteins may be differentially cleaved by picornaviruses in different cell lines (Chase and Semler, 2014). RNA-binding host protein, unr, has been shown to act synergistically with PTB to enhance human rhinovirus IRES-mediated translation but has minimum enhancement of poliovirus IRES translation (Hunt et al., 1999). This difference in host protein usage among enteroviruses suggests that these viruses may

utilize different cellular proteins to mediate the same viral functions, including the switch in viral translation to RNA replication.

PCBP2 is a host cell RNA-binding protein that functions as an ITAF for enterovirus translation and has been shown to be involved in the switch from viral translation to RNA synthesis. PCBP2 binds to poly(rC) regions of RNA and is expressed in both the nucleus and cytoplasm of the uninfected cell. During poliovirus infection, PCBP2 binds to RNA secondary structure stem-loop IV of the viral IRES, along with host splicing factor SRSF3, to help form the RNP complex necessary for viral translation (Bedard et al., 2007; Blyn et al., 1996; Blyn et al., 1997). PCBP2 can also bind stem-loop I in the 5' NCR of the poliovirus genome to form a ternary complex with viral precursor proteinase 3CD (Parsley et al., 1997). This complex is required for initiation of negative-strand RNA synthesis and has been suggested also to be involved in positive-strand RNA synthesis (Gamarnik and Andino, 1997; Parsley et al., 1997; Vogt and Andino, 2010). During poliovirus, coxsackievirus, or human rhinovirus infection of HeLa cells, PCBP2 is cleaved in the linker region between its K-homologous (KH) domains, KH2 and KH3, by viral proteinase 3C/3CD (Chase et al., 2014; Perera et al., 2007). Cleaved PCBP2 can no longer bind stem-loop IV or interact with SRSF3, resulting in inhibition of IRES-mediated translation, but it can still bind stem-loop I for viral RNA synthesis (Bedard et al., 2007; Chase et al., 2014; Perera et al., 2007). It has also been suggested that poliovirus 3CD binds to stem-loop I to increase the binding affinity of PCBP2 to stem-loop I, thus decreasing its availability for binding to stem-loop IV for translation (Gamarnik and Andino, 1998). Cleavage of PCBP2, along with the cleavage of other proteins such as PTB, can help mediate the switch from viral translation to RNA synthesis. Interestingly, cleavage of PCBP2 does not occur in human rhinovirus-infected human lung fibroblasts, WisL cells, while it does when they are infected with poliovirus, as determined by Western blot analysis

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(Chase and Semler, 2014). Such a differential cleavage pattern suggests that cleavage of specific host proteins may be required to mediate the switch to RNA synthesis only in certain cell types. It is also possible that the concentration of PCBP2 in WisL cells is low and below the level of detection of the Western blot analysis used in this study (Chase and Semler, 2014). The mechanism that brings about the switch from viral translation to RNA replication remains incompletely understood and will require future studies.

Host protein SRSF3, a shuttling protein involved in mRNA splicing and translation, contains an N-terminal RRM domain for RNA binding and a serine/arginine (RS)-rich domain in its C-terminus for nucleo-cytoplasmic shuttling and protein-protein interactions (Caceres et al., 1997; Caceres et al., 1998). During poliovirus or CVB3 infection, and to a lesser extent during human rhinovirus 16 infection, SRSF3 relocalizes to the cell cytoplasm (Fitzgerald et al., 2013; Fitzgerald and Semler, 2011). Poliovirus proteinase 2A activity is required for the redistribution of SRSF3 to the cell cytoplasm (Fitzgerald et al., 2013). Upon relocalization, SRSF3 enhances poliovirus translation by binding the KH3 domain of PCBP2 with its RS domain and recruiting ribosomes to the IRES for translation (Bedard et al., 2007). Whether SRSF3 recruits the ribosomes directly or indirectly to stem-loop IV for IRES-mediated translation remains to be determined. It is possible that additional undiscovered ITAFs are required to recruit ribosomes to the IRES for translation or that SRSF3 may recruit the ribosomes via direct interactions.

Following the initial rounds of translation, viral polyproteins are processed by the viral-encoded proteinases. The nonstructural viral proteins can go on to function in viral RNA synthesis. To allow for efficient viral RNA synthesis to occur, there is a switch from viral translation to RNA synthesis. As discussed above, this switch is currently thought to occur when host factors, such as PTB and PCBP2, are cleaved by enterovirus proteinase 3C/3CD. Cleavage of ITAFs inhibits

IRES-mediated translation but still allows viral RNA replication to proceed, since the presence of these cleaved proteins favors the clearing of ribosomes from the RNA template. Overall, viral translation and RNA replication are dependent on the modifications of host proteins by the viral-encoded proteinases. In addition to host protein modifications, the cellular environment becomes altered in picornavirus-infected cells so efficient viral RNA replication can occur.

Alteration of host cell membranes for viral RNA synthesis

For viral RNA replication to occur, cellular organelles must be modified to form virus-induced membranous vesicles that serve as sites of replication complexes for viral RNA synthesis (Caliguiri and Tamm, 1969; Dales et al., 1965). The specific localization of these membranous vesicles may physically separate RNA synthesis from IRES-mediated translation in the cytoplasm and increase the local concentrations of viral proteins required for viral replication. The virus-induced vesicles are derived from the endoplasmic reticulum (ER), Golgi, and from components of autophagic vesicles to form single- and double-walled vesicles (Bienz et al., 1987; Jackson et al., 2005; Schlegel et al., 1996).

The COPII complex components, Sec13 and Sec31, have been shown to colocalize with viral protein 2B, suggesting that COPII may be involved in the formation of replication complexes (Rust et al., 2001). COPII is a vesicle coat protein complex that transports proteins from the ER to the Golgi in the uninfected cell (Barlowe et al., 1994). COPII vesicle proteins are made in the ER with the help of COPII complexes, which include coat proteins. Once the COPII-coated vesicles are formed, they bud from the ER, lose their coat proteins, and fuse to the Golgi (Klumperman, 2000; Rust et al., 2001; Springer et al., 1999). During poliovirus infection, it has been shown that these ER-derived vesicles accumulate in the cytoplasm, and there is a transient increase in COPII vesicle budding from the ER (Bienz et al., 1987; Rust et al., 2001; Trahey et al., 2012). Alterations

in the secretory pathway also mediate the formation of viral replication complexes. During poliovirus infection, nonstructural viral protein 3A recruits guanine nucleotide exchange factor (GEF), GBF1, while viral proteinase 3CD recruits GEFs, BIG1, and BIG2, to membranes to activate the secretory pathway by converting the small GTPase Arf1 into its active form (Arf1-GTP) (Belov et al., 2007). Arf1-GTP can alter membrane curvature and recruit coat proteins to form secretory transport vesicles (Belov and Ehrenfeld, 2007). The activation of Arf1 leads to the production of phosphatidylinositol-4-phosphate (PI4P), a lipid with an important role in vesicle transport. Expression of CVB3 3A can lead to an accumulation of PI4P and PI4-kinase III β (PI4KIIIβ) (Hsu et al., 2010). During CVB3 infection, PI4KIIIβ has also been shown to colocalize with sites of viral RNA replication and to be required for both poliovirus and CVB3 replication (Hsu et al., 2010). 3A has been shown to associate with acyl coenzyme A [acyl-CoA]-binding protein domain 3 (ACBD3), a protein that binds to an integral Golgi protein known as giantin (Greninger et al., 2012). However, it was recently shown that although ACBD3 does interact with CVB3 3A and PI4KIIIβ directly, this interaction is not required for the recruitment of PI4KIIIβ to replication complexes (Dorobantu et al., 2014). Additionally, depletion of GBF1 and Arf1 by pharmalogical inhibition or small interfering RNA (siRNA) treatment in CVB3-infected cells did not inhibit PI4KIIIβ recruitment (Dorobantu et al., 2014). These contradictory findings reveal that the mechanism for virus-induced host membrane reorganization remains poorly understood and additional studies are required to dissect the involvement of the secretory pathway during enterovirus replication.

Host cell membrane organization throughout poliovirus infection has been observed by electron microscopy (Belov et al., 2012; Caliguiri and Tamm, 1970). At 3 hours post-infection, replication complexes appear to be single-membraned, while at 4 hours post-infection the

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complexes appear to be convoluted. At later times of infection, replication complexes appear to be double-membraned, illustrating the dynamic nature of viral-induced, membranous vesicles throughout the replication cycle. The convoluted membranes observed at peak times of infection resemble the crescent-shaped precursor membranes seen during autophagy. During poliovirus infection, LC3, a marker for autophagy, localizes to these membranous vesicles. This localization is induced by viral proteins 2BC and 3A (Jackson et al., 2005; Taylor and Kirkegaard, 2007). One hypothesis to explain these observations is that picornaviruses induce the formation of replication complexes via a mechanism similar to autophagasome formation (Kemball et al., 2010; Klein and Jackson, 2011; Suhy et al., 2000). However, a recent study using an antibody specific for double-stranded RNA (dsRNA), which is an RNA intermediate formed during viral RNA synthesis, to identify replication complexes found that dsRNA does not significantly colocalize with LC3 early during infection but does so at late times of infection (Richards et al., 2014). Although this contradicts previous studies suggesting that LC3 plays a role in replication complex formation, the authors of this report alternatively suggest that LC3 may have a role in viral replication, but not in complex formation (Richards et al., 2014). This apparent discrepancy in findings may be due to previous studies using antibodies against viral proteins to analyze the role of the autophagy pathway during viral RNA synthesis instead of antibodies specific for viral dsRNA. Although previous studies have attempted to elucidate the mechanisms utilized in picornavirus replication complex formation, there are many features of this process that remain to be determined.

Additional host proteins usurped for viral translation and replication

Picornaviruses require numerous host factors to carry out their viral replication cycles. It is apparent that the host factors described above are not sufficient to carry out translation, replication, and encapsidation of the viral RNA. In an attempt to comprehensively identify host

factors binding to the viral RNA during infection, several experimental approaches have been employed. A study using thiouracil cross-linking mass spectrometry (TUX-MS) identified host factors binding to poliovirus RNA during replication in HeLa cells (Lenarcic et al., 2013). In addition to previously characterized host factors known to interact with the viral RNA, 66 novel host proteins were identified using this methodology. From these 66, eight proteins were selected for validation. Knockdown of two of these proteins, NONO (non-POU-domain-containing octamer-binding protein) and CNBP (cellular nucleic acid-binding protein), decreased poliovirus replication similar to levels when PCBP2, La, PTB, or hnRNP C was knocked down. Further analysis of these two host proteins revealed that CNBP was required for efficient viral translation and NONO was required for efficient positive-strand RNA synthesis (Lenarcic et al., 2013). This methodology proved to be effective for identifying proteins associated with viral RNA during picornavirus replication, since the authors identified other already previously published proteins involved in the viral replication cycle. One of the proteins on this list is AU-rich binding factor 1 (AUF1) (Lenarcic et al., 2013), which has been previously identified via an RNA affinity screen for proteins interacting with the 5' NCR (Rozovics et al., 2012).

AUF1, also known as hnRNP D, is a cellular protein that binds to AU-rich elements in the 3' NCR of mRNAs in the uninfected cell (Zhang et al., 1993). It is involved in RNA stability and can target RNAs for degradation via an mRNA-decay pathway (Kiledjian et al., 1997). AUF1 has four isoforms produced by alternative splicing that contain tandem RRMs that bind RNA (Kajita et al., 1995). During poliovirus, CVB3, or EMCV infection, AUF1 relocalizes from the nucleus to the cytoplasm in a proteinase 2A-driven manner and colocalizes with the nonstructural viral protein 2B (Cathcart et al., 2013; Cathcart and Semler, 2014). AUF1 is cleaved by poliovirus or human rhinovirus 3CD and CVB3 3C but is not cleaved during EMCV infection (Cathcart and

Semler, 2014; Rozovics et al., 2012; Wong et al., 2013). AUF1 has also been shown to directly interact with the poliovirus 5' NCR, specifically full length 5' NCR and stem-loop IV (Cathcart et al., 2013; Rozovics et al., 2012). This interaction is inhibited by the cleavage of AUF1 by 3CD (Cathcart et al., 2013). AUF1 has been shown to interact with the 3' NCR of CVB3 RNA as well, via the AU-rich sequence at the 3' end (Wong et al., 2013). When AUF1 is genetically ablated or knocked down, poliovirus, human rhinovirus 16, or CVB3 viral titers increase, suggesting an inhibitory role for AUF1 during enterovirus infection (Cathcart et al., 2013; Wong et al., 2013). AUF1 can also decrease poliovirus translation *in vitro*, suggesting that AUF1 functions as an antiviral factor during enterovirus infection (Cathcart et al., 2013). It is possible that enteroviruses cleave AUF1 to disrupt the interaction of this protein with viral RNA as a mechanism to evade the cellular RNA decay pathway. Additionally, AUF1 can interact with other host factors involved in viral replication, including PCBP2, nucleolin, and PABP (Dempsey et al., 1998; Kiledjian et al., 1997; Lu et al., 2006). AUF1 cleavage may disrupt these host protein-protein interactions so that these host factors can bind to the viral RNA and stimulate viral translation and replication. Another possible role for AUF1 during enterovirus replication may involve the circularization of viral RNA through homo-multimerization of AUF1. This is supported by the observation that AUF1 binds both the 5' and 3' NCRs of the viral RNA and contains a dimerization domain at its N-terminus. Further studies are necessary to define the role of AUF1 during picornavirus infections and how the virus might evade the RNA degradation pathway initiated by this protein. Another recently identified host protein that is utilized during poliovirus replication is 5' tyrosyl-DNA phosphodiesterase 2 (TDP2) (Virgen-Slane et al., 2012). The function of TDP2 in the uninfected cell and during poliovirus infection will be discussed in further detail in the following two sections.

Most studies characterizing host proteins required for the picornavirus replication cycle have been carried out using the prototypic picornavirus, poliovirus. As discussed in this chapter, not all picornaviruses require the same host proteins for their viral replication cycles. This variation leaves room for other host proteins to be utilized during replication. Since there have been only a few reports of ITAFs required for EV71, a study was undertaken to identify cellular proteins bound to a biotinylated EV71 5'NCR. From this study, 12 cellular proteins were identified to interact with the 5' NCR (Lin et al., 2008). Of these 12 proteins, previously identified proteins were purified, including PTB, poly(C)-binding protein 1 (PCBP1, also known as hnRNP E1), PCBP2, La, and Unr. In addition, proteins previously unidentified as EV71 5' NCR-binding proteins were reported, including hnRNP K, hnRNP A1, far-upstream element-binding protein 1 (FBP1), and FBP2 (Lin et al., 2008). More recently, these latter proteins have been shown to redistribute from the cell nucleus to the cytoplasm and stimulate EV71 infection (Huang et al., 2011; Lin et al., 2008; Lin et al., 2009). Although the roles of hnRNP K, hnRNP A1, FBP1, and FBP2 during enterovirus 71 infection remain to be determined, this finding suggests that although picornaviruses have a highly conserved genome, they utilize several different host factors to enhance their viral replication. A summary of the key host factors discussed in this section can be found in **Table 1.2**.

Host protein	Viral Protein	Function of host protein	Role in viral replication	References
Nup62, Nup98, Nup153	PV and HRV 2A	Nucleo-cytoplasmic trafficking	Concentrates host proteins in the cytoplasm	Park et al., 2010; Park et al., 2008; Watters and Palmenberg, 2011; Castello et al., 2009 Gustin and Sarnow, 2001
La	PV 3C	Maturation of RNA pol III transcripts	Binds IRES for translation	Shiroki et al., 1999
PTB	PV and HRV14 3C/3CD	Alternative splicing	Full length PTB enhances viral translation; cleaved PTB mediates switch to RNA synthesis	Back et al., 2002
PCBP2	PV, CVB3, and HRV 3C/3CD	RNA binding; mRNA stability	Full length PCBP2 binds SLIV to enhance translation; full length PCBP2 binds SLI to initiate RNA synthesis; cleaved PCBP2 binds SLI for RNA synthesis	Perera et al., 2007; Chase et al., 2014
SRSF3 (SRp20)	PV 2A	Shuttling RNA binding protein; mRNA splicing	Interacts with PCBP2 to enhance translation	Fitzgerald et al., 2013
COPII	PV 2B	Protein transport from the ER to Golgi	Forms replication complexes	Rust et al., 2001
GBF1, BIG1/2	PV 3A and 3CD	Guanine nucleotide exchange factors	Activates Arf1 to produce PI4KIIIβ for replication complex formation	Belov et al., 2007
LC3	PV 2BC and 3A	Autophagy	Forms replication complexes	Jackson et al., 2005; Taylor and Kirkegaard, 2007
AUF1	PV and HRV 3CD; CVB3 3C	mRNA decay; RNA stability	Negative regulator of translation	Rozovics et al., 2012; Wong et al., 2013; Cathcart et al., 2013
TDP2	none known	DNA repair	Cleaves VPg from 5' end	Virgen-Slane et al., 2012

Table 1.2. Use and abuse of host cell functions for picornavirus translation and RNA replication. Enterovirus proteins alter host proteins to stimulate viral translation and RNA replication. Viral proteinases 2A, 3C, and 3CD can mediate cleavage of host proteins to change their canonical functions to non-canonical activities to aid in viral replication. Furthermore, some nonstructural proteins, including 2B, 2BC, and 3A, can modify the microenvironment of the cytoplasm to generate replication complexes so that viral RNA synthesis can be carried out.

5' tyrosyl-DNA phosphodiesterase 2

TDP2, also known as TRAF and TNF receptor-associated protein (TTRAP) and ETS1-associated protein II (EAPII) will be the main focus of this dissertation. TDP2 primarily functions as a DNA repair enzyme that can remove stalled topoisomerase adducts from DNA. Topoisomerases are essential enzymes that regulate DNA topology during replication and transcription in the uninfected cell by cutting and religating the phosphate backbone of either single-stranded or double-stranded DNA. Humans have six topoisomerases but the main focus of this section will be on topoisomerase II (TOPII). TOPII functions as a homodimer and creates double-stranded DNA breaks allowing for duplex DNA to pass through these breaks. The tyrosine residue of TOPII functions as a nucleophile to generate a covalent tyrosine-nucleic acid catalytic intermediate known as a cleavage complex [reviewed in (Pommier et al., 2014)]. This cleavage complex occurs on the 5' end of the scissile phosphate. The double stranded breaks are then religated when the deoxyribose hydroxyl ends attack the tyrosyl-phosphodiester bonds of the cleavage complex [reviewed in (Pommier et al., 2014)]. However, these TOPII-mediated cleavage complexes can be trapped by both endogenous and exogenous DNA lesions, mediated by reactive oxygen species, base pair mismatches, abasic sites, TOPII poisons (i.e., etoposide), and UV lesions [reviewed in (Pommier et al., 2014)]. TDP2 repairs TOPII-mediated damage by hydrolyzing the 5' phosphotyrosyl DNA linkages between TOPII and the DNA to remove TOPII from the DNA (Cortes Ledesma et al., 2009). It was first suggested that TDP2 recognizes this phosphotyrosyl linkage between TOPII and the DNA following ubiquitination and proteasomal degradation of the DNA adducts (Mao et al., 2001) and later confirmed that proteolytic degradation of TOPII occurs to allow TDP2 access to the phosphotyrosyl DNA-TOPII linkage (Gao et al., 2014). Although TDP2 has been shown to possess weak 3'-tyrosyl DNA phosphodiesterase activity, the

phosphodiesterase 3'-tyrosyl DNA phosphodiesterase 1 (TDP1) is not capable of repairing TOPII-induced DNA damage (Zeng et al., 2012). These findings suggest that TDP2 is the predominant phosphodiesterase that functions on 5' phosphotyrosyl bonds.

In addition to its DNA repair role, TDP2 has multiple roles in the uninfected cell, such as transcriptional regulation, the survival inflammatory response, cell proliferation, and signal transduction, through its multiple cellular binding partners, including CD40, TNF receptor-associated factors (TRAFs), and ETS1 (Li et al., 2011; Pei et al., 2003; Pype et al., 2000). These roles are outlined in **Table 1.3**. TDP2 was first identified as a CD40 binding protein that could interact with TRAF6 and inhibit the NF-kB pathway (Pype et al., 2000). Moreover, a yeast two-hybrid screen revealed that TDP2 interacts with ETS1 and other ETS proteins, which are DNA binding proteins that function as transcription factors. In this study the authors found that TDP2 negatively regulates ETS1 transcriptional activity (Pei et al., 2003). Another study found that TDP2 also interacts with promyelocytic leukemia nuclear body (PML NB) proteins PML3, Sp100, and DAXX using a yeast mating assay (Xu et al., 2008). Like most PML NB proteins, TDP2 expression is upregulated by expression of interferon gamma, a cytokine important for both innate and adaptive immunity against viral infections (Xu et al., 2008). Due to these latter findings, another group set out to determine if TDP2 played a role during human immunodeficiency virus (HIV) infection. TDP2 was shown to interact with HIV-1 integrase via its N-terminus using a yeast two-hybrid screen and was able to stimulate viral integration (Zhang et al., 2009). Additionally, TDP2 has been shown to have functional roles during other viral infections, such as human papillomavirus, and hepatitis B virus (HBV) (Edwards et al., 2013; Koniger et al., 2014). For example, TDP2 can hydrolyze the tyrosyl-DNA phosphodiester bond between the HBV viral

polymerase P protein from the virus relaxed circular DNA during HBV closed circular DNA biogenesis, a viral persistence mechanism used by HBV.

TDP2, a predominantly nuclear protein, is found in both the nucleus and cytoplasm of the uninfected cell (Pei et al., 2003). It has a molecular mass of 40.9 kDa with two additional isoforms predicted to be generated by alternative splicing (44.3 and 32.1 kDa). The N-terminal domain of TDP2 contains a ubiquitin-associated protein-like (UBA) domain, which is thought to be responsible for its multiple protein-protein interactions. The UBA domain is hypothesized to be necessary for detecting the phosphotyrosyl linkages in trapped TOPII-DNA cleavage complexes decorated with ubiquitin. TDP2 has SUMO-interacting motifs at the N-terminus that allow TDP2 to associate with PML NBs (Vilotti et al., 2012). Also in the UBA domain, TDP2 is phosphorylated by extracellular signal-regulated kinase 3 (ERK3), an atypical member of the MAPK family (Bian et al., 2016). This post-translational modification leads to an upregulation of its phosphodiesterase activity present at its C-terminus (Bian et al., 2016). The C-terminus contains a conserved catalytic domain that bears the hallmark motifs of the Mg^{2+}/Mn^{2+}-dependent phosphodiesterase superfamily [reviewed in (Li et al., 2011)]. The closest relative to TDP2 in this superfamily based on sequence identity is the DNA repair enzyme apurinic/apyrimidinic endonuclease-1 (APE-1) (Rodrigues-Lima et al., 2001). Structural data obtained from small-angle x-ray scattering analysis revealed that the UBA domain may possibly form an intramolecular interaction with the catalytic domain (Schellenberg et al., 2012), implicating a link between its post-translational modification and phosphodiesterase activity. Three-dimensional structures of TDP2 from mouse, *C. elegans,* and zebrafish reveal a narrow single-stranded DNA-binding groove that leads to the catalytic site formed by four conserved amino acid residues (mouse: E162, H359, N274, and D272) (Gao et al., 2012; Schellenberg et al., 2012; Shi et al., 2012). This catalytic tetrad shares similarities with

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DNase I and APEI (Shi et al., 2012). Previous work that characterized the biochemical properties of TDP2 revealed that the enzyme's preferred substrates are single-stranded DNA or duplex DNA with four base pair overhangs and that substrates as short 5 nucleotides long can be processed (Gao et al., 2012). Although TDP2 has been shown to function on single-stranded RNA, its preferred substrate is tyrosyl-DNA linkages (Gao et al., 2014). TDP2 is highly specific to phosphotyrosyl linkages and can even hydrolyze 5' digoxigenin-DNA adducts as long as the phosphotyrosyl linkage is present (Gao et al., 2012). While TDP2 enzymatic activity is Mg^{2+}/Mn^{2+}-dependent, it is also weakly active in the presence of Zn^{2+} or Ca^{2+}, due to its two-metal ion binding site present in its catalytic domain (Adhikari et al., 2012; Gao et al., 2012). However, crystallography studies only detect one metal ion in the active site (Schellenberg et al., 2012; Shi et al., 2012). In one of these studies, the authors proposed a mechanism for TDP2 catalytic activity, where the first metal ion along with a deprotonated water molecule necessary for the nucleophilic attack of the phosphate group, is coordinated by D262, H351, and N264 (Schellenberg et al., 2012). The second metal ion is coordinated by D122 and E152, functioning in the bridging of the metal binding sites of E152 and D262 (Schellenberg et al., 2012). Together these findings reveal that TDP2 catalytic activity is highly specific for phosphotyrosyl linkages from single-stranded DNA/RNA.

TDP2 binding partners	Function	References
CD30, CD40, TNF-75, and TRAFs 2, 3, 5, and 6	CD40 signaling pathway: inhibits transcription of NF-κB (regulates immune response)	Pype *et al.*, 2000
ETS-1, ETS-2, and FL-1	Ras/ERK signaling pathway: modulates ETS1 transcriptional activity and inhibits cell migration in cancer	Pei *et al.*, 2003
PML, DAXX, and Sp100	Interacts with PML nuclear bodies: many functions including transcriptional regulation, apoptosis, ribosome biogenesis	Xu *et al.*, 2008
ALK4 and Smad3	Nodal-Activin/TGF-β signaling pathway: vertebrate development	Eseguerra *et al.*, 2007
HIV-1 integrase	HIV integration	Zhang *et al.*, 2009
Human papillomavirus	HPV episome stability	Edwards *et al.*, 2013
HBV DNA	HBV close circular DNA biogenesis	Koniger *et al.*, 2014

Table 1.3. Summary of the binding partners of TDP2 in the uninfected and infected cell.

TDP2 has multiple binding partners in both the uninfected and infected cell. The proteins TDP2 has been shown to interact with are listed. A summary of the function of these proteins and the role TDP2 has when bound to these proteins is provided.

VPg unlinkase/TDP2

TDP2 was shown to harbor the cellular activity, discovered decades ago, that cleaves the phosphotyrosyl bond between the picornavirus-encoded protein VPg and the 5' end of poliovirus virion RNA (Ambros and Baltimore, 1978; Ambros et al., 1978; Virgen-Slane et al., 2012). This activity was initially referred to as VPg unlinkase and was shown to be present in both the nucleus and the cytoplasm of uninfected and poliovirus infected cells (Ambros et al., 1978). As mentioned in the previous section, TDP2 is a predominantly nuclear protein but is also found in the uninfected cell cytoplasm at lower concentrations. During poliovirus infection, TDP2 re-localizes from the nucleus to the cytoplasm of the cell (Virgen-Slane et al., 2012), most likely due to the alteration of nucleo-cytoplasmic trafficking via viral proteinases. Following its cytoplasmic accumulation, TDP2 appears to be excluded from putative sites of RNA replication and genome packaging during peak times of poliovirus infection. Significantly, the levels of VPg unlinkase activity remain unchanged in crude cytoplasmic extracts harvested throughout the course of poliovirus infection, suggesting that TDP2 activity is modulated by its cytoplasmic location or transient interactions with host or viral gene products (Rozovics et al., 2011; Virgen-Slane et al., 2012). This modulation may be required to ensure that, early during picornavirus infections, viral RNAs destined for translation do not have VPg linked to their 5' ends while at late times during infection, TDP2/VPg unlinkase is sequestered away from progeny RNAs to allow them to maintain VPg on their 5' ends. The latter scenario appears to be a requirement for progeny virion formation, since only VPg-linked viral RNAs are packaged (Fernandez-Munoz and Lavi, 1977). Early work showed that VPg is absent from ribosome-associated viral RNA suggesting that VPg needs to be removed for viral translation to occur (Hewlett et al., 1976; Nomoto et al., 1977b; Nomoto et al., 1976). However, previous studies showed that VPg-linked viral RNA can form a translation initiation complex *in*

vitro (Golini et al., 1980). Limitations of the experiments in this latter study include that they were done in a cell-free system using RRL, a lysate deficient in VPg unlinkase activity (Rozovics et al., 2011), and that the sucrose gradients could not determine if the VPg linked RNA represented a small or larger population of the viral RNA associated with the ribosomes.

In accordance with the early findings described above, a study using a replicon with an uncleavable bond between VPg and the viral RNA and a reporter gene shows that following transfection of this mutated CVB3 or poliovirus RNA, viral translation and replication are unaltered (Langereis et al., 2014). Although these results suggest that cleavage of the VPg-RNA linkage is not required for viral translation and replication, the caveats of the study must also be considered. The viral RNA harboring this uncleavable bond and reporter gene was transfected into the cells, thus bypassing the normal receptor-mediated entry pathway and the uncoating step. These steps that occur during normal infection may be essential in determining the orientation of viral RNA during uncoating and its initial exposure to the cell cytoplasm following uncoating. It is possible that since the viral RNA has been transfected into the cell and thus only undergoes primary rounds of translation and replication, the cleavage of VPg by VPg unlinkase/TDP2 may be necessary to determine the fate of nascent viral RNAs to either be encapsidated in progeny virus particles or undergo an additional round of translation and replication. Since the VPg unlinkase activity of TDP2 is not absolutely required for input viral translation and RNA replication, its regulation during viral infection suggests a distinct function in coordinating the fate of cytoplasmic viral RNAs, making TDP2 an attractive target for antiviral therapeutic development. We hypothesize that the role of TDP2 during enterovirus infections is to unlink VPg to distinguish viral RNA for use in translation, RNA synthesis, or encapsidation.

Conclusions

Due to their limited coding capacity, picornaviruses hijack host cell functions to stimulate viral translation and replication. This is typically carried out by cleaving cellular proteins to modify their canonical functions. Picornaviruses modify proteins involved in cellular nucleo-cytoplasmic trafficking, translation, and transcription to make these proteins available for viral translation and RNA replication. The viral proteins can further modify specific host proteins to mediate the switch from viral translation to RNA synthesis. Nonstructural viral proteins without proteolytic activity also alter cellular functions, such as membrane reorganization for viral replication complex formation. Additionally, picornaviruses can evade the host antiviral or stress response to ensure efficient replication. These modifications are outlined in **Figure 1.2**. Although it is known that picornaviruses can modify the host functions in multiple ways, many of the mechanisms remain unclear. Thus, it is necessary to elucidate these mechanisms and identify viral specific protein-protein interactions so that antivirals may be generated targeting either the host proteins or viral proteins involved. Although antivirals against cellular proteins can be potentially toxic to the cell, targeting a non-canonical function of a host protein or a novel protein-protein interface may circumvent such an issue. Importantly, identifying cellular targets required for picornavirus replication mitigates the generation of resistant viral variants due to the high mutation rates of viral RNA-dependent RNA polymerases. Such a prospect holds considerable promise for development of broad-spectrum antiviral therapies to treat picornavirus infections.
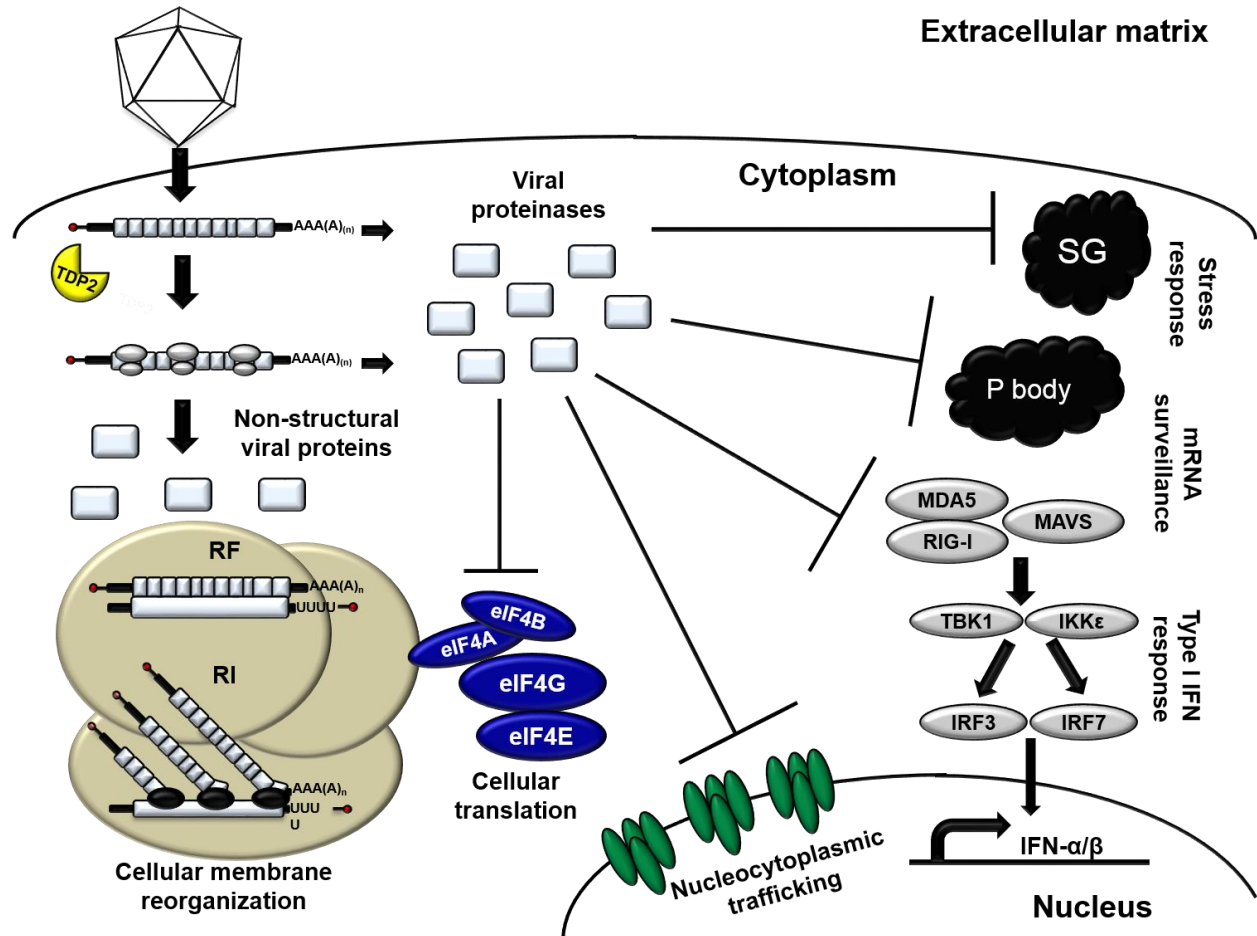
Figure 1.2. Summary of picornavirus-mediated host modifications to enhance viral translation and RNA replication. Picornaviruses modify cellular functions to stimulate viral replication. Upon release into the cytoplasm, the positive-strand viral RNA is translated. It has been suggested that cellular enzyme TDP2 cleaves the covalently linked viral protein, VPg, from the 5' end of the RNA to allow for polysome association. Following translation of the viral genome, the viral polyprotein is proteolytically processed. The viral proteins can then alter a number of cellular proteins, resulting in the hijacking of host functions for viral replication. Enterovirus proteinases 2A, 3C, and 3CD are responsible for cleaving host proteins involved in cellular nucleo-cytoplasmic trafficking, translation, transcription, and the antiviral response. For cardioviruses, the majority of these cellular modifications are carried out by the L protein and viral

proteinase 3C. Although the manner in which picornaviruses inhibit the antiviral response was not discussed in detail in this dissertation, it has been summarized and depicted in this figure. Nonstructural viral proteins, such as 2B, 2BC, and 3A, are also responsible for inducing conformational changes in the host cytoplasmic membranes to serve as replication sites for viral RNA synthesis. Together these virus-induced modifications of cellular proteins result in an altered microenvironment that allows the virus to replicate efficiently.

CHAPTER 2

The role of TDP2 during enterovirus infections

Summary

To determine the role of TDP2 during enterovirus infections, we took advantage of a mouse embryonic fibroblast (MEF) cell line lacking TDP2 (MEF-TDP2$^{-/-}$) (Zeng et al., 2012). We initially verified that the MEF-TDP2$^{-/-}$ cell line lacked VPg unlinkase activity using our rapid *in vitro* VPg unlinkase assay (Rozovics et al., 2011). To determine if TDP2 is required for enterovirus replication, we determined the growth kinetics of poliovirus, CVB3, and HRV during infections in the absence of TDP2. We found the requirement for TDP2 during infection differs among the enteroviruses. High-level growth of CVB3 was exclusively dependent on TDP2 expression, while levels of poliovirus and HRV replication were reduced by one to two orders of magnitude in the absence of TDP2. Viral yields were reduced by one order of magnitude when wild type and TDP2$^{-/-}$ mouse cells were infected with a chimeric poliovirus encoding the CVB3 5' noncoding region (NCR) in place of the poliovirus 5' NCR, suggesting that the phenotype observed during CVB3 infection in the absence of TDP2 is not solely mediated by the 5' NCR. Western blot analysis confirmed that viral protein accumulation was greatly reduced in lysates from poliovirus-infected cells lacking TDP2 and was not detectable in lysates from CVB3-infected cells lacking TDP2. In contrast, viral protein accumulation was detected at severely reduced levels in the absence of TDP2 following infection of the poliovirus-CVB3 chimera. Although we found that CVB3 replication and protein accumulation following infection were not detectable by plaque assay and Western blot analysis, respectively, in the absence of TDP2, detectable (albeit reduced) levels of CVB3 replication were observed following transfection of an infectious CVB3 RNA encoding a reporter protein. A similar phenotype was observed following transfection of an infectious poliovirus RNA

encoding a reporter. In addition, viral RNA replication was similar between the wild type and TDP2$^{-/-}$ mouse cells following transfection of a non-infectious poliovirus RNA with a reporter lacking the sequence for the capsid proteins. These findings suggest that the viral capsid proteins and encapsidation of the newly synthesized viral RNAs are playing a role in the reduced viral replication observed in the absence of TDP2. Taken together our data show that TDP2 activity potentiates enterovirus infections and, in the case of CVB3, is a required host function following virus infection of mouse cells. Our data support the hypothesis that TDP2 may be playing a role in viral translation, RNA replication, and encapsidation of newly synthesized viral RNAs.

Introduction

As discussed in **Chapter 1**, the picornavirus RNA genome lacks a 7-methylguanosine cap at the 5' end, requiring these viruses to initiate cap-independent translation of their polyprotein via an IRES in the 5' NCR. Instead of a cap, picornavirus genomes possess a viral protein that is 20-22 amino acids in length (depending on the picornavirus) known as VPg (Flanegan et al., 1977; Lee et al., 1977). VPg is covalently linked to the 5' terminus of viral RNA via an O^4-(5'-uridylyl)tyrosine phosphodiester bond as a result of viral RNA synthesis (Ambros and Baltimore, 1978; Rothberg et al., 1978). Since the viral RNA polymerase ($3D^{pol}$) cannot initiate viral RNA synthesis *de novo*, picornaviruses have evolved to use a uridylylated VPg as a protein primer for the initiation of viral RNA synthesis (Paul et al., 1998). Early work had suggested that VPg is cleaved from the 5' end of the genome to allow for polysome association and translation to occur but remains attached to the negative-strand RNA of the double-stranded replicative form (RF), as well as the positive-strand of the replicative intermediate (RI), and newly synthesized virion RNA (vRNA) (Fernandez-Munoz and Darnell, 1976; Fernandez-Munoz and Lavi, 1977; Hewlett et al., 1976; Nomoto et al., 1977a; Nomoto et al., 1977b; Nomoto et al., 1976; Pettersson et al., 1978). It has also been reported that VPg can be detected on the 5' end of the positive-strand RNA of RF molecules (Wu et al., 1978). Newly synthesized vRNAs are then either encapsidated into progeny virions (all encapsidated vRNA is VPg-linked) or can undergo additional rounds of translation and RNA replication (Fernandez-Munoz and Lavi, 1977; Flanegan et al., 1977; Nomoto et al., 1977a). The different forms of viral RNA species are depicted in **Figure 2.1.**
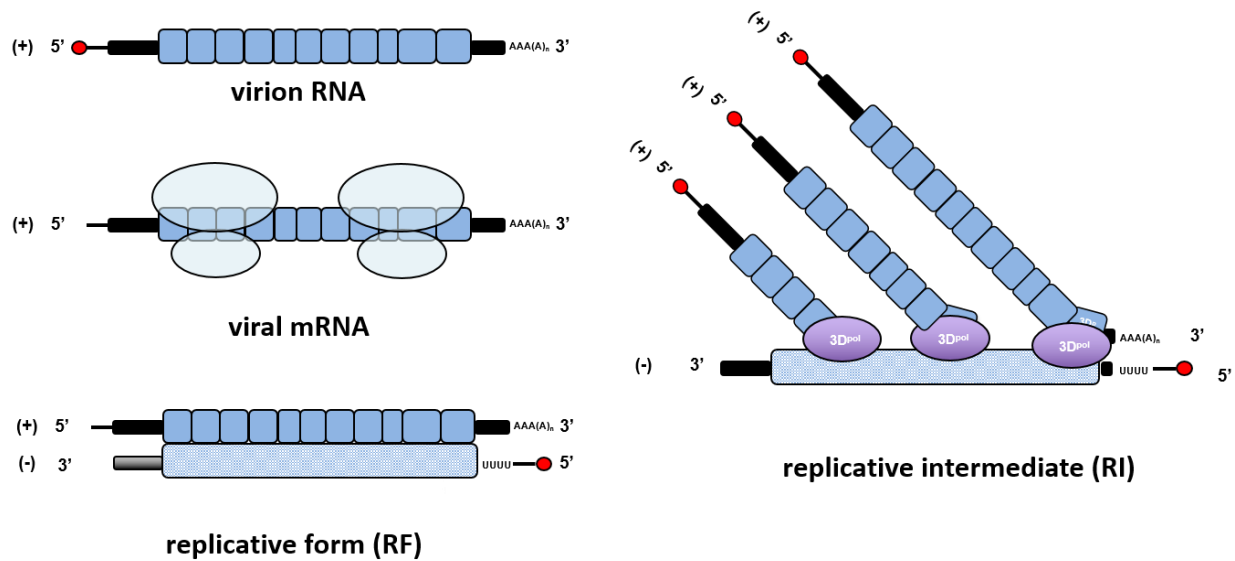
Figure 2.1. The presence or absence of VPg on the different picornavirus RNA species. The different picornavirus RNA species during the viral RNA replication cycle are depicted. VPg is present on virion RNA (vRNA). VPg is absent from ribosome-associated viral mRNA. VPg is present on the negative strand of the replicative form (RF) since it serves as a protein-primer for viral RNA synthesis and on the positive strand of the replicative intermediate (RI) of the newly synthesized viral RNAs. VPg is also present on encapsidated vRNA.

In 1978 it was first discovered that a cellular activity, referred to as VPg unlinkase, cleaved the conserved phosphodiester bond between VPg and viral RNA (Ambros et al., 1978). VPg unlinkase activity was found to be present in both the nucleus and cytoplasm of uninfected HeLa cell extracts (Ambros and Baltimore, 1980). The activity of the protein was shown to be Mg^{2+}- or Mn^{2+}-dependent but was inhibited in the presence of vanadate, SDS, Zn^{2+}, and EDTA, all of which are hallmarks of a *bona fide* enzyme (Ambros et al., 1978; Sangar et al., 1981). More than three decades later, VPg unlinkase activity was shown to be mediated by the cellular DNA repair enzyme, 5' tyrosyl-DNA phosphodiesterase 2 (TDP2) (Virgen-Slane et al., 2012). TDP2, also known as TTRAP and EAPII, is a predominantly nuclear enzyme, although it is present in the cytoplasm of the cell at lower concentrations (Pei et al., 2003). As a DNA repair enzyme, TDP2 hydrolyzes the 5' tyrosine-phosphodiester bond of single-stranded DNA in topoisomerase-mediated double-stranded DNA breaks (Cortes Ledesma et al., 2009). As discussed in **Chapter 1**, TDP2 has multiple roles in the uninfected cell, such as transcriptional regulation and signal transduction, through its multiple cellular binding partners, including ETS1, TRAFs, and CD40 (Li et al., 2011; Pei et al., 2003; Pype et al., 2000). Additionally, TDP2 has been shown to have functional roles during other viral infections, such as human immunodeficiency virus, human papillomavirus, and hepatitis B virus (Edwards et al., 2013; Koniger et al., 2014; Zhang et al., 2009).

During poliovirus infection, TDP2 re-localizes from the nucleus to the cytoplasm of the cell (Virgen-Slane et al., 2012), most likely due to the alteration of nucleo-cytoplasmic trafficking that occurs as a result of enterovirus proteinase-mediated cleavage of the nuclear pore complex. This alteration leads to an increased concentration of a number of nuclear-resident proteins within the cytoplasm, some of which have been shown to be used for viral translation or RNA replication

[reviewed in (Chase and Semler, 2012)]. Following its cytoplasmic accumulation, TDP2 appears to be excluded from putative sites of RNA replication and genome packaging during peak times of poliovirus infection. Significantly, the levels VPg unlinkase activity remain unchanged in crude cytoplasmic extracts harvested throughout the course of poliovirus infection, suggesting that TDP2 activity is modulated by its cytoplasmic location or transient interactions with host or viral gene products (Rozovics et al., 2011; Virgen-Slane et al., 2012). This modulation may be required to ensure that, early during enterovirus infections, viral RNAs destined for translation do not have VPg linked to their 5' ends while at late time during infection, TDP2/VPg unlinkase is sequestered away from progeny RNAs to allow them to maintain VPg on their 5' ends. This latter scenario appears to be a requirement for progeny virion formation, since only VPg-linked viral RNAs are packaged. However, previous studies have shown that VPg-linked viral RNA can form a translation initiation complex *in vitro* and that VPg-linked RNA can be translated and replicated *in vitro* (Golini et al., 1980; Langereis et al., 2014). Since the VPg unlinkase activity of TDP2 is not absolutely required for viral translation and RNA replication, its regulation during viral infection suggests a distinct function in coordinating the fate of cytoplasmic viral RNAs. We hypothesize that the role of TDP2 during enterovirus infections is to unlink VPg to distinguish viral RNA for use in translation, RNA synthesis, or encapsidation. The fine balance regulated by TDP2 that must exist during picornavirus replication is depicted in **Figure 2.2**.
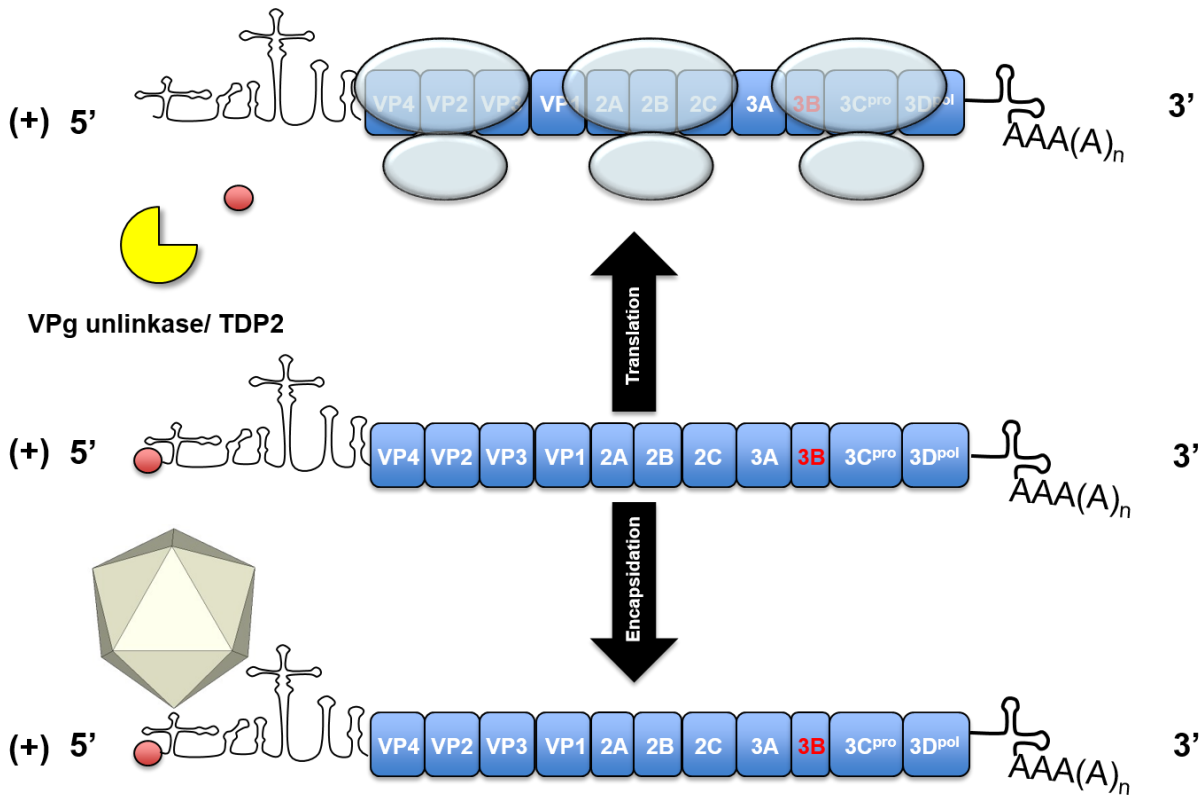
Figure 2.2. VPg unlinkase/TDP2-mediated balance during picornavirus infections.

Following uncoating, the viral RNA is released into the cell cytoplasm where we propose that VPg unlinkase/TDP2 hydrolyzes the phosphotyrosyl bond between VPg and the 5' NCR of the picornavirus RNA. However, TDP2 is relocalized to the cell periphery during peak times of infection, in sites distinct from replication and encapsidation, allowing the newly synthesized VPg-linked viral RNA to become encapsidated to form progeny virions. TDP2 activity functions as a way of marking the newly synthesized viral RNA for either additional rounds of viral translation or encapsidation.

Results

Mouse embryonic fibroblasts genetically ablated for TDP2 lack VPg unlinkase activity

To determine if TDP2 is required during enterovirus infections, we measured picornavirus growth kinetics in the absence of TDP2. Since cells depleted of TDP2 through RNAi may still result in low levels of VPg unlinkase activity, we utilized a mouse embryonic fibroblast (MEF) cell line genetically ablated for TDP2 (MEF-TDP2$^{-/-}$) (Zeng et al., 2012). To verify that the wild type MEF cells (MEF-TDP2$^{+/+}$) expressed VPg unlinkase activity and that the MEF-TDP2$^{-/-}$ cells lacked VPg unlinkase activity, we utilized a rapid *in vitro* VPg unlinkase assay previously described by Rozovics *et al.* (Rozovics et al., 2011). This assay allows for the detection of VPg unlinkase activity utilizing an ^{35}S-methionine radiolabeled virion RNA (vRNA) substrate and a source of VPg unlinkase. ^{35}S-methionine radiolabeled substrate was generated by radiolabeling cells infected with a mutated poliovirus (W1-VPg31) containing two methionines in the VPg sequence (Kuhn et al., 1988). The products of unlinkase reactions were resolved on a high percentage polyacrylamide gel and visualized by autoradiography. Since the full-length VPg-RNA substrate is too large to enter the gel, only the free VPg species can migrate to the bottom of the gel. To rule out non-specific nuclease activity, the radiolabeled substrate was incubated with either no source of VPg unlinkase, RNase A (which specifically degrades single-stranded RNA), or purified, recombinant GST-TDP2 (**Figure 2.3 A**). Previous reports demonstrated that RNase treatment of the VPg-linked RNA cannot remove the final nucleotide attached to VPg (Flanegan et al., 1977; Nomoto et al., 1977b), resulting in a slower migrating VPg species (VPg-pUp) (**Figure 2.3 A, lane 2**) compared to the free VPg species produced by incubation with recombinant TDP2 (**Figure 2.3 A, lanes 3 and 4**).

To verify that our MEF-TDP2$^{-/-}$ cell line did not exhibit VPg unlinkase activity, increasing concentrations of crude, whole cell extracts from HeLa cells, MEF-TDP2$^{+/+}$ cells, and MEF-TDP2$^{-/-}$ cells were incubated with the radiolabeled VPg-RNA substrate. VPg was unlinked from the radiolabeled substrate when incubated with increasing concentrations of HeLa cell or MEF-TDP2$^{+/+}$ cell crude extract (**Figure 2.3 B, lanes 3-6**). However, VPg remained linked to the viral RNA when incubated with up to 50 times more MEF-TDP2$^{-/-}$ cell crude extract (**Figure 2.3 B, lanes 7-12**). These results confirm that the MEF-TDP2$^{+/+}$ cell-line possesses VPg unlinkase activity while the MEF-TDP2$^{-/-}$ cells lack VPg unlinkase activity as determined by the *in vitro* VPg unlinkase assay.
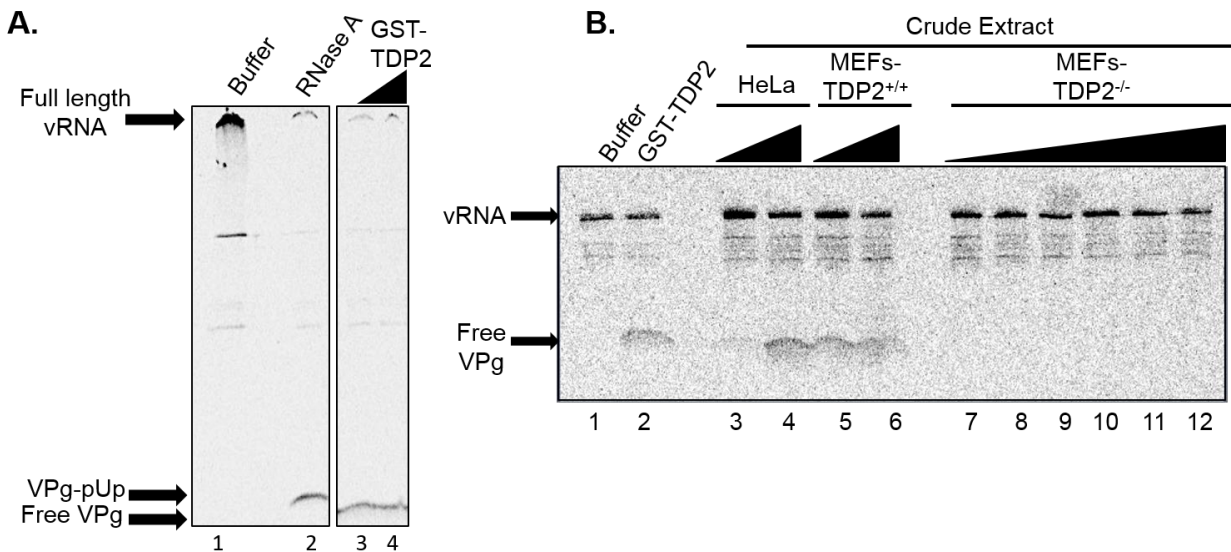
Figure 2.3. TDP2 in mouse embryonic fibroblasts is required for VPg unlinking. (A) Radiolabeled poliovirus VPg-vRNA was incubated with buffer only, RNase A, or increasing concentrations of GST-TDP2 (1 pmol or 8.5 pmol) to separate VPg species generated by non-specific nuclease activity or VPg unlinkase activity. The slower migrating VPg-pUp species generated by RNase and free VPg species generated by GST-TDP2 are denoted with black arrows at the bottom of the gel. The image of the Tris-Tricine polyacrylamide gel was cropped. **(B)** Radiolabeled poliovirus VPg-vRNA substrate from the same preparation in **(A)** was incubated with increasing concentrations of HeLa cell (2 or 20 µg), MEF-TDP2^{+/+} (2 or 20 µg), or MEF-TDP2^{-/-} (2-100 µg) crude extract to detect radiolabeled free VPg cleaved from the poliovirus vRNA substrate. The radiolabeled VPg-vRNA was also incubated with recombinant GST-TDP2 (1 pmol) to verify VPg unlinking from poliovirus RNA.

TDP2 is required for efficient poliovirus replication following transfection of poliovirus virion RNA in mouse embryonic fibroblasts

A previous study demonstrated that TDP2 not only hydrolyzes the phosphotyrosyl linkage between VPg and the 5' end of the poliovirus RNA but also re-localizes from the nucleus to the cytoplasm following poliovirus infection, suggesting that TDP2 is used during poliovirus replication (Virgen-Slane et al., 2012). Additionally, this same study showed that TDP2 is relocalized to the cell periphery in foci distinct from putative viral replication and encapsidation sites during peak times of poliovirus replication, suggesting that TDP2 activity is modulated during the course of poliovirus infection (Virgen-Slane et al., 2012). However, a recent report showed that removal of VPg from the 5' end of enterovirus RNA is not required for *in vitro* translation and RNA replication (Langereis et al., 2014). To determine if TDP2 activity is required for replication of the prototypic enterovirus, poliovirus, we characterized the viral growth kinetics and quantified the viral yields of poliovirus-infected MEF cells lacking TDP2. Since mouse cells do not express the poliovirus receptor (PVR), they are not naturally susceptible to poliovirus infection. To bypass this limitation, we purified poliovirus vRNA (VPg-RNA) and transfected the vRNA into HeLa, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers. Poliovirus vRNA was transfected into HeLa cells as a positive control to measure maximum viral yields. We quantified viral yields by plaque assay and plotted titers [plaque-forming units (PFU) per cell] on a logarithmic scale. Poliovirus replicated to approximately 2 log$_{10}$ units lower in MEF-TDP2$^{+/+}$ cells than in HeLa cells, showing that poliovirus replicates less efficiently in this mouse cell line compared to HeLa cells (**Figure 2.4**). Significantly, poliovirus yields in mouse cells are reduced by up to 2 log$_{10}$ units in the absence of TDP2. These results demonstrate that TDP2 is necessary for efficient poliovirus replication following poliovirus vRNA transfection into MEF cells.
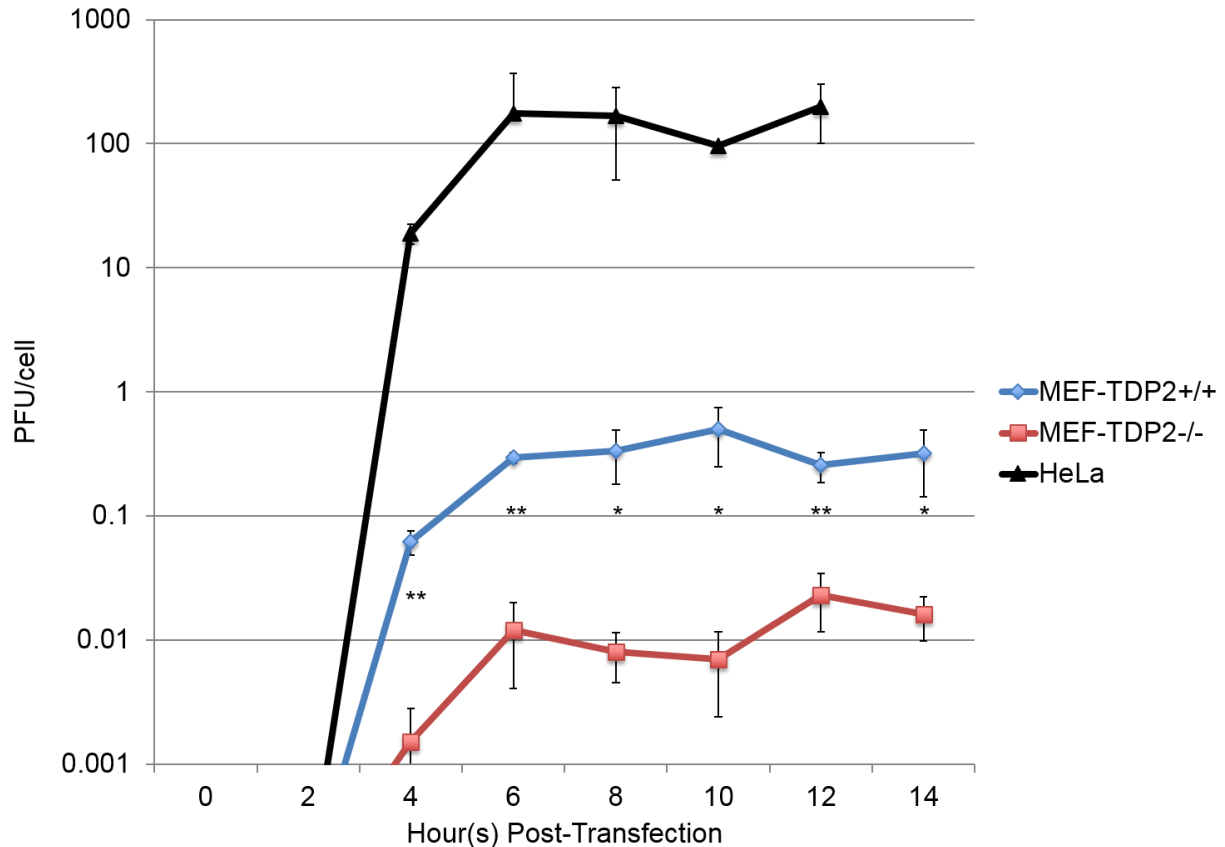
Figure 2.4. TDP2 is used for efficient poliovirus replication following poliovirus vRNA transfection in mouse embryonic fibroblasts. Single-cycle growth analysis was carried out in HeLa cells, MEF-TDP2$^{+/+}$, or MEF-TDP2$^{-/-}$ cell monolayers following DEAE-dextran transfection of 1 µg of purified poliovirus VPg-vRNA. Cells and supernatant were harvested every 2 hours up to 14 hours post-transfection and subjected to 5 freeze-thaw cycles to release virus particles. Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to transfection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviation of the results from triplicate plaque assays. An asterisk (*) (Student's t test; $P < 0.05$) or a double asterisk (**) (Student's t test; $P < 0.01$) indicates statistical significance between MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ data points.

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TDP2 is required for efficient poliovirus replication in mouse embryonic fibroblasts stably expressing the human poliovirus receptor

Although poliovirus yields were significantly reduced in the absence of TDP2 following vRNA transfection, there are experimental caveats that may contribute to reduced viral yields. In particular, transfection of viral RNA bypasses normal cellular receptor-mediated entry pathways. It is likely that the uncoating of the vRNA following a normal infection determines the cytoplasmic delivery site of the vRNA and which end of the vRNA is initially exposed to the cytoplasm (Harutyunyan et al., 2014). To circumvent this potential limitation, we generated MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cell lines stably expressing the human poliovirus receptor (PVR) (Mendelsohn et al., 1986; Mendelsohn et al., 1989) under blasticidin selection. Due to ambiguous results when attempting to verify PVR protein expression in the stable cell lines by Western blot analysis (data not shown), PVR mRNA expression was determined by reverse-transcription (RT)-PCR analysis (**Figure 2.5 A**). Low levels of PVR mRNA were detected in the MEF-TDP2$^{-/-}$ cell line stably expressing PVR compared to the MEF-TDP2$^{+/+}$ cell line stably expressing PVR (**Figure 2.5 A, lanes 4 and 6**). However, a previous report demonstrated that two different transgenic mouse lines expressing the human PVR were similar in poliovirus susceptibility despite differences in PVR RNA and protein expression (Deatly et al., 1998). MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ stable cell lines with PVR were infected with poliovirus, and viral yields were quantified by plaque assay. We found that poliovirus replicates somewhat less efficiently in the MEF-TDP2$^{+/+}$ cells expressing PVR compared to HeLa cells (**Figure 2.5 B**). Interestingly, poliovirus did not replicate in the MEF-TDP2$^{-/-}$ cell line stably expressing PVR (**Figure 2.5 B**), suggesting that TDP2 is required for productive poliovirus infection. Additionally, we did not detect nonstructural viral protein 3A and its precursor 3AB accumulation in the poliovirus-infected MEF-TDP2$^{-/-}$ cell line stably expressing

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PVR by Western blot analysis (data not shown). Since Western blot analysis may not be sensitive enough to detect low levels of viral protein synthesis, we infected the HeLa cells and MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cells stably expressing PVR with poliovirus and radiolabeled the viral proteins with ^{35}S-methionine. Following infection, we immunoprecipatated poliovirus proteinase 3C and its precursors 3C' and 3CD from the lysates generated from the infected cells using an antibody against 3C (**Figure 2.5 C**). As expected, we found that the viral proteinases 3C, 3C', and 3CD were highly expressed in the poliovirus-infected HeLa cells by 5 h.p.i. (**Figure 2.5 C, lane 4**). The viral proteinase 3C, 3C', and 3CD expression was reduced but detectable at 5 and 6 h.p.i. in the poliovirus-infected MEF-TDP2$^{+/+}$ cell line stably expressing PVR (**Figure 2.5 C, lanes 7 and 8**). In contrast, no viral proteinase 3C, 3C', or 3CD expression was detected in the MEF-TDP2$^{-/-}$ cells stably expressing PVR (**Figure 2.5 C, lanes 11 and 12**) suggesting that viral proteins were not synthesized at detectable levels in our MEF-TDP2$^{-/-}$ stable cell line expressing PVR. No viral protein expression was detected in the control MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell lines not expressing PVR by 6 hours post-infection (**Figure 2.5 C, lanes 5 and 9**). To verify that the MEF-TDP2$^{-/-}$ stable cell line was fully susceptible to poliovirus infection, we infected the MEF stable cell lines with a 5-fold increase in multiplicity of infection (MOI) and quantified the viral yields by plaque assay (**Figure 2.5 D**). Similar results were observed at an MOI of 100 compared to an MOI of 20, confirming that TDP2 was required for poliovirus infection in these stable cells expressing low levels of PVR.
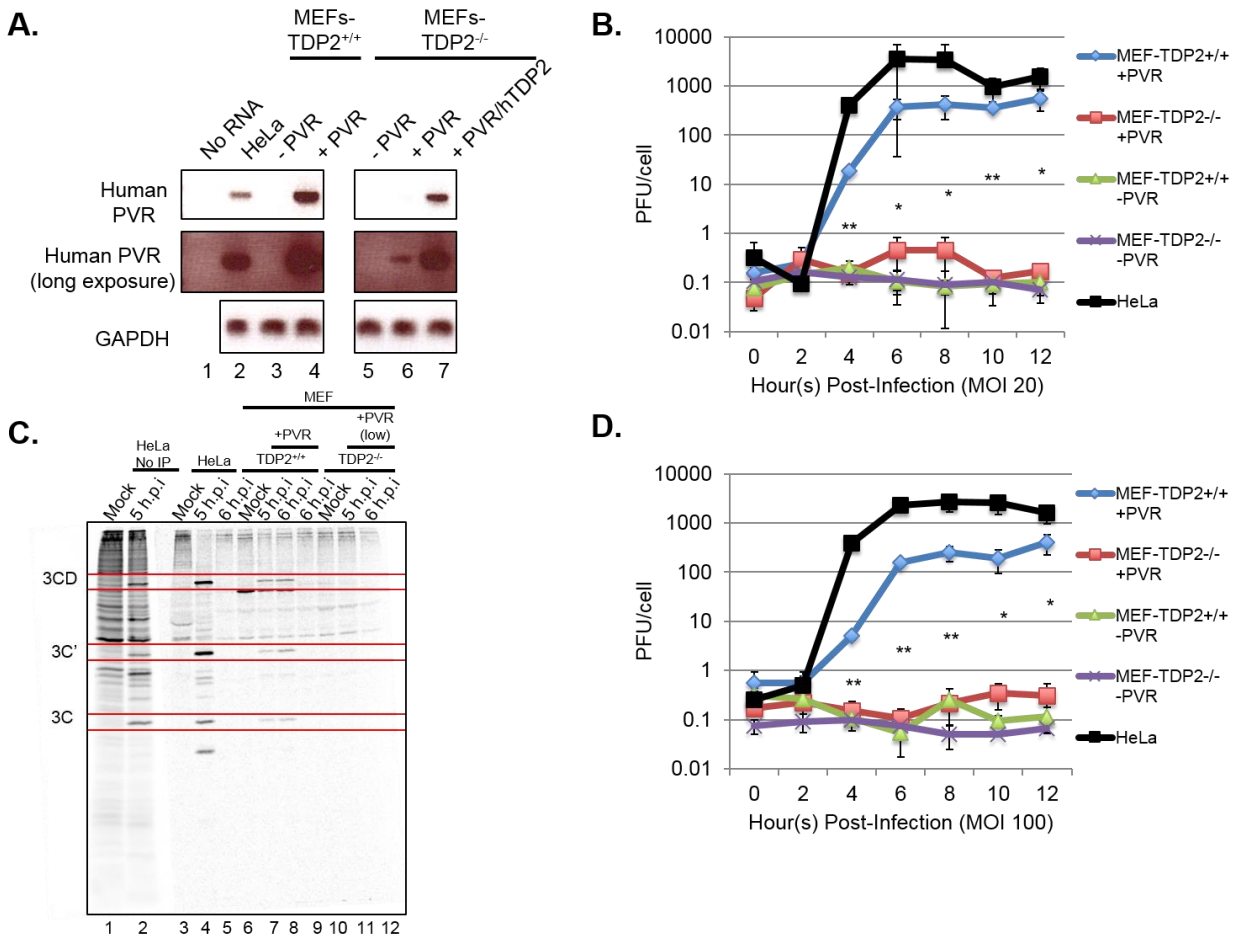
Figure 2.5. TDP2 is required for poliovirus infection in mouse embryonic fibroblasts expressing low levels of poliovirus receptor. (A) Human PVR mRNA expression in the MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ stable cell lines was verified by RT-PCR analysis. PCR products were separated by electrophoresis on an ethidium bromide stained 1% agarose gel. A longer exposure of the gel is shown to verify PVR mRNA expression in the MEFs-TDP2$^{-/-}$ stable cell line (lane 6). The image of the gel depicting human PVR mRNA expression of the MEF-TDP2$^{+/+}$ stable cell line co-expressing PVR and human TDP2 has been cropped. Single-cycle growth analysis was carried out in HeLa cells, MEF-TDP2$^{+/+}$, or MEF-TDP2$^{-/-}$ stable cell line monolayers following poliovirus infection at an MOI of 20 **(B)** or an MOI of 100 **(D)**. Cells and supernatant were collected every 2 hours up to 12 hours post-infection (h.p.i.). Virus yields (PFU) were quantified

by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviation of the results from triplicate plaque assays. An asterisk (*) (Student's t test; $P < 0.05$) or a double asterisk (**) (Student's t test; $P < 0.01$) indicates statistical significance between MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ stable cell lines. (**C**) Viral protein synthesis analysis was assayed in HeLa cells, MEF-TDP2$^{+/+}$, or MEF-TDP2$^{-/-}$ stable cell line monolayers either mock-infected or infected with poliovirus at an MOI of 20 and radiolabeled with ^{35}S-methionine following a 2 hour methionine starvation post-infection. Cells were collected at either 5 or 6 h.p.i. and used to generate RIPA lysates. Lysates were subjected to immunprecipitation using an antibody to detect the poliovirus proteinase 3C and its precursor proteinases 3C' and 3CD. Immunoprecipitated lysates were subjected to SDS-PAGE and the viral proteins were visualized by autoradiography.

Although a previous report indicated that variable PVR mRNA and protein expression does not significantly affect poliovirus susceptibility (Deatly et al., 1998), we wanted to further verify that the phenotype we observed in **Figures 2.5 B-D** was due to the absence of TDP2 and not to low levels of PVR. We generated two additional MEF-TDP2$^{-/-}$ cell lines stably expressing higher levels of PVR mRNA than our original transformants, referred to as medium and high PVR-expressing cell lines based upon the quantified band intensity of PVR mRNA on ethidium-stained agarose gels following RT-PCR analysis (**Figure 2.6 A, lanes 7 and 8**). We also generated a MEF-TDP2$^{-/-}$ stable cell line co-expressing PVR and human TDP2 (hTDP2) (Pei et al., 2003) to confirm that the phenotype we observed in the MEF-TDP2$^{-/-}$ cell lines was due to the absence of TDP2 and not an off target effect of TDP2 gene ablation (**Figure 2.6 A, lane 9**). TDP2 expression was verified in the MEF-TDP2$^{-/-}$ stable cell line co-expressing PVR and hTDP2 by RT-PCR analysis (**Figure 2.6 A, lane 9**). Additionally, mouse TDP2 was confirmed to be absent from the MEF-TDP2$^{-/-}$ stable cell lines (**Figure 2.6 A, lanes 5-9**).

To determine if TDP2 is required for poliovirus infection, each of the cell lines described above was infected with poliovirus and viral yields were quantified by plaque assay (**Figure 2.6 B**). Similar to the previous single-cycle growth analysis in **Figure 2.5**, poliovirus growth kinetics were reduced in the MEF-TDP2$^{+/+}$ cells expressing PVR compared to HeLa cells. In contrast to the previous single-cycle growth analysis of poliovirus in the MEF-TDP2$^{-/-}$ cell line expressing low levels of PVR mRNA, poliovirus replicates in the two additional MEF-TDP2$^{-/-}$ stable cell lines expressing medium and high levels of PVR mRNA, albeit with a significant (1-2 $\log_{10}$ unit) reduction in viral yields compared to the MEF-TDP2$^{+/+}$ cells expressing PVR. The 0.5 $\log_{10}$ unit increase observed over the time course in the MEF-TDP2$^{-/-}$ cell line expressing low levels of PVR mRNA does not represent a delay in poliovirus growth kinetics since we confirmed no increase in

viral yields at 24 hours post-infection (**Figure 2.6 C**). To confirm that the reduced viral titers observed were due to the absence of TDP2, we quantified the viral yields from poliovirus-infected MEF-TDP2$^{-/-}$ cells co-expressing PVR and hTDP2 and found that poliovirus yields could be rescued by hTDP2 expression (**Figure 2.6 B**). Additionally, we imaged the poliovirus-infected HeLa cells and MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ stable cell lines expressing PVR to determine if we could observe a difference in the cytopathic effect (CPE) by 24 hours post-infection. (**Figure 2.6 D**). CPE is observed when there is an apparent change in cell morphology, such as cell lysis caused by viral infection. As expected, we found the infected HeLa cell lines to exhibit full CPE by 12 hours post-infection. The MEF-TDP2$^{+/+}$ stable cell line expressing PVR showed minimal CPE at 24 hours post-infection. Interestingly, the MEF-TDP2$^{-/-}$ stable cell lines expressing variable levels of PVR showed no CPE by 24 hours post-poliovirus infection, possibly due to reduced viral replication occurring in the absence of TDP2 or inefficient virus egress in the absence of TDP2. Together, these results demonstrate that although virus yields are affected by the levels of PVR mRNA in transformed MEF cells, TDP2 is required for efficient poliovirus replication in mouse cells.

A.

	No RNA	HeLa	MEFs-TDP2+/+		MEFs-TDP2-/-				
			- PVR	+ PVR	- PVR	+ PVR (low)	+ PVR (medium)	+ PVR (high)	+ PVR/hTDP2

Human PVR

Human PVR (long exposure)

Human TDP2

Mouse TDP2

GAPDH
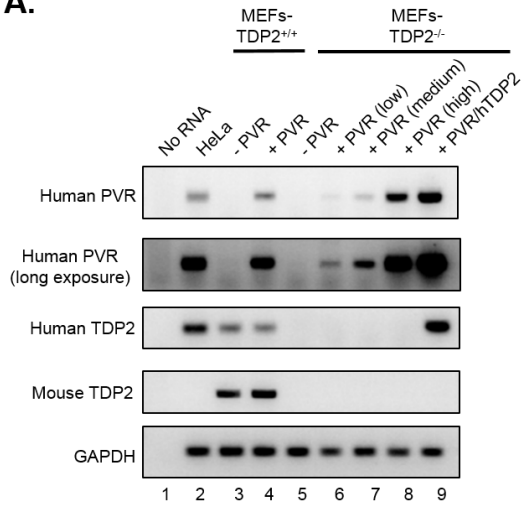
	1	2	3	4	5	6	7	8	9
Band Intensity (%):	0	100	0	90	0	2.8	27	306	403

B.

C.

D.

HeLa

Mock   12 h.p.i

MEF-TDP2⁺/⁺

Mock   24 h.p.i

+PVR

MEF-TDP2⁻/⁻

Mock   24 h.p.i

+ PVR (low)

+ PVR (medium)

+ PVR (high)
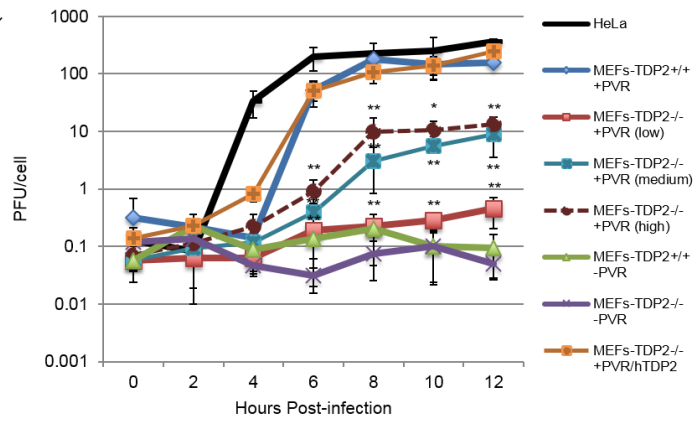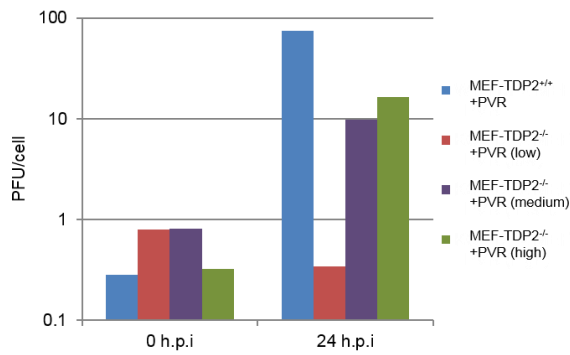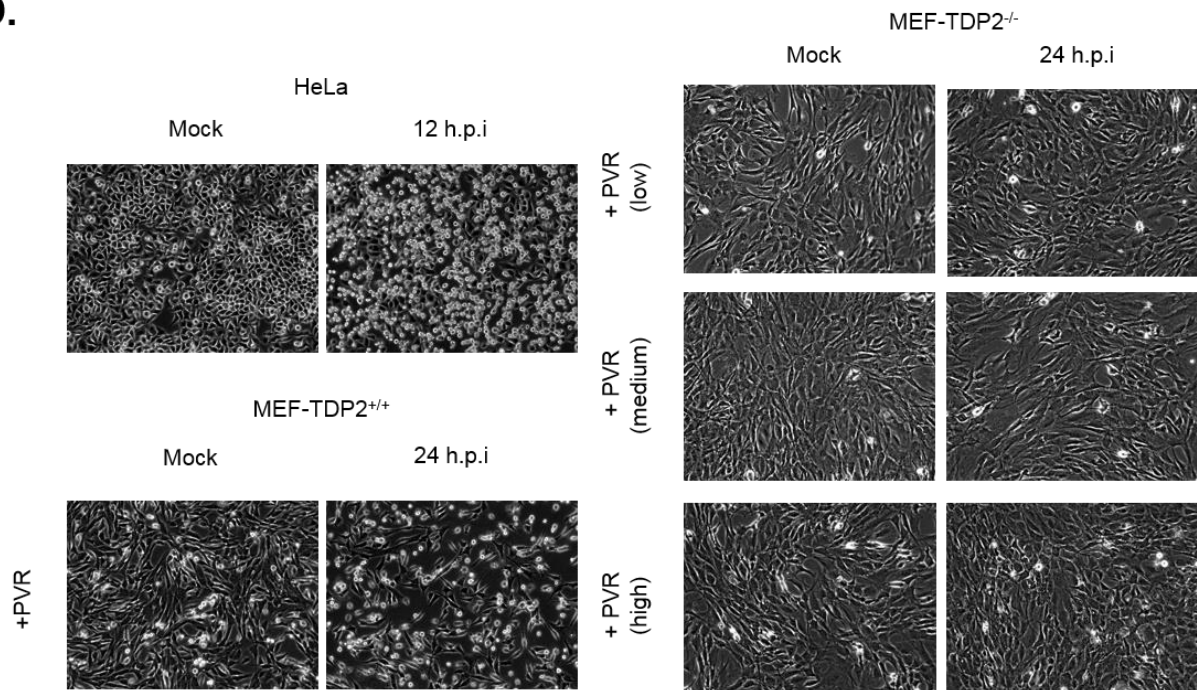
Figure 2.6. TDP2 is used for efficient poliovirus replication following infection of mouse embryonic fibroblasts stably expressing the poliovirus receptor. (A) Human PVR and human and mouse TDP2 mRNA expression in the MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ stable cell lines was verified by RT-PCR analysis. PCR products were separated by electrophoresis on an ethidium bromide stained 1% agarose gel. A longer exposure of the gel depicting human PVR mRNA expression is shown. Human PVR band intensity was quantified using Quantity One software (Bio-Rad). The panels shown in the figure were cropped from the original images. **(B)** Single-cycle growth analysis was carried out in HeLa cells, MEF-TDP2$^{+/+}$, or MEF-TDP2$^{-/-}$ stable cell line monolayers following poliovirus infection at an MOI of 20. Cells and supernatant were collected every 2 hours up to 12 hours post-infection (h.p.i.). Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviation of the results from triplicate plaque assays. An asterisk (*) (Student's t test; P <

0.05) or a double asterisk (**) (Student's t test; $P < 0.01$) indicates statistical significance between virus titers produced in MEF-TDP2$^{+/+}$ versus MEF-TDP2$^{-/-}$ stable cell lines. **(C)** MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ stable cell line monolayers were infected with poliovirus at an MOI of 20. Cells and supernatant were collected at 0 and 24 h.p.i.. Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were averaged from duplicate plaque assays and were plotted on a logarithmic scale. **(D)** HeLa and MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ stable cell lines expressing variable levels of PVR were infected with poliovirus at an MOI of 20. HeLa cells were imaged at 12 h.p.i. and the MEF-TDP2 stable cell lines were imaged at 24 h.p.i. using phase-contrast microscopy.

To determine if TDP2 has an impact on poliovirus translation or RNA replication, we analyzed viral protein accumulation in the presence or absence of TDP2. We performed Western blot analysis using an antibody against poliovirus non-structural protein 3A and its precursor 3AB, which are involved in poliovirus RNA replication (Giachetti et al., 1992; Towner et al., 1996) (**Figure 2.7 A**). Viral proteins 3A and 3AB accumulation was reduced at 8 h.p.i. in the MEFs-TDP2$^{+/+}$ cells expressing PVR compared to poliovirus-infected HeLa cells (**Figure 2.7 A, lanes 3 and 7**). Viral proteins 3A and 3AB were undetectable in the poliovirus-infected MEFs-TDP2$^{-/-}$ expressing low levels of PVR (**Figure 2.7 A, lane 11**) and detectable at reduced levels in the MEFs-TDP2$^{-/-}$ cell lines expressing medium and high levels of PVR at 8 h.p.i. (**Figure 2.7 A, lanes 14 and 17**). The lack of accumulation of viral proteins 3A and 3AB was rescued in the MEF-TDP2$^{-/-}$ cells co-expressing PVR and hTDP2 (**Figure 2.7 A, lane 20**) to wild-type levels. Collectively, these results agree with the single-cycle viral growth analyses in **Figure 2.4 and Figure 2.7 B**, showing that viral protein accumulation is reduced in the absence of TDP2, just as viral yields are markedly reduced in the absence of TDP2.

To further characterize the role of TDP2 during viral RNA translation and replication, we used a previously published infectious poliovirus construct encoding a *Renilla* luciferase gene (RLuc-PV-PPP) at the N-terminus of the coding region, followed by a 3CD proteinase cleavage site, to generate *in vitro* transcribed viral RNA (Liu et al., 2010). The non-VPg linked viral RNA was transfected into MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell monolayers in the presence or absence of the enterovirus negative-strand RNA synthesis inhibitor, guanidine HCl (GuHCl) (Barton and Flanegan, 1997; Pincus et al., 1986; Pincus and Wimmer, 1986). We then measured luciferase expression at 6 and 12 hours post-transfection and found that in the presence of GuHCl, luciferase expression was similar between MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cells, but in the absence of GuHCl,

luciferase expression was reduced in the MEF-TDP2$^{-/-}$ (**Figure 2.7 B**). To determine if the RNA replication phenotype observed in MEF-TDP2$^{-/-}$ in **Figure 2.7 B** was due to the premature encapsidation of the newly synthesized VPg-linked viral RNA, we investigated poliovirus translation and RNA replication in the absence of capsid proteins (**Figure 2.7 C**). To accomplish this, we used a previously published non-infectious poliovirus construct encoding a *Firefly* luciferase gene (FLuc-PV-PP) in place of the capsid proteins sequence to generate *in vitro* transcribed viral RNA (Andino et al., 1993). The non-VPg linked viral RNA was transfected into MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell monolayers in the presence or absence of GuHCl. We next measured luciferase expression at 6 and 12 hours post-transfection and found that in the presence of GuHCl, luciferase expression was similar between MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cells despite not being able to be visualized in the figure. A similar phenotype was observed in the absence of GuHCl by 12 hours post-transfection, confirming that in the absence of capsid proteins, viral replication is similar in the presence or absence of TDP2. However, we did find that viral RNA replication was slightly reduced at 6 hours post-transfection in the absence of TDP2, suggesting that TDP2 is also playing a role during viral RNA replication. These results demonstrate that an encapsidation incompetent reporter replicates similarly in both MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cells. This opens up the possibility that premature encapsidation may be occurring in the absence of TDP2 and contributing to the observed phenotype during poliovirus infection in the absence of TDP2.
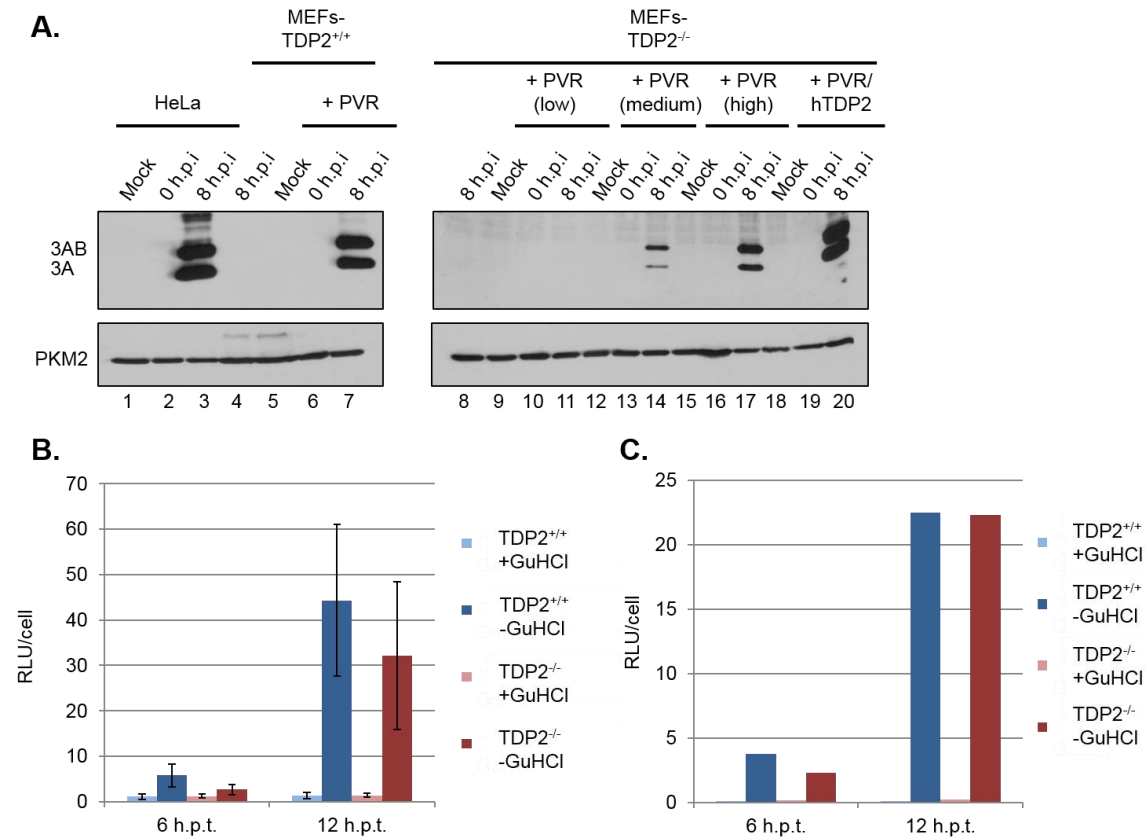
Figure 2.7. Poliovirus RNA translation and replication are reduced in the absence of TDP2.

(**A**) HeLa cell monolayers, MEF-TDP2^{+/+} and MEF-TDP2^{-/-} stable cell line monolayers were mock- or poliovirus-infected at an MOI of 20. Cells and supernatant were collected at 0 or 8 hours post-infection (h.p.i.) and used to generate NP-40 lysates. NP-40 lysate protein concentration was determined by Bradford assay. Mock- or poliovirus-infected NP-40 lysate was subjected to SDS-PAGE and Western blot analysis using anti-poliovirus 3A antibody or anti-PKM2 antibody (loading control) to visualize proteins. (**B**) Infectious poliovirus RNA encoding a *Renilla* luciferase (RLuc) reporter gene sequence following the 5' NCR was *in* vitro transcribed from the RLuc-PV-PPP construct (Liu et al., 2010). MEF-TDP2^{+/+} and MEF-TDP2^{-/-} cell monolayers were transfected with 1 µg of infectious poliovirus RNA in the presence or absence of 5 mM GuHCl. Cells were washed and lysed with 1X Renilla luciferase lysis buffer at 6 or 12 hours post-transfection (h.p.t.).

Samples were collected and subjected to the *Renilla* luciferase assays to measure RLuc values. RLuc values (RLU/s) were divided by the total cell count prior to transfection (RLU/cell). The increase of RLU values in the absence of GuHCl indicates the contribution of viral RNA replication. The error bars indicate standard error of the mean of the results from three biological replicate experiments. The difference in RLU/cell values between the cells treated with GuHCl and those not treated with GuHCl indicate that viral RNA synthesis is inhibited in the presence of GuHCl. **(C)** Non-infectious poliovirus RNA encoding a *Firefly* luciferase (FLuc) reporter sequence inserted in the place of the structural proteins region was *in* vitro transcribed from the FLuc-PV-PP construct (Andino et al., 1993). MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cell monolayers were transfected with the RNA in the presence or absence of 5 mM GuHCl. Cells were washed and lysed with 1X Passive Lysis Buffer (Promega) at 6 or 12 h.p.t. Samples were collected and subjected to the luciferase assays to measure FLuc values. FLuc values (RLU/s) were divided by the total cell count prior to transfection (RLU/cell). The increase of RLU/cell values in the absence of GuHCl indicates the contribution of viral RNA replication. The values were derived from the mean of two biological replicate experiments. No standard error of the mean or standard deviation was calculated for the mean of the two biological replicate experiments.

CVB3 replication is severely impaired in the absence of TDP2 in mouse embryonic fibroblasts

We next determined if TDP2 was required during infection by the closely related enterovirus, CVB3. Mouse cells, like human cells, are naturally susceptible to CVB3 infection because they express the coxsackievirus adenovirus receptor (CAR). We infected MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cell monolayers with CVB3 and found that the viral growth was delayed and reduced in the MEF-TDP2$^{+/+}$ cells compared to HeLa cells (**Fig. 2.8 A**). Significantly, viral yields did not increase in the absence of TDP2 over the 16-hour time course, while CVB3 increased up to 2 log$_{10}$ in the MEFs-TDP2$^{+/+}$, showing that TDP2 is essential for CVB3 replication (**Fig. 2.8 A**).

To determine the effect of TDP2 on viral translation and RNA synthesis, we analyzed viral protein production in the MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cells by Western blot analysis using an antibody against the CVB3 non-structural protein 3A and its precursor 3AB (Cornell et al., 2006). We found that 3A and 3AB accumulation is reduced in the CVB3-infected MEFs-TDP2$^{+/+}$ at 8 h.p.i. compared to HeLa cells (**Fig. 2.8 B, lane 3 versus 6**). In agreement with our single-cycle growth analysis of CVB3 in the MEF-TDP2$^{-/-}$ cell line, we found that 3A and 3AB do not accumulate to detectable levels in the absence of TDP2 (**Fig. 2.8 B, lane 9**). Since Western blot analysis may not be sensitive enough to detect low levels of viral protein accumulation, we transfected full-length CVB3 RNA encoding a *Renilla* luciferase (RLuc) reporter gene, generated from an infectious RLuc-CVB3 clone (Lanke et al., 2009), into MEF-TDP2$^{+/+}$ cells and MEF-TDP2$^{-/-}$ cells in the presence or absence of the viral RNA synthesis inhibitor guanidine HCl (GuHCl) and measured luciferase expression over a 12-hour time course. Since the transfected RNA was synthesized *in vitro* using the bacteriophage T7 RNA polymerase and a promoter element, it lacked an authentic VPg linkage to the 5' end of the RNA, and we therefore predicted

that transfected RNA would be translated in a TDP2-independent manner. As expected, in the presence of GuHCl, viral RNA was translated in the presence or absence of TDP2 (**Fig. 2.8 C**). Although still detectable, in the absence of the viral RNA synthesis inhibitor GuHCl, viral RNA replication was reduced in the MEF-TDP2$^{-/-}$ cell line at 8 and 12 hours post-transfection compared to the wild type MEF cell line (**Fig. 2.8 C**).
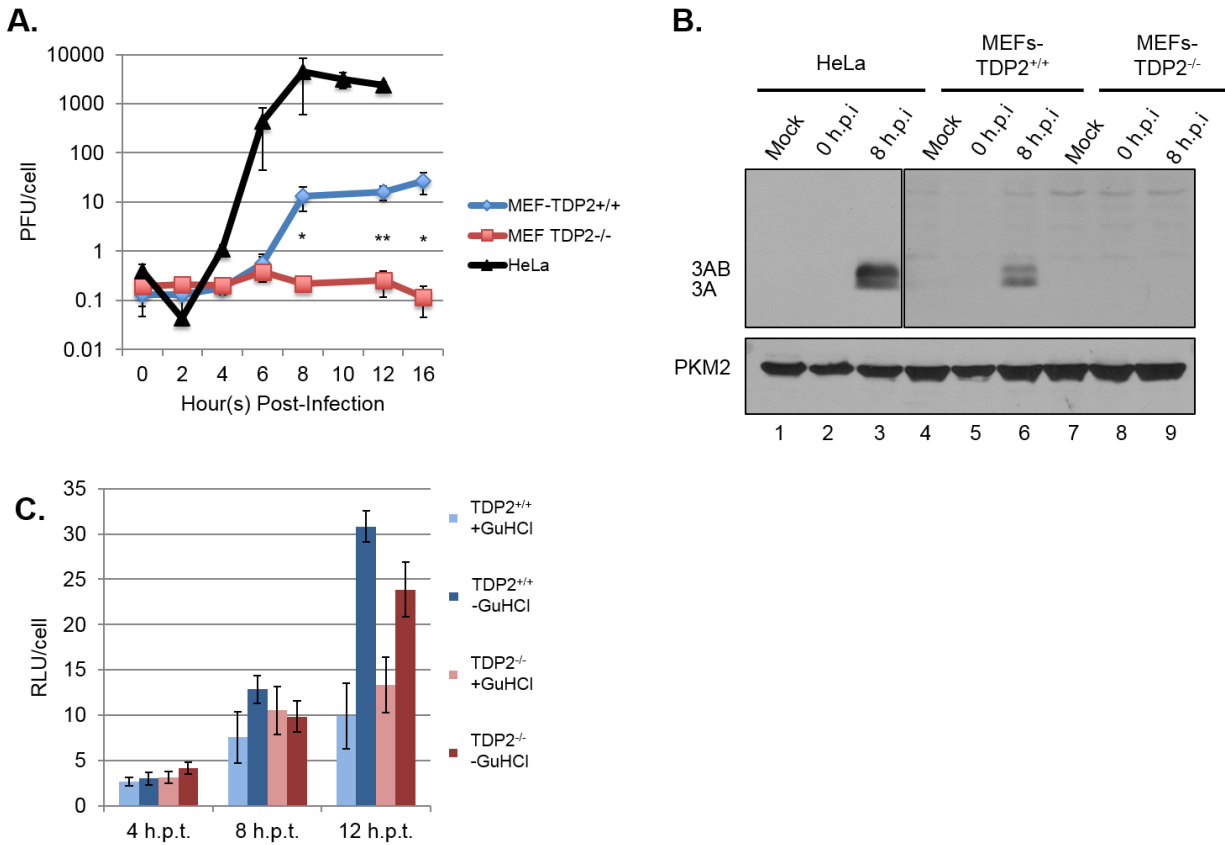
Figure 2.8. TDP2 is required for CVB3 infection in mouse embryonic fibroblasts. (A) Single-cycle growth analysis was carried out in HeLa cells, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ monolayers following infection with CVB3 at an MOI of 20. Cells and supernatant were collected every 2 hours up to 16 hours post-infection (h.p.i.). Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviation of the results from triplicate plaque assays. An asterisk (*) (Student's t test; $P < 0.05$) or a double asterisk (**) (Student's t test; $P < 0.01$) indicates statistical significance between MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ data points. **(B)** The HeLa, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers were mock- or CVB3-infected at an MOI of 20. Cells and supernatant were collected at 0 or 8 h.p.i. and used to generate NP-40 lysates. NP-40 lysate protein concentration was

determined by Bradford assay. Mock- or CVB3-infected NP-40 lysate was subjected to SDS-PAGE and Western blot analysis using an anti-CVB3 3A antibody or anti-PKM2 antibody (loading control) to visualize proteins. The image in **(B)** was cropped due to a longer exposure of the SDS-containing polyacrylamide gel resolving the NP-40 lysates from CVB3-infected MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$cells. **(C)** Infectious CVB3 RNA encoding a *Renilla* luciferase (RLuc) reporter gene was *in vitro* transcribed from an infectious RLuc-CVB3 clone (p53CB3/T-7-Rluc) (Lanke et al., 2009). RLuc-CVB3 RNA (2 µg) was transfected into HeLa cell, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers in the presence or absence of 2.5 mM GuHCl. Cells were washed and lysed with 1X Renilla luciferase lysis buffer at 4, 8, or 12 hours post-transfection (h.p.t.). Samples were collected and subjected to the *Renilla* luciferase assays to measure RLuc values. RLuc values (RLU/s) were divided by the total cell count prior to transfection (RLU/cell). The increase of RLuc/cell values in the absence of GuHCl indicates the contribution of viral RNA replication. The error bars indicate standard error of the mean of the results from three biological replicate experiments.

Since viral RNA replication occurs in the absence of TDP2 following CVB3 RNA transfection (**Figure 2.8 C**), we wanted to verify that CVB3 replication was not merely delayed in the absence of TDP2. We quantified viral yields in CVB3-infected MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cells at 24 hours post-infection and found that viral yields did not increase at 24 hours post-infection in the absence of TDP2 (**Fig. 2.9 A**). These data show that CVB3 replication was not delayed in the MEF-TDP2$^{-/-}$ cell line. Additionally, we monitored the CPE during CVB3 infection of the MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cells at 24 hours post-infection by phase-contrast microscopy (**Figure 2.9 B**). We found that no CPE occurred in either of the CVB3-infected MEF-TDP2$^{+/+}$ or TDP2$^{-/-}$ cell monolayers, suggesting that CVB3 does not induce CPE during infection of mouse embryonic fibroblasts. Alternatively, since there is a reduced level of viral replication occurring in these cell lines, these cells may not show changes in cell morphology. Collectively, our results show that CVB3 RNA can be translated and replicated in the absence of TDP2 following transfection of CVB3 RNA; however, viral replication following CVB3 infection accumulation is dramatically impaired in the absence of TDP2. Furthermore, reduced replication in the MEF-TDP2$^{-/-}$ cells does not delay the onset of CVB3 replication.
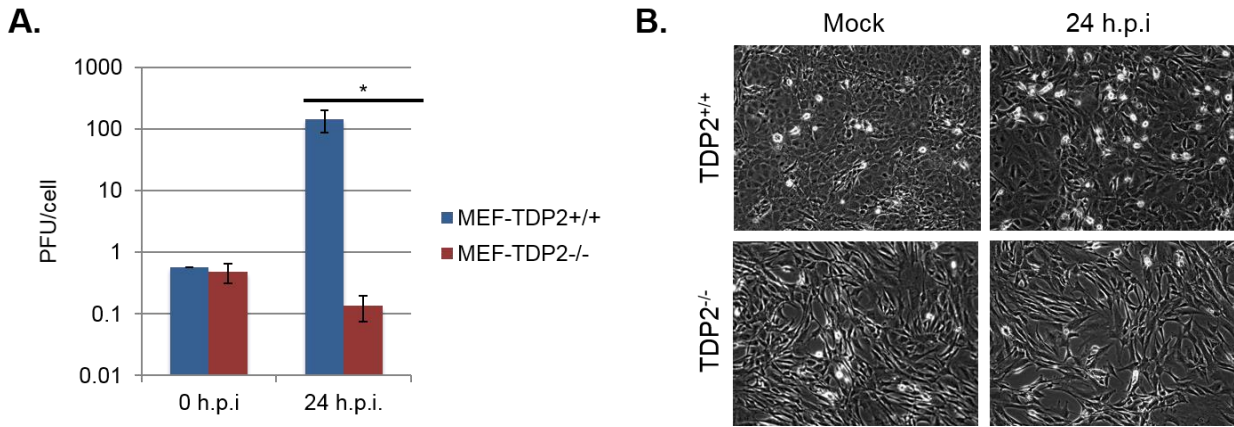
Figure 2.9. CVB3 replication is not delayed in the absence of TDP2. (A) MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cell monolayers were infected with CVB3 at an MOI of 20. Cells and supernatant were collected at 0 and 24 hours post-infection (h.p.i.). Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviation of the results from triplicate plaque assays. An asterisk (*) (Student's t test; $P < 0.05$) indicates statistical significance between MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ data points. **(B)** MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cell monolayers were either mock-infected or CVB3-infected at an MOI of 20. The mouse cell lines were imaged at 24 h.p.i. using phase-contrast microscopy.

The CVB3 5' NCR is not solely responsible for the severe decrease in CVB3 yields in the absence of TDP2

To further characterize the role of TDP2 during CVB3 infection, we used a previously published chimeric poliovirus (PCV-305), which contains the CVB3 5' NCR in place of the poliovirus 5' NCR (Johnson and Semler, 1988), to determine if the RNA stem-loops present within the CVB3 5' NCR are TDP2-dependent for efficient viral translation and replication. We used HeLa cells and MEF-TDP2$^{+/+}$and MEF-TDP2$^{-/-}$ cells stably expressing PVR for our single-cycle growth analysis since the chimeric virus encodes the poliovirus capsid proteins, which are responsible for binding PVR. We used the MEF-TDP2$^{-/-}$ stable cell line expressing the highest level of PVR mRNA to carry out these experiments since it is most susceptible to poliovirus infection. We infected the three cell lines with PCV-305 at an MOI of 20 and quantified the viral yields every 2 hours post-infection for up to 12 hours (**Figure 2.10 A**). We found that PCV-305 yields were reduced in the MEF-TDP2$^{+/+}$ cells in comparison to the HeLa cells as observed with poliovirus and CVB3. Interestingly, the chimeric virus replicated in the absence of TDP2, but viral yields were reduced ~1 log$_{10}$ unit over the 12 hour time course. These results suggest that the CVB3 5' NCR alone is not responsible for the dramatic decrease in viral yields observed in the absence of TDP2 during CVB3 infection since we find that the chimeric virus can replicate in the absence of TDP2, albeit at reduced levels.

Next, we examined the role of the CVB3 5' NCR during viral translation and RNA replication. We performed a Western blot analysis using antibodies against poliovirus nonstructural protein 3A and its precursor 3AB. We found that viral protein accumulation was slightly reduced in the PCV-305-infected MEF-TDP2$^{+/+}$ cells stably expressing PVR in comparison to HeLa cells (**Figure 2.10 B, lanes 7 and 8**), despite having a ~1.5 log$_{10}$ unit decrease

in viral yields. We observed a dramatic decrease in viral protein accumulation in the absence of TDP2 at both 8 and 10 hours post-infection (**Figure 2.10 B, lanes 11 and 12**), despite its ~1 $\log_{10}$ unit reduction in titers. Although we cannot rule out that the CVB3 5' NCR is playing a role in reduced viral titers and viral protein accumulation during CVB3 infection, it is not exclusively responsible for the dramatic phenotype observed during CVB3 infection in the absence of TDP2.
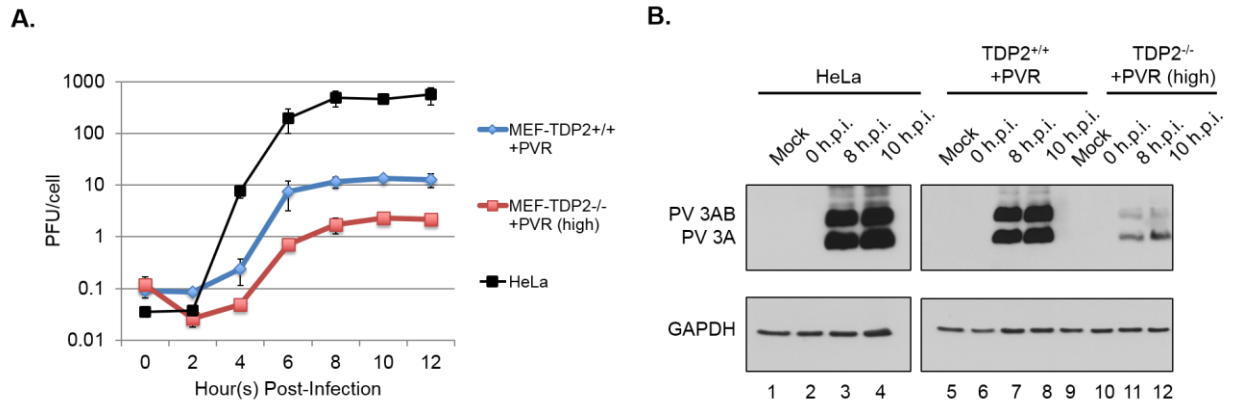
Figure 2.10. PCV-305 viral replication and protein accumulation is reduced in the absence of TDP2. (A) HeLa cells and MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cells stably expressing PVR at high levels were infected with PCV-305 at an MOI of 20. Cells and supernatant were collected every 2 hours over a 12 hour time course. Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviation of the results from triplicate plaque assays. **(B)** HeLa, MEFs-TDP2$^{+/+}$, and MEFs-TDP2$^{-/-}$ cell monolayers were mock- or PCV-305-infected at an MOI of 20. Cells and supernatant were collected at 0, 8, or 10 hours post-infection (h.p.i.) and used to generate NP-40 lysates. NP-40 lysate protein concentration was determined by Bradford assay. Mock- or PCV-305-infected NP-40 lysate was subjected to SDS-PAGE and Western blot analysis using an anti-PV 3A antibody or anti-GAPDH antibody (loading control) to visualize proteins. It is important to note that these stable cell lines have been passaged several times before being frozen down for tissue culture following the experiments done in **Figures 2.6 and 2.7** and thus may not be expressing as high of PVR mRNA levels as initially verified.

CVB3 infectivity is extinguished in the absence of TDP2

We next attempted to generate a CVB3 variant that could replicate in the absence of TDP2. We serially passaged CVB3 in MEF-TDP2$^{+/+}$ or MEF-TDP2$^{-/-}$ cell monolayers. Twenty blind serial passages were carried out in their respective cell line and viral yields were quantified for the first and twentieth blind passage in the MEF-TDP2$^{+/+}$ or TDP2$^{-/-}$ cell lines (**Figure 2.11**). We found that viral yields were increased by 1 log$_{10}$ unit when CVB3 was serially passaged in the MEF-TDP2$^{+/+}$ cell line. In contrast to the wild type cells, CVB3 infectivity was completely abolished by the twentieth serial blind passage in the absence of TDP2. Our results show that TDP2 is absolutely required for CVB3 infection in these mouse embryonic fibroblasts.

Human rhinovirus uses TDP2 in mouse embryonic fibroblasts for efficient viral replication

Finally, we determined if TDP2 was required for the replication of another picornavirus classified as an enterovirus, human rhinovirus (HRV). Since mouse embryonic fibroblasts lack the major HRV group receptor, ICAM-1, but express the minor HRV group receptor, low-density lipoprotein (LDLR), we infected the MEF-TDP2$^{+/+}$ cell line and TDP2$^{-/-}$ cell line with the minor rhinovirus group A member, HRV1a. We observed that viral yields were reduced by slightly more than 1 log$_{10}$ unit in the absence of TDP2 at peak viral titers (**Fig. 2.12**). These results show that TDP2 potentiates HRV1a replication in mouse embryonic fibroblasts; however this effect is less pronounced than what was observed for poliovirus and CVB3 replication.
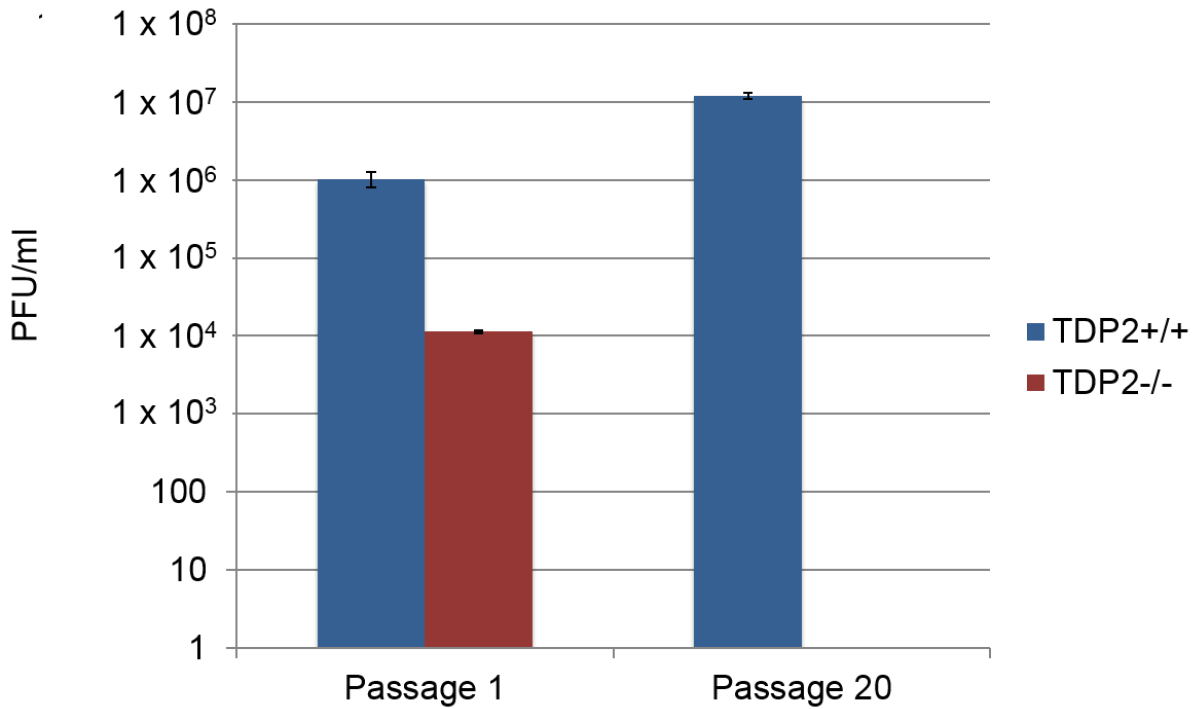
Figure 2.11. TDP2 is required for CVB3 infectivity. MEF-TDP2^{+/+} and MEF-TDP2^{-/-} cell monolayers were infected with CVB3 at an MOI of 20. Total cells and supernatant were collected 24 hours post-infection. Samples were subjected to 5 freeze-thaw cycles. 200 µl of the sample was then used to infect MEF-TDP2^{+/+} and MEF-TDP2^{-/-} cell monolayers. The cycle of using infected cells and supernatant was carried out over 20 passages in their respective cell line. Virus yields from the first and twentieth blind passage were quantified by plaque assay performed on HeLa cell monolayers. The error bars indicate standard deviation of the results from triplicate plaque assays.
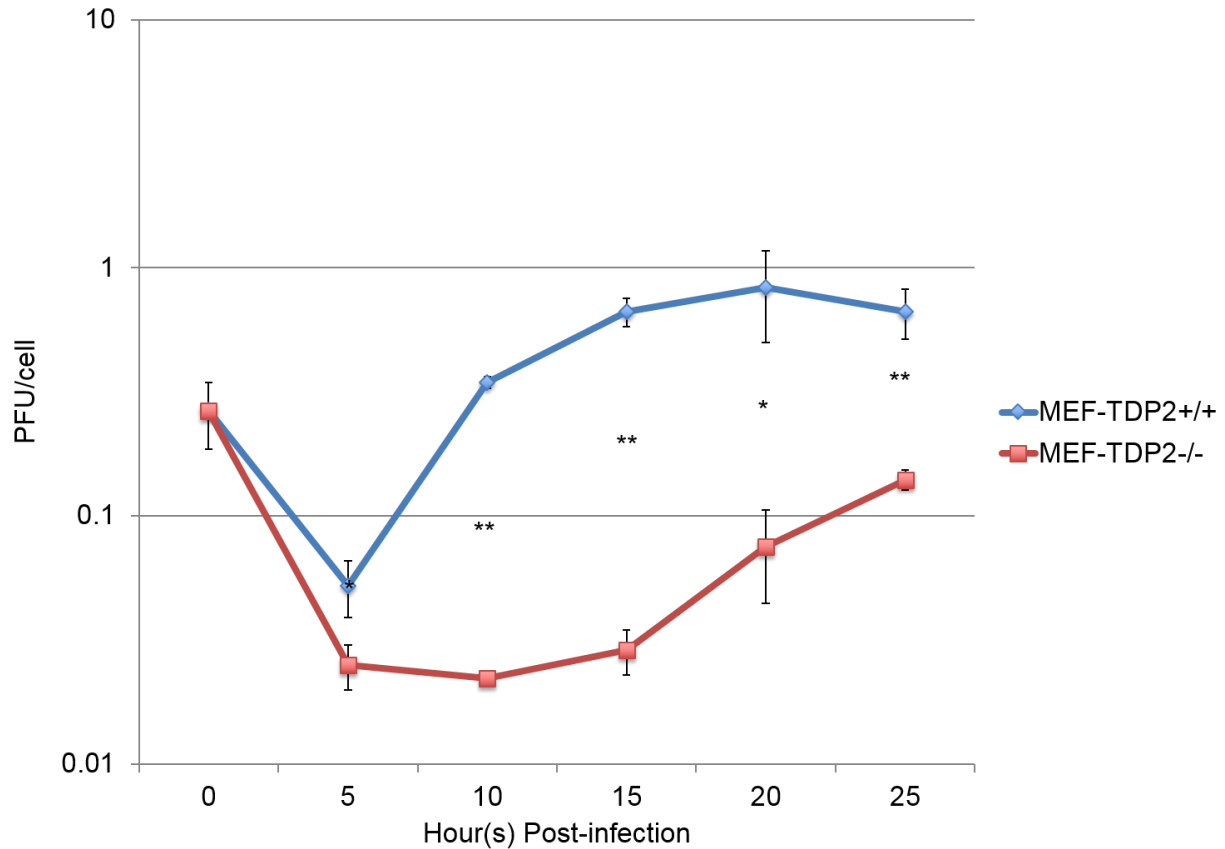
Figure 2.12. TDP2 is used for efficient rhinovirus replication in mouse embryonic fibroblasts. Single-cycle growth analysis was carried out in MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ monolayers following infection with the minor group A human rhinovirus (HRV1a) at an MOI of 20. Cells and supernatant were collected every 5 hours up to 25 hours post-infection. Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviation of the results from triplicate plaque assays. An asterisk (*) (Student's t test; $P < 0.05$) or a double asterisk (**) (Student's t test; $P < 0.01$) indicates statistical significance between MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ data points.

Discussion

In this chapter, we showed that the VPg unlinking activity of host cell DNA repair enzyme TDP2 is utilized during the course of enterovirus infections. This cellular activity was shown to hydrolyze the phosphotyrosyl linkage between the small viral protein VPg and the 5' end of poliovirus RNA (Ambros et al., 1978). Before the identification of VPg unlinkase as TDP2 (Virgen-Slane et al., 2012), VPg unlinkase was characterized as having hallmarks of a *bona fide* enzyme, its activity unaffected by the presence of exogenous VPg, and if assayed in crude extracts, its unlinking activity was unchanged over the course of poliovirus infection in HeLa cells (Ambros and Baltimore, 1980; Rozovics et al., 2011). However, following the identification of TDP2 as VPg unlinkase, TDP2 was shown to relocalize to the cell periphery in areas distinct from putative viral replication and encapsidation sites during peak times of poliovirus infection (Virgen-Slane et al., 2012). This relocalization pattern at peak times of infection suggests that TDP2 activity is used early on during infection, in agreement with previous studies showing that VPg is absent from ribosome-associated viral RNA (Fernandez-Munoz and Darnell, 1976; Hewlett et al., 1976; Nomoto et al., 1977b; Nomoto et al., 1976). Collectively, these previous findings suggest that TDP2 activity is modulated during the course of poliovirus infection. It is possible that the relocalization of TDP2 to the cell periphery at peak times of infection is a regulatory mechanism used by the virus to avoid premature removal of VPg from newly synthesized viral RNAs destined to become encapsidated. Although the functional role of TDP2 during the course of enterovirus infections remains unknown, we hypothesized that TDP2/VPg unlinkase activity or the subsequent masking of that activity marks viral RNA as a template for viral translation, RNA synthesis, or encapsidation.

Our findings provide evidence that TDP2 activity is required for efficient poliovirus replication. We found that a mouse embryonic fibroblast cell line genetically ablated for TDP2 lacks VPg unlinkase activity, using a previously described *in vitro* VPg unlinkase assay (Rozovics et al., 2011). Although it is possible that our *in vitro* assay is not sensitive enough to detect low levels of VPg unlinkase activity in the MEF cells lacking TDP2 due to another source of VPg unlinkase activity, previous studies from our laboratory showed that another phosphodiesterase that hydrolyzes 3' phosphotyrosyl bonds [3' tyrosyl-DNA phosphodiesterase 1 (TDP1)], as well as other phosphodiester bonds, lacks VPg unlinkase activity (Rozovics et al., 2011). Using the mouse cell line lacking TDP2/VPg unlinkase activity, we first examined the effect of TDP2 during the course of infection of the prototypic enterovirus, poliovirus. We used RNA transfections of mouse cells lacking the gene for TDP2 or infections using these same cells stably transformed with a cDNA expressing the human poliovirus receptor. In both cases, viral yields were reduced by approximately 90-99% in the absence of TDP2. Importantly, the decrease in poliovirus yields observed in the cell lines lacking TDP2 but stably expressing high levels of PVR was verified to be due to the absence of TDP2, because poliovirus yields could be rescued in the mouse cells stably co-expressing PVR and human TDP2.

We found that viral protein accumulation was severely reduced in the absence of TDP2, suggesting that TDP2 plays a role in poliovirus translation, RNA synthesis, or encapsidation, since these processes are tightly coupled during infection (Novak and Kirkegaard, 1994; Nugent et al., 1999). In the absence of TDP2, poliovirus RNA can be translated and replicated, albeit at reduced levels, as confirmed by our luciferase assay following transfection of the infectious poliovirus reporter RNA. These findings agree with previous reports that show that VPg-linked poliovirus RNA can form a translation initiation complex *in vitro* and VPg-linked poliovirus RNA can be

translated and replicated *in vitro* assuming that TDP2 removal of VPg from viral RNA is its primary function during viral replication (Golini et al., 1980; Langereis et al., 2014). From these studies, the activity of TDP2 did not appear to be solely involved in translation initiation or specific steps in viral RNA synthesis. To further explore if TDP2 plays a role during encapsidation, we measured viral RNA replication in the absence of TDP2 and the capsid proteins. Interestingly, we found that viral replication was initially slightly reduced in the absence of TDP2, but by peak times of replication, was equal to wild type levels. These results indicate that premature encapsidation of newly synthesized RNAs or the presence of capsid proteins may play a role in the observed phenotype we see during poliovirus infection in the absence of TDP2. Not only does this finding support our hypothesis that TDP2 marks viral RNA for viral encapsidation, but indirectly supports previous studies showing that only newly synthesized VPg-linked viral RNA can be encapsidated (Caliguiri and Tamm, 1970; Nomoto et al., 1977b; Nugent et al., 1999). VPg has never been directly shown to function as an encapsidation signal, but previous work suggests that the presence of VPg on the 5' end of newly synthesized viral RNA does play a positive role during viral encapsidation (Franco et al., 2005; Reuer et al., 1990). It also remains to be determined how the fate of viral RNA is altered if VPg remains linked to ribosome-associated viral RNA in the absence of TDP2. Considering that poliovirus can still replicate in the absence of TDP2, although with reduced efficiency, our results could suggest that reduced levels of ribosome-associated, VPg-linked RNA would be detected in the absence of TDP2. The detection of ribosome-associated, unlinked RNA in the absence of TDP2 would indicate that another cellular protein has redundant VPg unlinkase activity.

In contrast to poliovirus, we found that the closely related enterovirus, CVB3, does not appear to replicate in the absence of TDP2 in murine cells. Our single-cycle growth analysis and

Western blot assays confirmed that viral yields do not increase and viral proteins do not accumulate, respectively, in the absence of TDP2. Despite these results, CVB3 RNA can be translated and replicated at reduced levels. It is possible that the observed translation and replication of CVB3 RNA is due to the absence of VPg from input RNA or because the RNA was transfected versus infected. The latter explanation seems unlikely since we found a similar dependency on TDP2 following RNA transfection or poliovirus infection of MEF cells. Additionally, our results agree with a previous study that shows that VPg unlinkase-resistant CVB3 VPg-RNA can undergo *in vitro* translation and replication (Langereis et al., 2014). If CVB3 RNA can be translated and replicated in the absence of TDP2, why is there no increase in viral yields or viral protein accumulation? To further explore the role of TDP2 during CVB3 infection, we took advantage of a previously published poliovirus-CVB3 chimera, where the 5' NCR of poliovirus was replaced with the CVB3 5' NCR. This chimeric virus was used to determine if the CVB3 5' RNA stem-loop structures were responsible for the phenotype observed during CVB3 infection in the absence of TDP2. We found that the chimeric virus could replicate in the absence of TDP2, but with reduced yields and viral protein accumulation. These findings suggested that the CVB3 5' NCR was not exclusively responsible for the phenotype observed during infection in the absence of TDP2. However, the titer reduction observed with this virus in the MEF-TDP2$^{-/-}$ stable cell line expressing PVR does confirm that reduced viral yields and protein production could be partially attributed to decreased CVB3 RNA translation and replication. Additionally, we found that CVB3 infectivity was extinguished in the absence of TDP2 when we serially passaged the virus in MEF-TDP2$^{-/-}$ cell monolayers. These results further confirm that TDP2 is essential for efficient CVB3 replication in our murine cell model. Unfortunately our results do not identify the specific role(s) for TDP2 during CVB3 infection. The greater dependence of CVB3 on TDP2 for

VPg unlinkase activity compared to other enteroviruses could be related to CVB3-specific requirements for host factors involved in partitioning translation/replication complexes for early versus late stage steps in the viral life cycle. The putative interaction of TDP2 with such factors may be required to promote the assembly of progeny virions. It is also possible that CVB3 circumvents the mouse host immune response less efficiently than poliovirus. This weakened ability to counter the host immune response, in combination with reduced levels of CVB3 replication in the mouse embryonic fibroblasts, could lead to the phenotype observed in the MEF-TDP2$^{-/-}$ cells.

Previous reports showed that VPg unlinkase and recombinant TDP2 can hydrolyze the phosphotyrosyl linkage between human rhinovirus VPg and poliovirus RNA derived from a chimeric HRV-poliovirus (Rozovics et al., 2011; Virgen-Slane et al., 2012). Our results show that human rhinovirus yields are reduced by up to 90% in the absence of TDP2. Similar to poliovirus, rhinovirus can still replicate in the absence of TDP2, but with reduced yield. Collectively our results show that enteroviruses have disparate dependencies on TDP2 for replication. We find that CVB3 requires TDP2 for any detectable replication in murine cells, whereas poliovirus and human rhinovirus have a less strict dependence on TDP2. The fact that all of the enteroviruses we tested have some degree of dependency on TDP2 is consistent with our hypothesis that TDP2 has a role in the regulation of enterovirus infections. We propose that in the absence of TDP2, reduced viral translation and RNA synthesis can occur; however, nascent viral RNAs do not undergo additional rounds of translation but can instead become prematurely encapsidated. Our model can be found in **Chapter 4**. This idea is supported by our experiments measuring viral RNA replication in our MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cells in the presence versus absence of capsid proteins. Premature encapsidation of newly synthesized viral RNAs could lead to a decrease in overall levels of viral

translation and viral RNA synthesis in infected cells, thus resulting in a decrease in viral yields. In addition, a decrease in viral translation would lead to a decrease in capsid protein production that is necessary for efficient encapsidation of newly synthesized viral RNAs.

Since the steps in viral replication are tightly coupled, the exact role(s) that TDP2 plays during infection may prove difficult to dissect (Novak and Kirkegaard, 1994; Nugent et al., 1999). Understanding this role should be facilitated by first defining the mechanism that enteroviruses employ to modulate TDP2 activity. As previously noted in **Chapter 1** and our introduction in this chapter, TDP2 has multiple binding partners involved in different aspects of cell signaling and transcriptional regulation. Since enteroviruses have been shown to modulate cellular proteins via their viral-encoded proteinases, it is possible that enterovirus proteinases directly modify TDP2 or indirectly modify the activity of TDP2 by altering its binding partners. Although our results show that TDP2 potentiates enterovirus replication, it remains to be determined if TDP2 is required for other picornaviruses that differ from the closely related members of the enterovirus genus, such as cardioviruses and aphthoviruses. Since TDP2 is required for CVB3 replication and may be required for other picornavirus infections, a small molecule inhibitor targeting the VPg unlinkase activity of TDP2 could be developed to serve as a broad spectrum antiviral. A previous study has identified toxoflavin, its derivatives, and deazaflavins as the first sub-micromolar, selective TDP2 inhibitors by high-throughput screening (Raoof et al., 2013). Although further studies will have to be carried out to test the effects of small molecule inhibitors on picornavirus infections, TDP2 is an attractive potential target for novel antiviral therapeutics.

Materials and Methods

Cell culture and virus stocks

Wild type TDP2 mouse embryonic fibroblast (MEF-TDP2$^{+/+}$) and knockout TDP2 mouse embryonic fibroblast (MEF-TDP2$^{-/-}$) cell lines were described by Caldecott and colleagues (Zeng et al., 2012). MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells were grown as monolayers in DMEM supplemented with 8% newborn calf serum (NCS) or in suspension culture in suspension minimal essential medium (S-MEM) supplemented with 8% NCS or methionine-free DMEM.

The W1-VPg31 virus (Kuhn et al., 1988) was used to infect HeLa cells in suspension for the purification of poliovirus virion RNA (vRNA) substrate. Virus stock for HRV1a was kindly provided by Dr. Yury Bochkov at the University of Wisconsin, Madison. The PCV-305 chimeric virus was expanded from a previously published virus stock (Johnson and Semler, 1988). All virus stocks were expanded by three serial passages in HeLa cells.

^{35}S-methionine-labeled poliovirus virion RNA substrate generation and VPg unlinkase assay

Radiolabeled poliovirus vRNA substrate was prepared from the W1-VPg31 virus (Kuhn et al., 1988) as previously described (Rozovics et al., 2011). In brief, HeLa cells were grown in suspension. Following infection with W1-VPg31 (MOI 20), HeLa cells were starved of methionine for 2 hours at 37° C. 2.5 mCi of ^{35}S-methionine was added to the infected cell suspension and further incubated for 3 hours. The radiolabeled, infected cells were pelleted and subjected to the previously described poliovirus vRNA purification scheme (Rozovics et al., 2011). The final volume of the radiolabeled substrate was adjusted to 1000 cpm/µl (160 ng/µl).

The *in vitro* VPg unlinkase assay was carried out as previously described (Rozovics et al., 2011; Virgen-Slane et al., 2012). For our experiments, a 20 µl reaction containing 1000 cpm [160 ng] of radiolabeled poliovirus vRNA was incubated with RNase A [10 µg] or a source of VPg unlinkase activity in the presence of 2 mM $MgCl_2$ in unlinkase buffer (20 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 5% (vol/vol) glycerol) at 30° C for 30 minutes. The reactions were resolved by electrophoresis on an SDS-containing 13.5% polyacrylamide gel in Tris-tricine for 2.5 hours (for VPg unlinkase activity) or 16 hours (to resolve the VPg-pUp species). The gel was dried for 1 hour and visualized by autoradiography using a phosphor screen. VPg unlinkase sources included: recombinant GST-TDP2 that was expressed from the pGEX-2TK2-GST-EAPII plasmid, kindly provided by Runzhao Li, formerly of Emory University (Pei et al., 2003) and purified as described (Virgen-Slane et al., 2012), and extracts from MEF-TDP2^{+/+} and MEF-TDP2^{-/-} cell monolayers that were re-suspended in PDEG10 buffer (20 mM phosphate buffer, pH 7.0, 5 mM DTT, 1 mM EDTA, 10% (vol/vol) glycerol). RNase A was used to generate the VPg-pUp species.

Poliovirus virion RNA transfection

Poliovirus vRNA was generated as previously described (Dewalt and Semler, 1987). Poliovirus vRNA was incubated with 1 mg/ml DEAE-dextran in TS buffer (137 mM NaCl, 4.4 mM KCl, 0.7 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.68 mM $CaCl_2$, 25 mM Tris [pH 7.5]). MEF-TDP2^{+/+}, MEF-TDP2^{-/-}, and HeLa cell monolayers were plated into 20 cm^2 plates. Monolayers were washed with 1X PBS. Transfection mixture (250 µl) containing 1 µg vRNA was added, drop-wise, per plate. Transfection mixtures were incubated on cell monolayers for 30 minutes at room temperature. DMEM (3 ml) with 10% FBS was added to the MEF cells and 3 ml of DMEM with 8% NCS was added to HeLa cells. Transfected cells were incubated at 37° C. Cells and supernatant were collected at specific time points and subjected to 5 freeze-thaw cycles. Viral yields were quantified

by plaque assay on HeLa cell monolayers. Viral yields are reported as PFU per cell and plotted on a logarithmic scale. Plaque assays were performed in technical triplicate. Error is reported as standard deviation. Student's t test were performed using the graphpad software.

Generation of stable mouse embryonic fibroblast cell lines expressing human PVR or human TDP2

MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ monolayers were seeded on 10 cm^2 plates. Cells were co-transfected with pcDNA 6/TR (Life Technologies) for rapid blasticidin selection and the human PVR cDNA expression construct pSVL-H20A (Ren et al., 1990) kindly provided by Vincent Racaniello at Columbia University, New York, or pBR322.1 as a control vector using jetPRIME (Polypus transfection). Blasticidin-resistant cell colonies were isolated and expanded. Cells were maintained in DMEM supplemented with 10% FBS and 5 µg/ml of blasticidin. PVR mRNA expression was verified by reverse-transcription (RT) PCR analysis. Forward [5'-TATTGGTGCCCTCAAGCCAG-3'] and reverse [5'-CCTAGGGCATTGGTGACGTT-3'] primers specific for human PVR were used.

To generate the MEF stable cell line co-expressing PVR and human TDP2 (hTDP2), hTDP2 was cloned into a mammalian expression vector. hTDP2 was excised from the pGEX-2KT2-GST-EAPII plasmid with BamHI and EcoRI and gel-purified. The purified TDP2 product was amplified by PCR using the 5' EcoRI forward primer [5'-AGGAAGGAATTCCATGGAGTTGGGGAGTTGCCTGGAGGGCGGGAGGGAGGCG-3'] and 3' SalI reverse primer [5'-TGCAACGTCGACAATCAGGGCAAAACCCACAC-3'], double-digested with EcoRI and SalI, and gel-purified. The N-terminal pFlag-CMV expression plasmid was double digested with EcoRI and SalI, gel-purified, phosphatase treated, and incubated with the gel-purified TDP2 PCR product in the presence of T4 DNA ligase. Products of the ligation

reaction were transformed into DH10ß competent *Escherichia coli*. pFlag-CMV-TDP2 was isolated by mini-prep (Qiagen), sequenced, and cesium chloride purified for transfection. The MEF-TDP2$^{-/-}$ stable cell lines co-expressing PVR and human TDP2 were generated as described above. Human TDP2 mRNA expression was verified by RT-PCR analysis using forward [5'-TGTCTGGGAGTTTTTGGGCA-3'] and reverse [5'-GAAGGTCCAAACTTCGGGGA-3'] primers. Mouse TDP2 forward [5'-AGGCTCCAGATTCAACCACG-3'] and reverse [5'-GTTAGCCCTGAGATACGCCC-3'] primers were used for RT-PCR analysis to verify that the MEF-TDP2$^{-/-}$ stable cell line did not express TDP2.

Virus infections and single cycle growth analysis

HeLa and MEF cell monolayers were seeded on 20 cm^2 plates. The monolayers were washed with 1X PBS and infected with poliovirus, CVB3, PCV-305, or HRV1a at an MOI of 20. Poliovirus and PCV-305 was adsorbed at room temperature for 30 minutes, while CVB3 and HRV1a were adsorbed for 1 hour at room temperature. Following adsorption, the cells were washed with 1X PBS 3 times and overlaid with 3 ml of DMEM with their respective serum (8% NCS or 10% FBS). Poliovirus-. PCV-305-, and CVB3-infected cells were incubated at 37°C, while HRV1a-infected cells were incubated at 34°C. Cells and supernatant were collected and subjected to 5 freeze-thaw cycles. Viral yields were quantified by plaque assay on HeLa cell monolayers. Viral yields are reported as PFU per cell and plotted on a logarithmic scale. Plaque assays were performed in technical triplicate. Error is reported as standard deviation. Student's t test was calculated using the graphpad software.

For the CVB3 serial blind passage experiment, MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell monolayers were seeded on 20 cm^2 plates, washed with 1X PBS and infected with CVB3 at an MOI of 20 as described above for the initial infection. The infected cells were incubated at 37^{0}C for 24 hours.

Cells and supernatant were collected and subjected to 5 freeze-thaw cycles. 200 µl of the freeze-thawed virus sample was then used to infect MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell monolayers. This serial passaging was repeated 20 times. Viral yields were quantified by plaque assay on HeLa cell monolayers at 1 and 20 blind passages. Plaque assays were performed in technical triplicate. Error is reported as standard deviation.

Cytopathic effect observation in infected cells

Mock, poliovirus, and CVB3 infections were carried out in HeLa, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers as described above. The cells were imaged using phase-contrast microscopy with the Carl Zeiss Axiovert 200 inverted microscope.

Preparations of lysates from uninfected and infected cells and Western blot analysis

Poliovirus, CVB3, and PCV-305 infections were carried out as described above. Cells were harvested and pelleted at 0, 8, or 10 hours post-infection. Samples were washed with 1X PBS 3 times and pelleted. The pellet was resuspended in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40) and incubated on ice for 30 minutes. Cell debris was pelleted and the supernatant collected. Protein concentration was measured by a Bradford assay. For Western blot analysis, 50 µg of NP-40 lysates from poliovirus-infected cells, 75 µg of NP-40 lysates from CVB3-infected cells, or 10 µg of PCV-305 were subjected to electrophoresis on SDS-containing 12.5% polyacrylamide gels. Proteins were then transferred to a PVDF membrane. Antibodies against poliovirus protein 3A at a dilution of 1:2000 or CVB3 protein 3A at a dilution of 1:5000 were used to detect viral protein accumulation in the NP-40 lysates. The mouse monoclonal antibody to poliovirus protein 3A was kindly provided by George Belov at the University of Maryland. The mouse monoclonal antibody to CVB3 protein 3A was kindly provided by J. Lindsay Whitton at the Scripps Research Institute, La Jolla, CA. Antibody PKM2

(Bethyl) at a dilution of 1:2000 was used to detect endogenous PKM2 as a protein loading control. Antibody GAPDH (abcam) at a dilution of 1:10,000 was used to detect endogenous GAPDH as a protein loading control. Protein bands were visualized by ECL Western Blotting Substrate (Life Technologies).

^{35}S-methionine pulse labeling and immunoprecipitation of newly synthesized poliovirus proteins

HeLa and MEF cell monolayers were seeded on 20 cm^2 plates. The monolayers were washed with 1X PBS and mock- or poliovirus-infected at an MOI of 20. Poliovirus was adsorbed at room temperature for 30 minutes. Following adsorption, the cells were washed with 1X PBS 3 times and overlaid with 2 ml of DME, without methionine, with L-Glutamine, with their respective serum (8% NCS or 10% FBS), and 2 mM Guanidine HCl (final concentration). Cells were methionine starved for 1 hour at 37°C. Medium was aspirated and replaced with 2 ml of above medium (DME, -methionine, +L-Glutamine, serum, and 2 mM GuHCl) and 50 µCi of ^{35}S-methionine (final concentration) per plate. Cells were incubated an additional 3 hours at 37°C. Cells were washed with cold 1X PBS 2 times and then collected and pelleted. Pelleted cells were resuspended with 400 µl of RIPA buffer [10 mM Tris-HCl, pH 8.0, 1% NP-40 alternative, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 2 µg aprotinin] and stored at -20°C.

Immunoprecipitation was carried out using the RIPA buffer resuspended lysates. Lysates were pre-cleared with 50 µl of 50% Protein A agarose (Roche) for 30 min at 0° C. Protein A agarose was removed by centrifugation. Pre-cleared lysate was then incubated with 10 µl of anti-3C antibody rotating at 4°C for 2 hours. Next, 50 µl of 50% Protein A agarose was added to the antibody-protein complex and incubated rotating at 4°C for 1 hour. Beads were washed with RIPA buffer three times, followed by NTE buffer (50 mM NaCl, 10 mM Tris, pH 7.2, 1mM EDTA) one

time. Samples were boiled for three minutes with 50 µl 2X LSB. Reactions were resolved on SDS-PAGE. Gel was washed with DMSO for 20 minutes three times, PPO/DMSO for 30 minutes, and ran under deionized water for 45 minutes. The gel was dried for 1 hour and visualized by autoradiography using a phosphor screen.

In vitro transcription of infectious reporter RNA

For generation of *in vitro* transcribed *Renilla* luciferase poliovirus (RLuc-PV-PPP) RNA transcripts, the RLuc-PV-PPP construct, pT7R-Luc-PPP (Liu et al., 2010) (kindly provided by Eckard Wimmer at Stony Brook University), was linearized with PVUI to generate a transcription template. For generation of *in vitro* transcribed *Firefly* luciferase poliovirus (FLuc-PV-PP) RNA transcripts, the FLuc-PV-PP construct, pRLuc31 (Andino et al., 1993) (kindly provided by Raul Andino at the University of California, San Francisco), was linearized with MluI to generate a transcription template. For generation of *in vitro* transcribed *Renilla* luciferase CVB3 (RLuc-CVB3) RNA transcripts, the RLuc-CVB3 construct, p53CB3/T-7-Rluc (Lanke et al., 2009) (kindly provided by Frank van Kuppeveld at the University of Utrecht, The Netherlands), was linearized with SalI to generate a transcription template. RNA transcription was performed using a MEGAscript T7 transcription kit (Ambion). RNA transcript was purified using the RNeasy kit (Qiagen).

Transfection of *in vitro* transcribed infectious reporter RNA and luciferase assays

HeLa, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers in 10 cm^2 plates were transfected with either 1µg of RLuc-PV-PPP or FLuc-PV-PP RNA transcript or 2 µg of RLuc-CVB3 RNA transcript per plate using the TransIT-mRNA transfection kit (Mirus Bio). For the translation assay, transfection was carried out in the presence of the viral RNA synthesis inhibitors GuHCl: 5 mM GuHCl for poliovirus reporter RNAs or 2.5 mM GuHCl for CVB3 reporter RNA. For the

replication assay, transfection was carried out in parallel in the absence of the viral RNA synthesis inhibitor. Cells were washed with 1X PBS and lysed in either 1X *Renilla* Luciferase Assay Lysis Buffer (Promega) or 1X Passive Lysis Buffer (Promega) for 15 minutes. Luciferase values were measured using either the *Renilla* Luciferase Assay System (Promega) or Luciferase Assay System (Promega) according to the manufacturer's protocol. Total RLU/s values were normalized by dividing the value by total cell count measured prior to transfection. Values were plotted as RLU/cell. Transfections were carried out in three separate biological replicates unless noted in the figure legend. The RLU/s values from the biological replicates were averaged and the standard error of the mean was calculated for the error bars.

CHAPTER 3

The role of TDP2 during cardiovirus infections

Summary

Cardioviruses are known to cause diseases in a myriad of animals despite being composed of only three species. Cardioviruses differ from enteroviruses in numerous ways, ranging from their genome sequence to how they modify host proteins during infection. Some of these differences include their much more highly structured 5' noncoding region encoding a type II IRES and their additional viral-encoded leader (L) protein. Additionally, cardioviruses interact with host proteins, such as PCBP2 and AUF1, differently than enteroviruses. In this chapter we determined if the host protein TDP2, which is divergently required during enterovirus infections, is necessary for cardiovirus, EMCV, infection. We found that the predominantly nuclear protein TDP2 (although present at low levels in the cytoplasm), is relocalized from the nucleus to the cell cytoplasm during EMCV infection, suggesting that EMCV utilizes TDP2 during infection. Additionally, EMCV yields are reduced about 0.5 to 1 $\log_{10}$ unit in the absence of TDP2, showing that TDP2 has a role for efficient viral replication. These results were confirmed when we observed a slight decrease in viral protein accumulation in the absence of TDP2 by Western blot analysis and a reduction in viral RNA replication by luciferase expression. Interestingly, we found that TDP2 is cleaved at peak times of EMCV infection in HeLa cells but not during enterovirus infections, suggesting an alternative mechanism for cardioviruses to regulate TDP2 activity during infection. Collectively, these results show that TDP2 is necessary for efficient EMCV replication, albeit at a much lower dependence than enteroviruses, and is possibly being excluded from putative replication and encapsidation sites by a different mechanism than that employed by enteroviruses.

Introduction

The cardiovirus genus of the *Picornaviridae* family is divided into three species, inlcuding EMCV and Theilovirus. EMCV has one serotype, Mengovirus, while Theilovirus has multiple serotypes, including Theiler's murine encephalomyocarditis virus (TMEV). EMCV is primarily known for the diseases caused in both wild and domestic animals worldwide, including mice, swine, and non-human primates; however, infections in humans have also been reported [reviewed in (Carocci and Bakkali-Kassimi, 2012)]. This wide range of animals susceptible to infection makes EMCV a potential zoonotic agent. Additionally, cultured human cells that express the sialoglycoprotein vascular cell adhesion molecule 1 (VCAM-1) that functions as the EMCV receptor are also susceptible to EMCV infection (Huber, 1994). EMCV infections in swine and rodents can lead to myocarditis, encephalitis, paralysis, type I diabetes, or even mortality [reviewed in (Carocci and Bakkali-Kassimi, 2012)]. No vaccine or antiviral therapeutic against EMCV infection is currently available. Since cardioviruses have such a wide host range and the ability to cause multiple diseases, it is important to further understand the viral mechanisms employed by cardioviruses for future antiviral therapeutic development.

EMCV, like enteroviruses, has a small positive-sense RNA genome (~7.8 kb). Although members of the picornavirus family share many similarities in their genome, the cardiovirus genome does have distinct features that lead to alternative viral replication and host modulation mechanisms. Following the covalently linked VPg (20 amino acids) via the highly conserved phosphotyrosyl linkage to the 5' end of the viral RNA is the 5' NCR. The 5' NCR of EMCV differs from that of enteroviruses in that it possesses a poly(C) tract, followed by pseudoknots with unknown functions, a type II IRES, and a pyrimidine rich tract [reviewed in (Wimmer et al., 1993)]. The approximately 430-nucleotide type II IRES is more highly structured than the type I

IRES that enteroviruses possess and is subdivided into five domains known as H-L. A schematic of the poliovirus type I IRES and EMCV type II IRES is depicted in **Figure 3.1**. The IRES allows for initiation of cap-independent translation of the viral polyprotein while viral RNA synthesis is initiated on the cis-acting replication element (CRE). The CRE functions as a template for VPg uridylylation by the RNA-dependent RNA polymerase 3D for viral RNA synthesis to occur. The CRE on the EMCV genome is located in the VP2 coding region, whereas in the enterovirus sequence it is located in the 2C-coding region (Goodfellow et al., 2000; Lobert et al., 1999). These differences in sequence and RNA secondary structure can alter how the viral RNA interacts with both host and viral proteins during EMCV infection.

In addition to sequence differences, the EMCV genome undergoes a unique translational event known as "StopGo" that results in the generation of two polyproteins: L-1ABCD-2A and 2BC-3ABCD (Loughran et al., 2013). The EMCV genome also encodes an additional viral protein known as the leader (L) protein, which is phosphorylated during infection and contains a N-terminal zinc finger domain (Cornilescu et al., 2008; Dvorak et al., 2001). The L protein, despite having no enzymatic activity, disrupts nucleo-cytoplasmic trafficking by playing a role in phosphorylating the Nup proteins and binding to Ran GTPase (Porter et al., 2006; Porter and Palmenberg, 2009). This is different from enterovirus-mediated disruption of nucleo-cytoplasmic trafficking, which is carried out by their viral proteinase 2A (Castello et al., 2011; Park et al., 2010). Another difference between the EMCV and enterovirus protein functions can be seen with the viral protein 2B. In contrast to poliovirus, EMCV 2B does not inhibit ER to Golgi protein trafficking, but instead disrupts ER Ca^{2+} homeostasis (de Jong et al., 2008). Another major difference between EMCV and enteroviruses is that the EMCV 2A protein does not function as a proteinase like the enterovirus 2A does. However, that does not mean that EMCV 2A does not

play an important role during viral infection. In contrast to enterovirus 2A cleaving eIF4G, EMCV 2A expression triggers hypophosphorylation of the translation initiation factor 4E-BP1, leading to inhibition of cap-dependent translation, albeit at a much slower rate than during poliovirus infection (Etchison et al., 1982; Jen et al., 1980; Svitkin et al., 1998). EMCV 2A was also shown to inhibit apoptosis (Carocci et al., 2011). Despite these differences, EMCV, like enteroviruses, is able to alter the cellular environment to promote viral replication.

Other ways EMCV enhances its replication include inhibiting the host antiviral response. Previous work shows that EMCV L protein interferes with IRF-3 dimerization, a step necessary for transcription of the interferon-alpha/beta genes (Hato et al., 2007). Additionally, EMCV and poliovirus have been shown to target the viral RNA sensor, RIG-I (Barral et al., 2009; Papon et al., 2009). It is important to note that not all host proteins function similarly during enterovirus and cardiovirus infections. For example the cellular mRNA decay factor AUF1 plays a negative role during poliovirus infection but does not alter EMCV titers (Cathcart et al., 2013; Cathcart and Semler, 2014; Rozovics et al., 2012). Another host protein used during enterovirus but not EMCV replication is PCBP2. Although PCBP2 has been shown to bind both type I and type II IRESs, PCBP2 is necessary for enterovirus translation but not for EMCV translation (Walter et al., 1999). Together, these previous findings show that although cardioviruses and enteroviruses alter their cellular environment in a similar manner, their viral-mediated mechanisms can vary.

In this chapter we consider the role that the host protein TDP2 plays during EMCV infection. Although it is not confirmed that TDP2 functions as VPg unlinkase during cardiovirus infections, previous studies show that a cellular activity from mouse ascites Krebs II cells can hydrolyze the VPg-RNA linkage found in both the poliovirus and EMCV RNA (Drygin Iu and Siianova, 1986; Drygin Yu et al., 1988). This activity was specifically a phosphodiesterase

predicted to be involved in repair of RNA and topoisomerase complexes (Gulevich et al., 2001, 2002). We were interested in determining if TDP2 plays a role during EMCV infection and if so, at what step during the viral replication cycle. We were also interested if TDP2 is being used differently during EMCV infection compared to enterovirus infections since previous work has shown a differential usage for host proteins during cardiovirus and enterovirus infections. Characterization of the role of TDP2 during EMCV infection can further determine if antiviral therapeutics targeting TDP2 can be used as a broad picornavirus therapeutic.
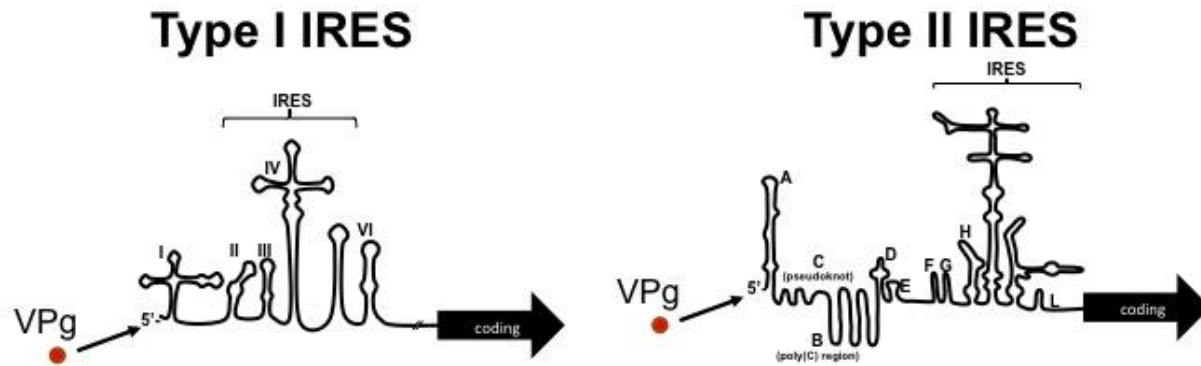
Figure 3.1. The differences in the type I and type II IRESs.

Poliovirus type I IRES is depicted on the left. The poliovirus 5' NCR (~740 nucleotides) is made up of 6 stem-loops. Stem-loops II-VI make up the poliovirus type I IRES that is approximately 450 nucleotides in length. The type I IRES is used by enteroviruses to initiate viral translation of their viral genome. EMCV type II IRES is depicted on the right. The EMCV 5' NCR (~860 nucleotides) is made up of 12 domains. Hairpin loops denoted as domains H-L make up the EMCV type II IRES that is approximately 430 nucleotides in length. The more highly structured type II IRES is used by cardioviruses to initiate viral translation of their viral genome. The IRES illustrations were made by Eric Baggs.

Results

TDP2 is relocalized from the nucleus to the cytoplasm during EMCV infection

TDP2, a predominantly nuclear protein (although present at lower concentrations in the cytoplasm), was shown to dramatically relocalize from the nucleus to the cytoplasm as early as 2 hours after poliovirus infection (Virgen-Slane et al., 2012). TDP2 was also shown to localize to the cell periphery in sites distinct from putative viral replication and encapsidation by peak times of poliovirus infection (Virgen-Slane et al., 2012). These findings suggest that TDP2 is being sequestered from viral RNA replication and encapsidation sites as a mechanism to modulate the catalytic activity of TDP2 throughout infection. To determine if this relocalization pattern is prevalent among picornaviruses, we examined TDP2 relocalization during EMCV infection by confocal microscopy. HeLa cells were either mock- or EMCV-infected and fluorescently labeled using antibodies against TDP2 or EMCV 3D. As shown in **Figure 3.2**, TDP2 is primarily localized to the nucleus in mock-infected cells and at 0 hours post-infection. At 2 and 4 hours post-infection, TDP2 increasingly relocalizes from the nucleus to the cytoplasm. At peak times of infection (6 hours) TDP2 is completely dispersed into the cell cytoplasm. Staining of the RNA-dependent RNA polymerase 3D was used as a marker for viral replication in EMCV-infected cells and is detected at peak times of infection (6 hours). TDP2 and EMCV 3D are closely localized in the cell cytoplasm. These results are in contrast to the distinctive pattern observed for TDP2 during poliovirus infection, suggesting an alternative mechanism for modulating TDP2 catalytic activity throughout EMCV infection.
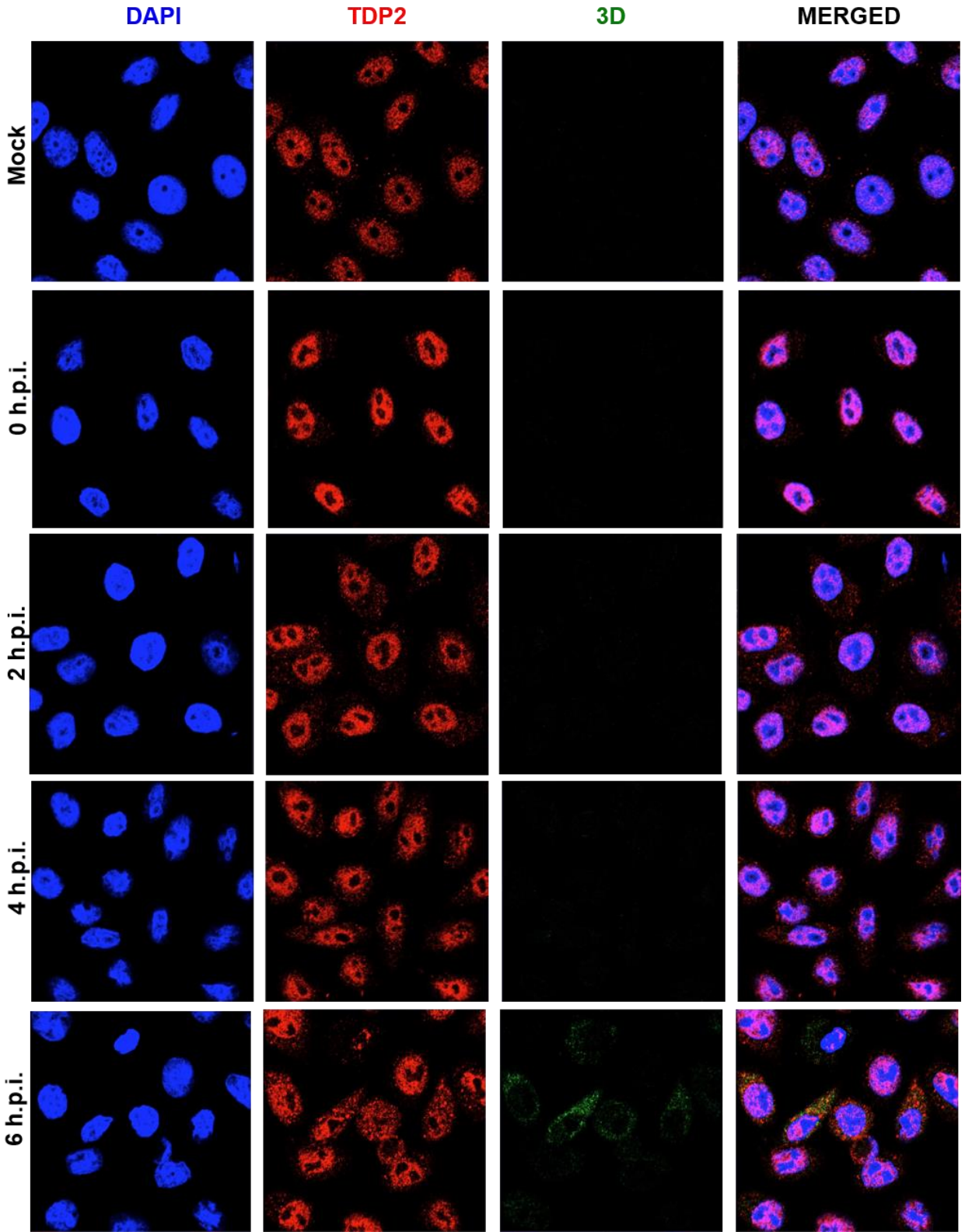
Figure 3.2. TDP2 is relocalized from the nucleus to the cytoplasm during EMCV infection. HeLa cells were seeded on coverslips and either mock- or EMCV-infected at an MOI of 20. Cells were then fixed with formaldehyde at 0, 2, 4, or 6 hours post-infection (h.p.i). The cells were visualized by confocal microscopy using antibodies against human TDP2 (red) or EMCV 3D (green). The nuclei were stained with DAPI. The cells were imaged by confocal microscopy and analyzed by z-stack analysis.

The absence of TDP2 during EMCV infection causes a decrease in viral yields

Since enteroviruses require TDP2 for efficient replication, we wanted to determine if TDP2 is required for cardiovirus infections. Because mice are naturally susceptible to EMCV infection due to the expression of the EMCV receptor VCAM-1 (Huber, 1994), also expressed in human cells, we infected HeLa and MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell monolayers with EMCV and quantified viral yields by plaque assay every 2 hours post-infection for 10 hours (**Figure 3.3**). We found that viral growth was reduced and delayed by 2 hours in the MEF-TDP2$^{+/+}$ cells in comparison to HeLa cells. Viral yields are reduced by 0.5 log$_{10}$ unit at 6 hours post-infection and approximately a little less than 1 log$_{10}$ unit by 8 hours post-infection in the absence of TDP2. However, by 10 hours post-infection viral yields are dramatically increased in the MEF-TDP2$^{-/-}$ compared to 6 and 8 hours post-infection, but are still reduced at 10 hours post-infection compared to MEF- TDP2$^{+/+}$. The results from this single-cycle growth analysis reveal that EMCV replication is indeed affected by the absence of TDP2; however, it is less dependent on TDP2 for its viral replication cycle than poliovirus, rhinovirus, or CVB3 (**Figure 2.6 B, Figure 2.12**, or **Figure 2.8 A**, respectively).
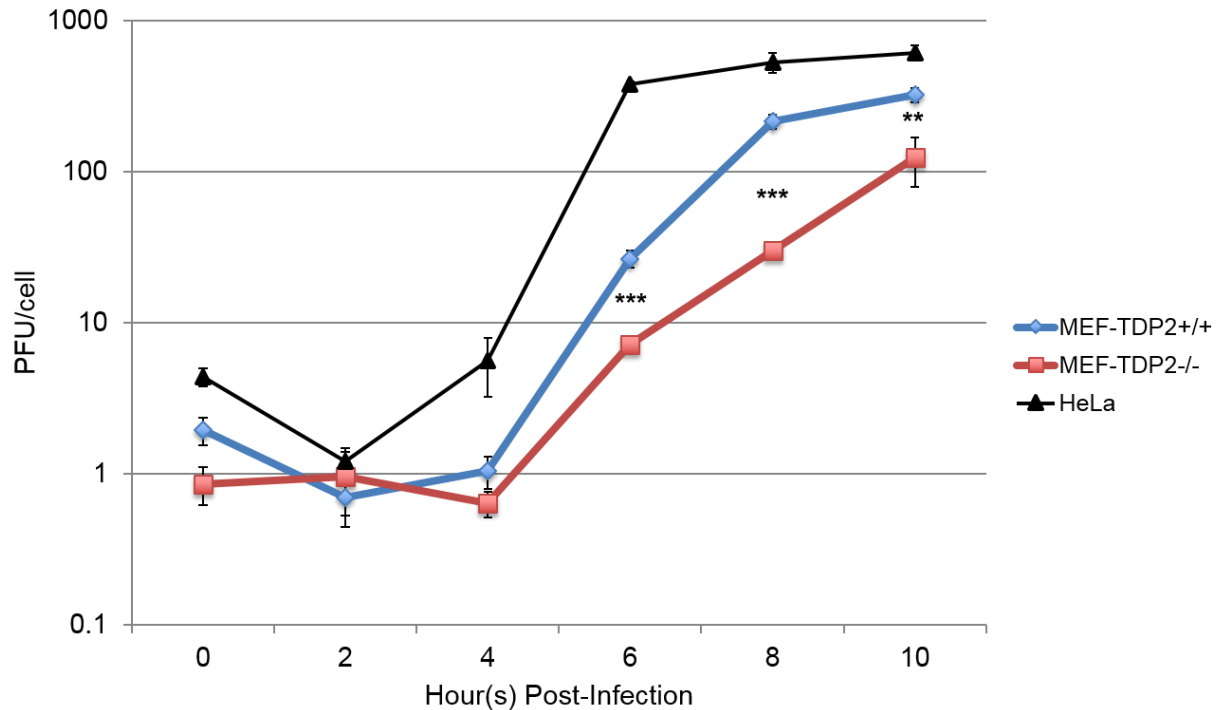
Figure 3.3. EMCV requires TDP2 for efficient viral replication. Single-cycle growth analysis was carried out in HeLa, MEF-TDP2$^{+/+}$, or MEF-TDP2$^{-/-}$ cell monolayers following EMCV infection at an MOI of 20. Cells and supernatant were collected every 2 hours up to 10 hours post-infection. Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviation of the results from triplicate plaque assays. An asterisk (*) (Student's t test; $P < 0.05$), a double asterisk (**) (Student's t test; $P < 0.01$), or triple asterisk (***) (Student's t test; $P < 0.001$) indicates statistical significance between MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cells.

TDP2 is necessary for efficient EMCV replication

Next, we investigated the role of TDP2 in EMCV translation and RNA synthesis. We first analyzed viral protein accumulation in MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cells by Western blot analysis using an antibody against the EMCV nonstructural protein 2A. We found that 2A accumulation was reduced in the EMCV-infected MEFs-TDP2$^{+/+}$ at 8 hours post-infection compared to HeLa cells (**Figure 3.4 A, lanes 3 and 7**). However, 2A protein accumulation is comparable between the MEF-TDP2$^{+/+}$ and HeLa cells by 10 hours post-infection (**Figure 3.4 A, lanes 4 and 8**). In contrast to what we expected based upon our single-cycle growth analysis of EMCV in the MEF-TDP2$^{-/-}$ cell line, we found that 2A production was severely reduced at both 8 and 10 hours post-infection in comparison to MEF-TDP2$^{+/+}$ (**Figure 3.4 A, lanes 7 and 8 versus 11 and 12**). We quantified the band intensity of 2A, normalized against the loading control at 8 and 10 hours post-infection in the MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell lysates, and set the 2A expression in the MEF-TDP2$^{+/+}$ at 8 hours post-infection equal to 1 (**Figure 3.4 B**). We found that 2A protein accumulation was decreased by ~20% at 8 hours post-infection and ~80% at 10 hours post-infection in the absence of TDP2. To verify that this decreased protein expression in the absence of TDP2 was not due to an overall decrease in IRES-driven translation, we measured type II IRES-driven translation in the MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell monolayers by co-transfecting an RNA replicon encoding a *Firefly* luciferase (FLuc) reporter gene flanked by the EMCV 5' NCR and 3' NCR (FLuc-5'EMCV3') and a capped RNA control replicon (RLuc-RstF) and measuring luciferase expression (**Figure 3.4 C**). We found that EMCV IRES-driven translation was not decreased in the absence of TDP2. To verify that the phenotype observed in the MEF-TDP2$^{-/-}$ in **Figure 3.4 B** was not due to degradation of 2A or sensitivity of the antibody in recognizing 2A, we measured viral protein accumulation of EMCV 3C and 3D in EMCV-infected HeLa, MEF-

TDP2$^{+/+}$, and MEF- TDP2$^{-/-}$ cell monolayers (**Figure 3.4 D**). Similar to 2A protein accumulation, the EMCV nonstructural proteins 3C and 3D expression increased between 8 and 10 hours post-infection in the EMCV-infected MEF-TDP2$^{+/+}$ even though this increase was not as dramatic as observed with 2A (**Figure 3.4 D, lanes 7 and 8**). The increase in 3C and 3D protein accumulation observed in the EMCV-infected MEF-TDP2$^{+/+}$ cells was also observed in EMCV-infected HeLa cells at 8 and 10 hours post-infection (**Figure 3.4 D, lanes 3 and 4**). In agreement with the single-cycle growth analysis, EMCV 3C and 3D expression was also slightly reduced in the MEF-TDP2$^{-/-}$ (**Figure 3.4 D lanes 11 and 12**).

We next compared EMCV translation and RNA replication using a more sensitive assay than Western blot analysis. We transfected full-length EMCV RNA encoding a *Gaussia* luciferase (GLuc) reporter, generated from an infectious GLuc-EMCV clone, into MEF-TDP2$^{+/+}$ cells and MEF-TDP2$^{-/-}$ cells in the presence or absence of the EMCV viral RNA synthesis inhibitor dipyridamole (Fata-Hartley and Palmenberg, 2005; Tonew et al., 1977) and measured luciferase expression at 5 and 10 hours post-transfection. As expected, in the presence of dipyridamole, the viral RNA was equally translated in the presence or absence of TDP2 (**Figure 3.4 E**). The RLU/cell values for the mouse cells transfected with GLuc-EMCV in the presence of dipyridamole in **Figure 3.4 E** were confirmed to be similar despite not being visible in the figure. Since the transfected RNA was synthesized *in vitro* using the bacteriophage T7 RNA polymerase and a T7 promoter element, it lacks an authentic VPg linkage to the 5' end of the RNA, and we would therefore predict that transfected RNA would be translated in a TDP2-independent manner, as observed with the other viral RNAs with reporters described in **Chapter 2**. In agreement with our previous findings (single cycle-growth and Western blot analysis), in the absence of dipyridamole, viral RNA replication occurred in the MEF-TDP2$^{-/-}$ cell line at 5 and 10 hours post-transfection, but at slightly

reduced levels compared to the wild type MEF cell line (**Figure 3.4 E**). Collectively, our results

show that EMCV RNA can be translated and replicated in the absence of TDP2 following either

transfection of infectious EMCV RNA or EMCV infection; furthermore, viral replication is

partially reduced in the absence of TDP2.
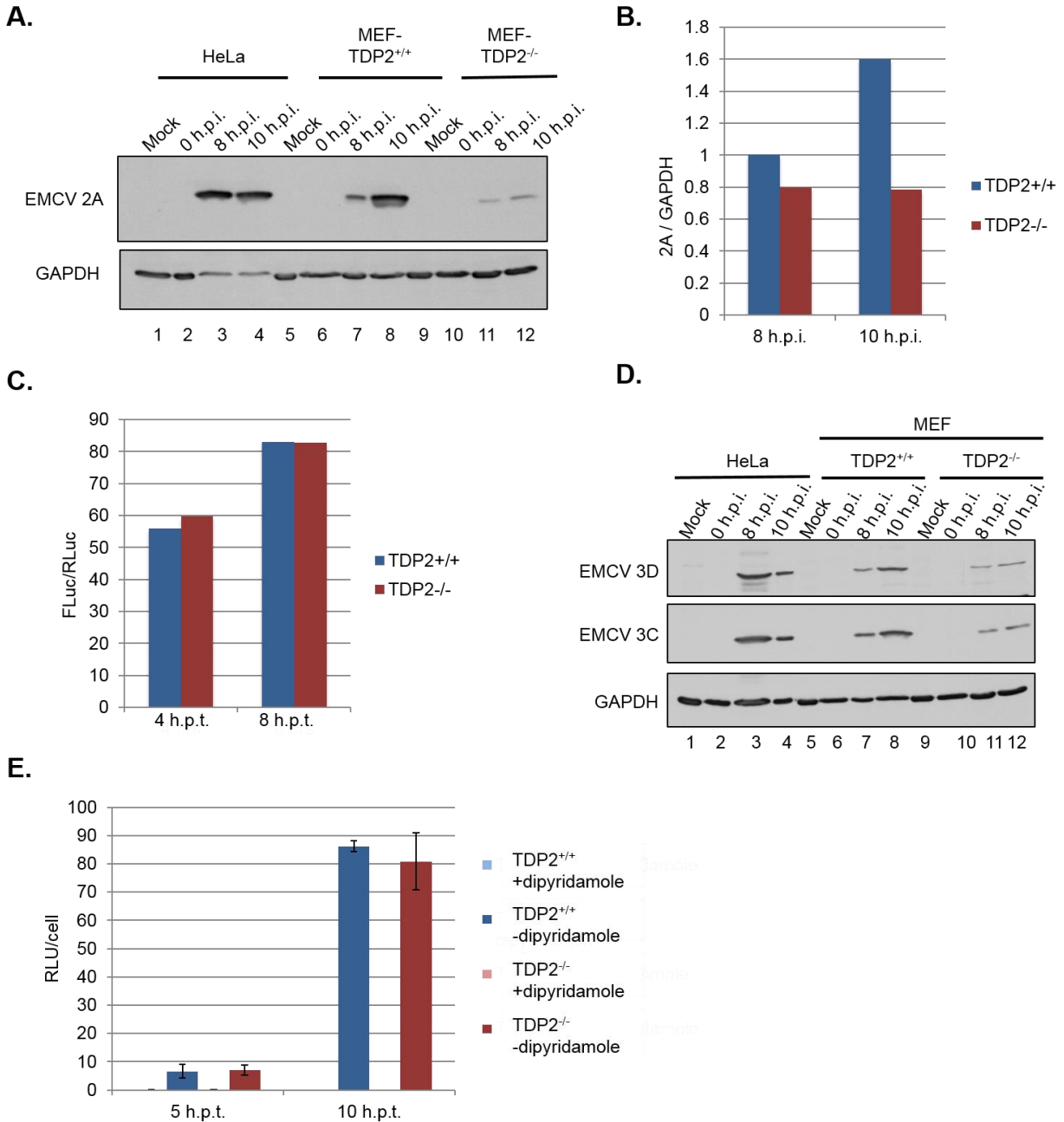
Figure 3.4. EMCV viral protein accumulation and RNA replication is reduced in the absence of TDP2. HeLa, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers were mock- or EMCV-infected at an MOI of 20. Cells and supernatant were collected at 0, 8, or 10 hours post-infection (h.p.i.) and used to generate NP-40 lysates. Mock- or EMCV-infected NP-40 lysate was subjected to SDS-PAGE and Western blot analysis using an **(A)** anti-EMCV 2A, **(D)** anti-EMCV 3C, anti-Mengo

3D antibody, or **(A, D)** anti-GAPDH antibody (loading control) to visualize proteins. **(B)** The EMCV 2A band intensity in **A** was quantified using the BioRad Quantity One software and normalized against the loading control band intensity. The normalized EMCV 2A band from the MEF-TDP2$^{+/+}$ at 8 h.p.i. was set as 1. **(C)** General EMCV type II IRES-driven translation was measured in the MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell lines by co-transfecting the FLuc-5'EMCV3' RNA and capped control RNA RLuc-RstF RNA. Luciferase values (RLU/s) were divided by the total cell count prior to transfection (RLU/cell). Transfections were carried out in biological duplicate experiments. FLuc-5'EMCV3' luciferase values (RLU/cell) were normalized against the cap control pRstF values (RLU/cell). **(E)** The infectious RLuc-EMCV RNA (2 μg) was transfected into HeLa cell, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers in the presence or absence of 100 μM dipyridamole, an EMCV viral RNA synthesis inhibitor. Cells were washed and lysed with 1X Renilla luciferase lysis buffer (Promega) at 5 or 10 hours post-transfection (h.p.t.). Samples were collected and subjected to the *Renilla* Luciferase Assay System (Promega) to measure GLuc values. GLuc values (RLU/s) were divided by the total cell count prior to transfection (RLU/cell). The increase of GLuc values in the absence of dipyridamole indicates the contribution of viral RNA replication. The error bars indicate standard error of the mean of the results from biological triplicate experiments.

TDP2 is modified during EMCV infection

Numerous studies showed that picornaviruses modify host proteins via their viral-encoded proteinases to aid in carrying out their viral replication cycle. These viral proteinases primarily do this by cleaving cellular proteins at specific amino acid residues. Enterovirus proteinase 3C and its active precursor 3CD recognize the residues QG, QA, QN, and QS with an amino acid with an aliphatic side chain in the P4 position (4th residue on the left from the cleavage site) as putative cleavage sites. Additionally, rhinovirus 3C and 3CD recognize EG as putative cleavage sites. In contrast, cardiovirus 3C proteinase recognizes QG, QS, QA, EN, and ES with a proline in the P2 or P2' position as putative 3C cleavage sites. To determine if TDP2 is being cleaved during picornavirus infections we analyzed both the human and mouse TDP2 sequence for putative 3C or 3CD cleavage sites since EMCV does not encode a proteinase 2A equivalent (Hellen et al., 1992). We found that human TDP2 contains two putative rhinovirus 3C/3CD cleavage sites and one cardiovirus 3C cleavage site (**Figure 3.5**). We next used the online ExPasy server (http://web.expasy.org/compute_pi/) (Artimo et al., 2012) to calculate the predicted molecular weights of the human TDP2 fragments that would result from these putative cleavage sites (**Table 3.1**). The first rhinovirus cleavage site (EG) would result in 1.8 and 39.1 kDa TDP2 protein fragments. The second rhinovirus cleavage site (EG) would result in 36.9 and 4.0 kDa TDP2 protein fragments. The cardiovirus cleavage site (ES) would result in 28.6 and 12.3 kDa TDP2 protein fragments. Mouse TDP2 encodes two putative cardiovirus 3C cleavage sites and one enterovirus 3C/3CD cleavage site (**Figure 3.5**). We also used ExPasy to calculate predicted molecular weights of the mouse TDP2 fragments that would result from these putative cleavage sites (**Table 3.1**). The first cardiovirus cleavage site (EN) would result in 8.1 and 32.9 kDa TDP2 protein fragments. The second cardiovirus/ first enterovirus cleavage site (QS) would result in 37.7

and 3.3 kDa TDP2 protein fragments. Next we predicted where the putative cleavage sites present in the human and mouse TDP2 catalytic domain would be located using the Visual Molecular Dynamics software and the previously published TDP2 structures deposited in the Protein Data Bank (**Figure 3.6**) (Humphrey et al., 1996; Schellenberg et al., 2012; Schellenberg et al., 2016).

To determine if TDP2 is being cleaved via the picornavirus 3C/3CD proteinases, we performed a Western blot analysis using a polyclonal antibody that recognizes epitopes located at the N-terminus of human TDP2 on lysates generated from poliovirus-, CVB3-, or EMCV-infected HeLa cells at 0, 4, and 6 hours post-infection (**Figure 3.7, A**). We found that TDP2 was cleaved at 6 hours post-EMCV infection (**Figure 3.7, A lane 12**), resulting in a fragment similar in molecular weight to the N-terminus fragment (~28.6 kDa) predicted from the putative ES cleavage site in **Table 3.1**. TDP2 was not cleaved during poliovirus and CVB3 infection as predicted in **Figure 3.5**. Additionally, we performed a Western blot analysis using an antibody against human TDP2 and human rhinovirus 16 (HRV16)-infected lysates from 0, 6, 8, and 10 hours post-infection (**Figure 3.7, B**) and human rhinovirus 1A (HRV1A)-infected lysates from 0 and 13 hours post-infection (data not shown). HRV16 belongs to the major receptor rhinovirus group and infects lower airway cells while HRV1A belongs to the minor receptor rhinovirus group (Gern et al., 1997). We found that TDP2 is not cleaved during rhinovirus infection despite encoding putative rhinovirus 3C/3CD cleavage sites. It was confirmed that TDP2 was not cleaved even as late as 13 hours post-HRV16 infection (data not shown). Together these results show that TDP2 is cleaved during peak times of EMCV infection in HeLa cells and not during enterovirus infections, suggesting an alternative mechanism for regulating TDP2 activity during cardiovirus infections.

Human TDP2

MELGSCLEGGREAAEE**EG**EPEVKKRRLLCVEFASVASCDAAVAQCFLAENDWEMERA
LNSYFEPPVEESALERRPETISEPKTYVDLTNEETTDSTTSKISPSEDTQQENGSMFSLIT
WNIDGLDLNNLSERARGVCSYLALYSPDVIFLQEVIPPYYSYLKKRSSNYEIITGHEEGYF
TAIMLKKSRVKLKSQEIIPFPSTKMMRNLLCVHVNVSGNELCLMTSHLESTRGHAAERMN
QLKMVLKKMQEAP**ES**ATVIFAGDTNLRDREVTRCGGLPNNIVDVWEFLGKPKHCQYTW
DTQMNSNLGITAACKLRFDRIFFRAAAE**EG**HIIPRSLDLLGLEKLDCGRFPSDHWGLLCN
LDIIL

Mouse TDP2

MASGSSSDAAEPAGPAGRAASAPEAAQAEEDRVKRRRLQCLGFALVGGCDPTMVPSV
LRENDWQTQKALSAYFELP**EN**DQGWPRQPPTSFKSEAYVDLTNEDANDTTILEASPSG
TPLEDSSTISFITWNIDGLDGCNLPERARGVCSCLALYSPDVVFLQEVIPPYCAYLKKRAA
SYTIITGNEEGYFTAILLKKGRVKFKSQEIIPFPNTKMMRNLLCVNVSLGGNEFCLMTSHL
ESTREHSAERIRQLKTVLGKMQEAPDSTTVIFAGDTNLRDQEVIKCGGLPDNVFDAWEF
LGKPKHCQYTWDTKANNNLRIPAAYKHRFDRIFFRAEEGHLIP**QS**LDLVGLEKLDCGRF
PSDHWGLLCTLNVVL

Figure 3.5. Putative picornavirus 3C and 3CD proteinase cleavage sites in the TDP2 sequence. The picornavirus 3C proteinases and its precursor 3CD recognize specific residues at putative cleavage sites. Enterovirus 3C/3CD recognize QG, QA, QN, and QS residues with an amino acid with an aliphatic side chain in the P4 position (residues colored in green). Rhinovirus 3C/3CD recognizes the same cleavage sites as the enterovirus cleavage sites as well as an additional site: EG, also with an aliphatic side chain in the P4 position (residues colored in purple). Cardiovirus 3C proteinases recognize QG, QA, QS, EN and ES residues with a proline preferred in the P2 or P2' position (cleavage sites colored in red). Putative cleavage sites are highlighted in their denoted color in both the human and mouse TDP2 sequences.

Predicted cleavage site	Picornavirus 3C/3CD	TDP2 host	N-terminus molecular weight	C-terminus molecular weight
EG	rhinovirus	human	1.8 kDa	39.1 kDa
EG	rhinovirus	human	36.9 kDa	4.0 kDa
ES	cardiovirus	human	28.6 kDa	12.3 kDa
EN	cardiovirus	mouse	8.1 kDa	32.9 kDa
QS	cardiovirus/ enterovirus	mouse	37.7 kDa	3.3 kDa

Table 3.1. Predicted TDP2 molecular weights of cleavage products generated from different 3C proteinase recognition sites. Fragment TDP2 molecular weights due to putative proteinase cleavage sites were predicted using ExPasy online software. The predicted cleavage sites in the human or mouse TDP2 sequence are listed.
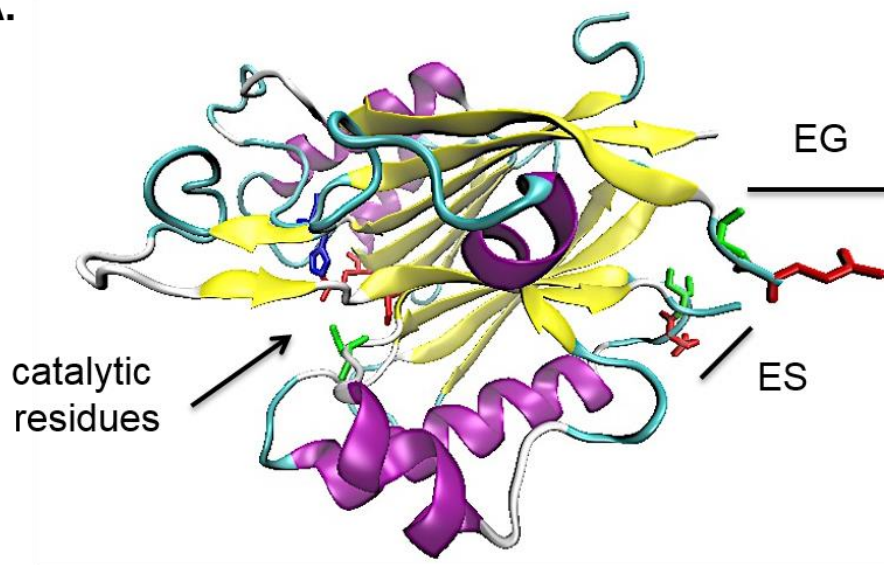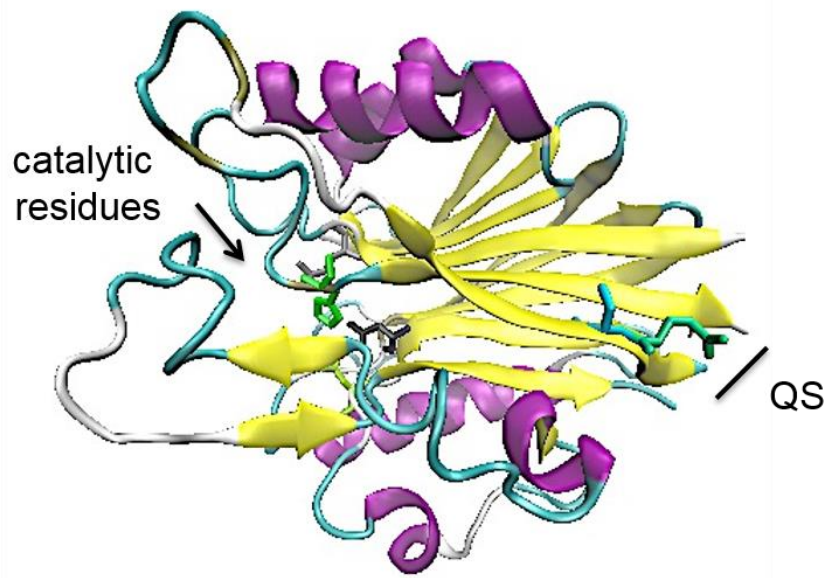
Figure 3.6. Putative proteinase cleavage sites in TDP2 catalytic domains. Ribbon models of the TDP2 catalytic domain were rendered using the Visual Molecular Dynamics (VMD) (Humphrey et al., 1996) software. **(A)** The C-terminal catalytic domain of human TDP2 was downloaded from PDB 5INO into the VMD software and is rendered using a ribbon model (Schellenberg et al., 2016). The secondary structures are color-coded (alpha helices in purple and beta sheets in yellow). The conserved catalytic residues E152, D262, N264, and H351 are represented using a stick model. The two putative 3C/3CD cleavage sites EG (rhinovirus) and ES (cardiovirus) are represented using a stick model. **(B)** The C-terminal catalytic domain of mouse TDP2 was downloaded from PDB 5INM into the VMD software and is represented using a ribbon model (Schellenberg et al., 2012). The secondary structures are color-coded as above). The conserved catalytic residues E162, D272, N274, and H359 are represented using a stick model. The putative cardiovirus or enterovirus 3C/3CD cleavage site QS is represented using a stick model.
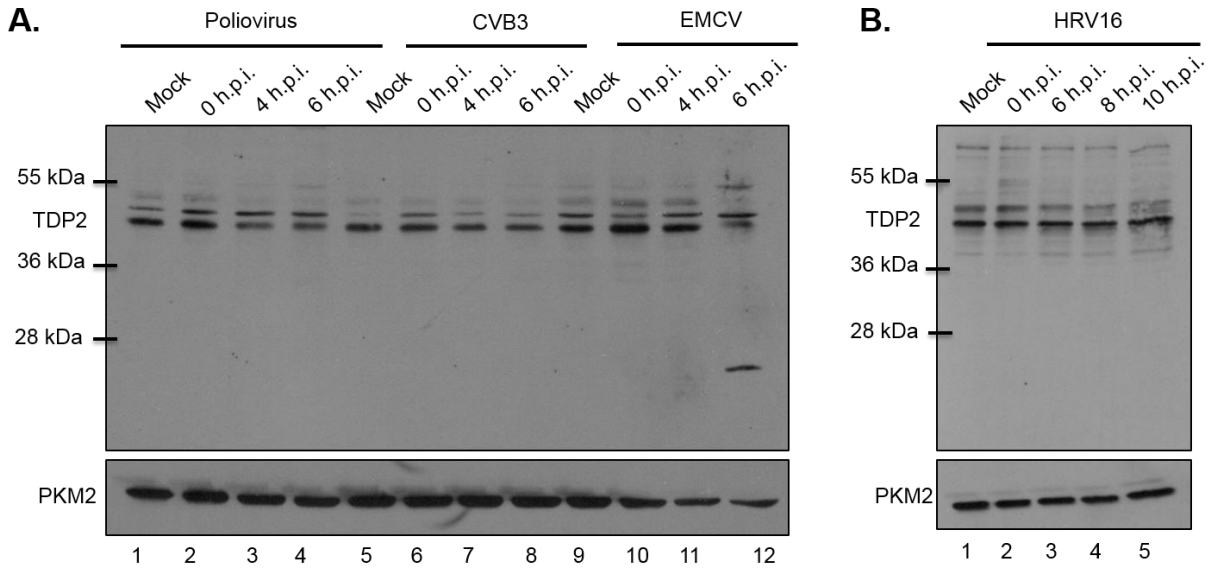
Figure 3.7. TDP2 is cleaved during EMCV infection. (A) HeLa cell monolayers were mock- or poliovirus-, CVB3-, or EMCV-infected at an MOI of 20. Cells and supernatant were collected at 0, 4, or 6 hours post-infection (h.p.i.) and used to generate NP-40 lysates. Mock- or virus-infected NP-40 lysate was subjected to SDS-PAGE and Western blot analysis using an anti-TDP2 or anti-PKM2 antibody (loading control) to visualize proteins. **(B)** HeLa cell monolayers were mock- or HRV16-infected at an MOI of 20. Cells and supernatant were collected at 0, 6, 8 or 10 h.p.i. and used to generate NP-40 lysates. Mock- or HRV16-infected NP-40 lysate was subjected to SDS-PAGE and Western blot analysis using an anti-TDP2 or anti-PKM2 antibody (loading control) to visualize proteins.

Discussion

As discussed in **Chapter 2**, enteroviruses are divergently dependent on TDP2 during their viral replication cycles (Maciejewski et al., 2016). TDP2, an enzyme primarily known as a DNA repair enzyme in the uninfected cell, was identified as possessing the cellular activity that hydrolyzes the highly conserved phosphotyrosyl bond between the third tyrosine of VPg and the 5' end of the viral RNA (Virgen-Slane et al., 2012). In the absence of this cellular protein, enterovirus yields are significantly reduced; with no increase in CVB3 yields over 24 hours post-infection. These decreases in viral yields are corroborated by an overall decrease in viral protein accumulation and viral RNA replication, with the most significant decrease observed during CVB3 infection. To determine whether TDP2 plays a positive role during infection of a picornavirus belonging to a different genus, we chose to characterize the role of TDP2 during cardiovirus infection, specifically EMCV. Previous work confirmed that a cellular activity, specifically a phosphodiesterase, could hydrolyze the phosphotyrosyl bond between VPg and the EMCV viral RNA (Gulevich et al., 2001). Although it is not known if TDP2 functions as VPg unlinkase during EMCV infection, we still wanted to characterize the role of TDP2 during EMCV infection.

A previous study carried out in our laboratory found that the primarily nuclear protein TDP2 was relocalized from the nucleus to the cytoplasm of the poliovirus-infected HeLa cells as early as 2 hours post-infection (Virgen-Slane et al., 2012). At peak times of poliovirus-infection (4 hours-post-infection), TDP2 is localized to the cell periphery in sites that do not colocalize with viral replication and encapsidation markers (3A and capsid, respectively) (Virgen-Slane et al., 2012). The authors concluded that this suggests a regulatory role for TDP2 and its catalytic activity during the course of poliovirus infection. To verify if TDP2 was relocalized during cardiovirus infection, we infected HeLa cells with EMCV and fluorescently labeled TDP2 and the EMCV

viral replication marker 3D. Similar to poliovirus infection, TDP2 relocalized from the nucleus to the cell cytoplasm as early as 2 hours post-EMCV infection. TDP2 cytoplasmic relocalization increased by 4 and 6 hours post-EMCV infection. In contrast to peak times of poliovirus-infection, we did not observe a distinct relocalization pattern to the cell periphery during peak times of EMCV infection. These results suggest that TDP2 is utilized during early times in EMCV infection, but is not being excluded from putative viral replication sites.

Next we were wanted to explore the dependence of EMCV on TDP2 during infection. We found that EMCV yields were reduced approximately 0.5 $\log_{10}$ unit at 6 hours post-infection, 1 $\log_{10}$ unit at 8 hours post-infection, and < 0.5 $\log_{10}$ unit at 10 hours post-infection in the absence of TDP2 using our wild type and TDP2$^{-/-}$ murine cell lines. Since peak titers are not achieved in the MEF-TDP2$^{+/+}$ cell line until 10 hours post-infection, the decrease in the viral titers in the absence of TDP2 is less pronounced than what we observed during enterovirus infections. However, our single-cycle growth analysis does reveal that EMCV viral replication is delayed in the absence of TDP2 and that TDP2 does play a pro-viral role during the cardiovirus replication cycle.

To further dissect the role of TDP2 during EMCV infection, we analyzed viral protein accumulation in the MEF-TDP2$^{+/+}$ and TDP2$^{-/-}$ cell lines. We initially detected viral accumulation of the nonstructural EMCV protein 2A during infection in the presence or absence of TDP2 and found that there was a significant decrease in 2A expression in EMCV-infected MEF-TDP2$^{-/-}$ cells. This could be due to degradation of the small viral protein in the absence of TDP2 or in sensitivity of the antibody in 2A recognition. To verify this phenotype, we analyzed viral protein accumulation of the nonstructural EMCV 3D and 3C proteins. We observed a less severe reduction in viral protein accumulation in the absence of TDP2 when we carried out Western blots for these

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larger viral proteins. This latter phenotype is also consistent with our single-cycle growth analysis of EMCV infection in the MEF-TDP2$^{-/-}$ cells. Interestingly, viral protein expression increases in the wild type mouse cells between the two time points, but remains unchanged between the two time points in the absence of TDP2. These results suggest that the MEF-TDP2$^{+/+}$ cells are translating the viral RNA for viral protein production in excess of what is necessary to carry out EMCV viral replication, or perhaps that reduced viral protein production results in the decrease in viral yields observed in the MEF-TDP2$^{-/-}$ cells.

To further characterize the role of TDP2 during EMCV infection, we took advantage of an infectious EMCV reporter construct (GLuc-EMCV) to generate *in vitro* transcribed infectious RNA. GLuc-EMCV RNA was transfected into the HeLa, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers. Viral translation (in the presence of viral RNA synthesis inhibitor dipyridamole) and RNA replication was measured in the presence and absence of TDP2. We found that viral translation was not affected by the absence of TDP2. We would predict that this is the case since the RNA does not contain VPg linked to the 5' end of the viral RNA. However we found that viral RNA replication was slightly reduced in the absence of TDP2. These results are consistent with our single-cycle growth analysis and Western blot analysis.

After confirming only modest dependence of EMCV on TDP2, we explored whether EMCV modified TDP2 activity during infection. Since we did not observe the distinct relocalization pattern of TDP2 to the cell periphery, away from potential viral replication sites as seen during poliovirus infection, we wanted to see if TDP2 was being modified during EMCV infection. Picornavirus proteinases are known to play a major role during infection by modifying host proteins to enhance their viral replication cycle. First we analyzed both the human and mouse TDP2 sequences to identify putative proteinase cleavage sites. We calculated the potential

molecular weights of TDP2 that would result from such cleavage events and then mapped the location of the putative cleavage sites on the catalytic domain. We found these cleavage sites to be not buried the TDP2 catalytic domain as predicted by our Visual Molecular Dynamics analysis, suggesting a possibility for cleavage of TDP2 during picornavirus infections. Our Western blot analysis confirmed that human TDP2 is cleaved during EMCV infection. The observed molecular weight of the TDP2 fragment was similar to the predicted molecular weight of the N-terminal fragment that would result following 3C proteinase cleavage as determined by the putative cleavage sites. This N-terminal fragment is also predicted to be recognized by the polyclonal antibody used for these studies. However, TDP2 cleavage was not observed during enterovirus infections in HeLa cells, despite identifying two putative rhinovirus cleavage sites. It is important to note that these putative cleavage sites are not preferred for rhinovirus 3C/3CD. It is possible that during rhinovirus infection the catalytic domain is hidden by putative TDP2 interactions with either viral or host proteins or is cleaved later than 13 hours post-infection. TDP2 cleavage is seen at 6 hours post-EMCV infection in HeLa cells, which is peak time for EMCV infection in this cell line. These results are not surprising since PCBP2, another host protein discussed in **Chapter 1**, is also differentially cleaved among picornavirus infections in WisL cells. PCBP2 is not cleaved during HRV infection of WisL cells but is cleaved during poliovirus infection of WisL cells (Chase and Semler, 2014). Since TDP2 is localized closely to EMCV 3D during peak times of EMCV infection, as confirmed by our confocal microscopy data, it is possible that EMCV cleaves TDP2 as a mechanism to regulate its catalytic activity during later times in the viral replication cycle. This could be an alternative mechanism for excluding TDP2/VPg unlinkase activity from viral RNA synthesis and encapsidation sites during enterovirus infection. These results support our hypothesis that TDP2 functions as a regulator of the viral replication cycle via its VPg unlinkase

activity and marks the viral RNA with the presence or absence of VPg for viral translation, RNA synthesis, or encapsidation. The presence or absence of VPg on the viral RNA is not absolutely required for these steps to occur; however, our data suggests that it is necessary for efficient cardiovirus replication.

Materials and Methods

Cell culture and virus stocks

Wild type TDP2 mouse embryonic fibroblast (MEF-TDP2$^{+/+}$) and knockout TDP2 mouse embryonic fibroblast (MEF-TDP2$^{-/-}$) cell lines were described by Caldecott and colleagues (Zeng et al., 2012). MEF-TDP2$^{+/+}$ and MEF-TDP2$^{-/-}$ cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells were grown as monolayers in DMEM supplemented with 8% newborn calf serum (NCS). The EMCV working stock was generated from the pEC9 construct kindly provided by Dr. Ann C. Palmenberg at the University of Wisconsin, Madison.

Virus infections and single cycle growth analysis

MEF-TDP2$^{+/+}$ or MEF-TDP2$^{-/-}$ cell monolayers were seeded on 20 cm^2 plates. The monolayers were washed with 1X PBS and infected with EMCV at an MOI of 20. EMCV was adsorbed at room temperature for 30 minutes. Following adsorption, the cells were washed with 1X PBS 3 times and overlaid with 3 ml of DMEM with 10% FBS. EMCV-infected cells were incubated at 37°C. Cells and supernatant were collected and subjected to 5 freeze-thaw cycles. Viral yields were quantified by plaque assay on HeLa cell monolayers. Viral yields are reported as PFU per cell (PFU/cell) and plotted on a logarithmic scale. Plaque assays were performed in technical triplicate. Error is reported as standard deviation. Student's t tests were performed using the graphpad software.

Immunofluorescence

HeLa cell monolayers were seeded on coverslips. The monolayers were washed with 1X PBS and infected with EMCV at an MOI of 20. EMCV was adsorbed at room temperature for 30 minutes. Following adsorption, the cells were washed with 1X PBS 3 times and overlaid with 3 ml of DMEM with 8% NCS. EMCV-infected cells were incubated at 37° C. The monolayers were washed with 1X PBS and fixed with 3.7% formaldehyde for 20 minutes at 0, 2, 4, or 6 hours post-infection. The fixed cells were permeabilized with 0.5% NP-40 and blocked with 1% goat serum diluted in 1X PBS. The cells were then incubated with either anti-Mengo 3D mouse monoclonal antibody (Santa Cruz), which reacts with EMCV 3D since they are both serotypically related, or anti-TDP2 rabbit polyclonal (Bethyl), both at a 1:200 dilution in 1% BSA for one hour at room temperature. The cells were then incubated with Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes) at a 1:250 dilution in 1% BSA for one hour at room temperature. The nuclei were stained with 4 ug/ml DAPI for 10 minutes at room temperature. Proteins were visualized and imaged with a Zeiss LSM700 laser scanning confocal microscope and Zen software.

Preparations of lysates from uninfected and infected cells and Western blot analysis

Infections with EMCV, poliovirus, and CVB3 were carried out as described above. Cells and their supernatants were harvested and pelleted at their respective time points post-infection. Samples were washed with 1X PBS 3 times and pelleted. The pellet was resuspended in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40) and incubated on ice for 30 minutes. Cell debris was pelleted and the supernatant collected. Protein concentration was measured using a Bradford assay. For Western blot analysis, 50 to 60 µg of NP-40 lysates generated from EMCV-infected cells were subjected to electrophoresis on SDS-containing 12.5%

polyacrylamide gels. Proteins were then transferred to a PVDF membrane. Membranes were incubated with the following antibodies: mouse monoclonal anti-Mengo 3D (Santa Cruz, 1:1000 dilution), mouse monoclonal anti-EMCV 2A (1:2000 dilution), or rabbit polyclonal anti-EMCV 3C (1:7500 dilution) to detect viral protein accumulation. Dr. Ann C. Palmenberg at the University of Wisconsin, Madison, kindly provided the mouse monoclonal antibody to EMCV protein 2A. Dr. T. Glen Lawson at Bates College kindly provided the rabbit polyclonal antibody to EMCV protein 3C. The rabbit polyclonal antibody anti-TDP2 (Bethyl, 1:2000) was used to detect TDP2 during enterovirus and cardiovirus infections. The rabbit monoclonal antibody anti-GAPDH (abcam, 1:10,000) was used to detect endogenous GAPDH as a protein loading control. The membranes were then incubated with goat anti-rabbit or goat anti-mouse IgG (H+L) HRP conjugate antibodies (Bethyl, 1:7500). Protein bands were visualized by ECL Western Blotting Substrate (Life Technologies).

In vitro transcription and transfection of 5'EMCVLuc3' RNA for luciferase assay

For the generation of *in vitro* transcribed EMCV 5' EMCV-Luc 3' NCR (5'EMCVLuc3') RNA transcripts, the previously published p5'EMCVLuc3' plasmid (Walter et al., 1999) was linearized with SalI to generate a transcription template. RNA transcription was performed using a MEGAscript T7 transcription kit (Ambion). For generation of the *in vitro* transcribed control capped RSTF (RLuc-RSTF) RNA transcripts, the previously published pRst-CVB3F plasmid (Jang et al., 2004) was linearized prior to the CVB3 5' NCR sequence with BlpI to generate a transcription template. RNA transcription was performed using a mMessage mMachine T7 transcription kit (Ambion). RNA transcripts were purified using the RNeasy kit (Qiagen). HeLa, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers in 10 cm^2 plates were co-transfected with 1.9 µg of 5' EMCV-Luc 3' NCR RNA and 100 ng of capped RSTF RNA transcript per plate using

the TransIT-mRNA transfection kit (Mirus Bio). Cells were washed with 1X PBS and lysed in 1X Passive Lysis Buffer (Promega) for 15 minutes. FLuc and RLuc values were measured using the Dual-Luciferase Reporter system (Promega) according to manufacturer's protocol. Transfections were carried out in biological duplicates. The FLuc-5'EMCV3' and RLuc-RSTF (RLU/s) values were divided by total cell count (RLU/cell). The RLU/cell values from FLuc-5'EMCV3' were normalized against the RLU/cell values from RLuc-RSTF.

In vitro transcription and transfection of GLuc-EMCV RNA for RNA translation and replication assays

For the generation of _in vitro_ transcribed _Gaussia_ luciferase EMCV (GLuc-EMCV) RNA transcripts, the infectious GLuc-Mengovirus clone, pdnGLuc-VFETQG-m16.1, was kindly provided by Dr. Frank van Kuppeveld at the University of Utrecht, The Netherlands. GLuc-EMCV was linearized with BamHI to generate a transcription template. RNA transcription was performed using a MEGAscript T7 transcription kit (Ambion). RNA transcript was purified using the RNeasy kit (Qiagen). HeLa, MEF-TDP2$^{+/+}$, and MEF-TDP2$^{-/-}$ cell monolayers in 10 cm^2 plates were transfected with 2 µg of RNA transcript per plate using the TransIT-mRNA transfection kit (Mirus Bio). For the translation assay, transfection was carried out in the presence of 100-µM dipyridamole (solubilized in ethanol), an EMCV viral RNA synthesis inhibitor (Fata-Hartley and Palmenberg, 2005; Tonew et al., 1977). For the replication assay, transfection was carried out in parallel in the absence of dipyridamole. Cells were washed with 1X PBS and lysed in 1X _Renilla_ Luciferase Assay Lysis Buffer (Promega) for 15 minutes. GLuc values were measured using the _Renilla_ Luciferase Assay System (Promega). RLU/s/cell was calculated and values were plotted as RLU/cell. Transfections were carried out in three separate biological replicates, with each of the replicates carried out as a technical duplicate. The GLuc values from the technical duplicates

were averaged and used as a representation of a single biological replicate. The GLuc values from the biological replicates were averaged and the standard error of the mean was calculated for the error bars.

Visual Molecular Dynamics analysis of TDP2 catalytic domain and cleavage sites

The human and mouse TDP2 catalytic domain structure was downloaded from the protein data bank (PDB 5INO and PDB 5INM) and visualized using the Visual Molecular Dynamics software (http://www.ks.uiuc.edu/Research/vmd/) as a ribbon model (Humphrey et al., 1996; Schellenberg et al., 2012; Schellenberg et al., 2016). The conserved catalytic residues E152, N264, D262, and H351 for human TDP2 and E162, N274, D272, and His359 were selected and represented as a stick model. The putative picornavirus 3C/3CD cleavage sites were represented as a stick model. Catalytic residues and putative cleavage sites were labeled.

CHAPTER 4

## Final conclusions and overall significance

Picornaviruses make up a family of RNA viruses containing multiple genera. Each genus can be as diverse as the enterovirus genus or small like the cardiovirus genus. Despite their genome sequence differences, all members encode small positive-sense RNA genomes that carry out their viral replication cycle in the cytoplasm of the infected cell. Due to their limited coding capacity, these viruses can alter the cellular environment, hijacking cellular protein functions to help carry out their viral replication cycle. One of the proteins usurped during infection, as discussed throughout this thesis, is the DNA repair enzyme TDP2. TDP2 was identified in our laboratory to posses the cellular activity VPg unlinkase that was discovered over three decades ago to hydrolyze the phosphotyrosyl bond between the 5' end of the viral RNA and the small viral protein VPg (Ambros and Baltimore, 1978; Ambros et al., 1978; Virgen-Slane et al., 2012).

Prior to its identification as TDP2, VPg unlinkase activity was partially characterized in both the uninfected and infected cell (Ambros and Baltimore, 1980; Rozovics et al., 2011; Sangar et al., 1981). The dominant question before its identification as TDP2 was what role does VPg unlinkase play during picornavirus replication? Early work showed that VPg is absent from ribosome-associated viral RNA but present on newly synthesized viral RNAs and encapsidated virion RNA (Flanegan et al., 1977; Hewlett et al., 1976; Nomoto et al., 1977b; Nomoto et al., 1976). A subsequent study showed that VPg-linked viral RNA can, however, form an *in vitro* translation initiation complex (Golini et al., 1980). This latter study was supported by more recent work that showed that input VPg-linked enterovirus RNA could be translated and replicated following transfection (Langereis et al., 2014). These differences in results can be attributed to the experimental approach. For example, performing an infection as done in the study showing non-

VPg linked viral RNA associated with ribosomes may not be equivalent to an *in vitro* approach as done in the subsequent studies. It is possible that receptor-mediated entry followed by uncoating can play a role in recognizing the VPg-linked viral RNA during a normal infection and this step is bypassed following transfection. Additionally, it is known that VPg serves as a protein-primer for viral RNA synthesis (Flanegan and Baltimore, 1977; Nomoto et al., 1977a; Paul et al., 1998). Together these findings show VPg present or absent at the 5' end of the viral RNA at specific steps during the viral replication cycle (**Figure 2.1**). Since TDP2 possesses the VPg unlinkase activity necessary for the removal of VPg from the 5' end of the viral RNA, it was hypothesized that TDP2 marks the viral RNA for viral translation, RNA synthesis, or encapsidation (**Figure 2.2.**). Confocal microscopy data showing TDP2 excluded from putative viral replication and encapsidation sites despite bulk VPg unlinkase activity being unchanged during the course of poliovirus infection further suggests that the catalytic activity of TDP2 is regulated through compartmentalization (Rozovics et al., 2011; Virgen-Slane et al., 2012).

In **Chapter 2** and **Chapter 3**, we showed that TDP2 is required for efficient picornavirus replication in murine cells. This conclusion is derived from several experiments carried out in wild type and knockout TDP2 mouse embryonic fibroblasts. We observed a reduction in viral yields, translation, and RNA replication in the absence of TDP2. We also confirmed that in the absence of both TDP2 and capsid proteins (thus encapsidation) that viral RNA replication is slightly reduced early in infection, but unchanged at peak times of replication. Since viral RNA replication is similar in the presence or absence of TDP2 without encapsidation occurring, these data suggest that premature encapsidation may contribute to the reduction of viral RNA replication levels in our knockout TDP2 mouse cells. However, additional experiments will be required to confirm these results, since there may be complications using a replicon lacking capsid protein coding

sequences. This issue can be addressed by mutating the poliovirus VP0 capsid protein maturation cleavage site residues to abolish or delay encapsidation (Ansardi and Morrow, 1995). The mutated RNA could then be transfected into wild and TDP2 knockout cells where viral RNA synthesis could be measured over a time course by quantitative real-time PCR (with or without guanidine HCl). Based on our results using the capsid-less replicon, we would expect viral RNA synthesis to be slightly decreased in the absence of TDP2 early on during infection and similar to wild type levels by peak times of infection. This experiment could help us determine if premature encapsidation in the absence of TDP2 is contributing to the reduced viral yields observed during infection. Our proposed model of the role of TDP2 is shown in **Figure 4.1**.
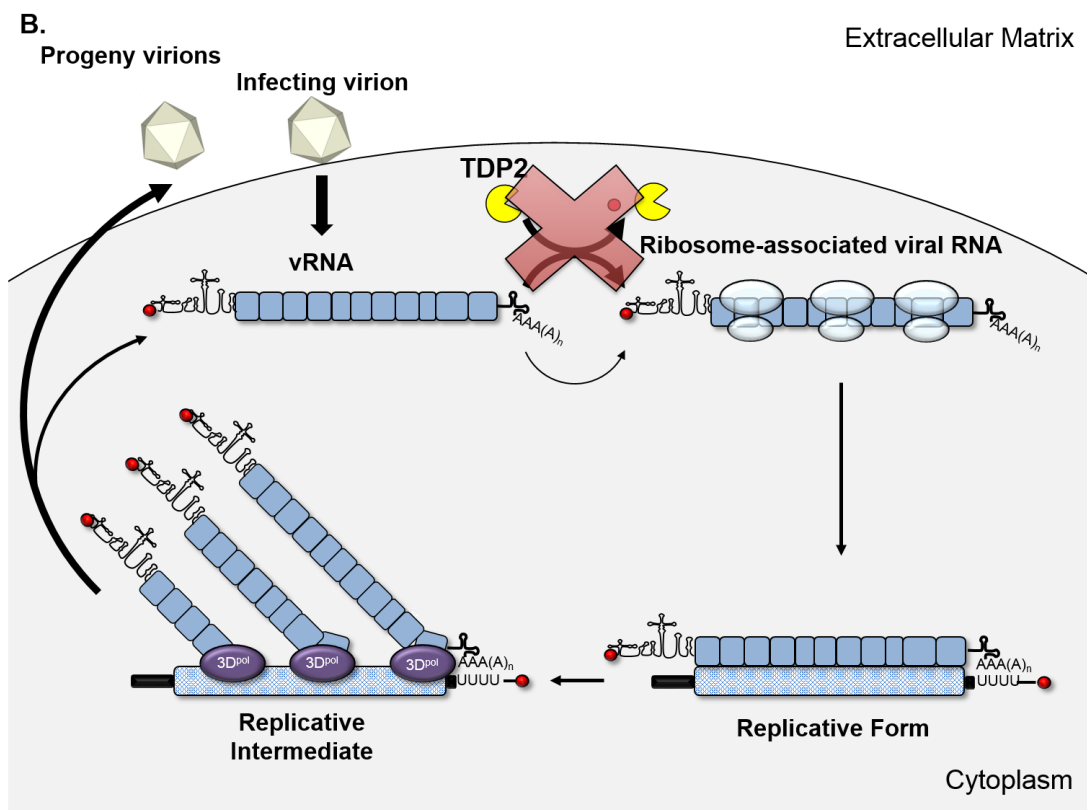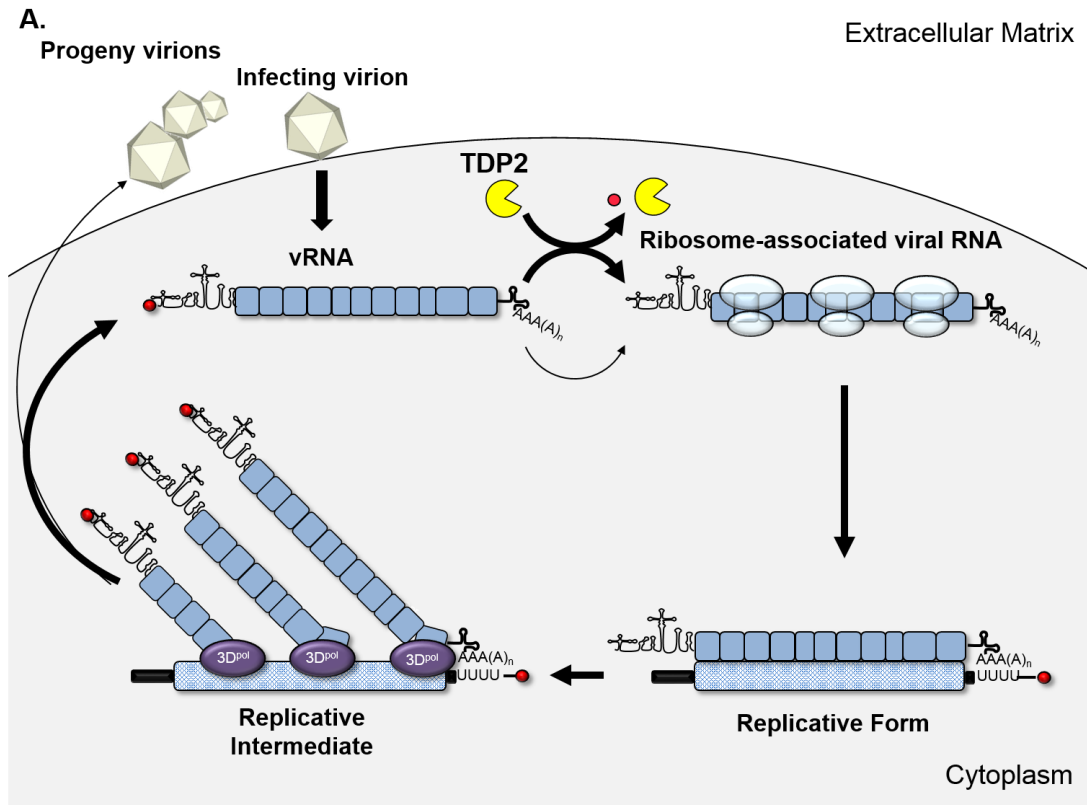
Figure 4.1. VPg marks picornavirus RNAs for viral translation, RNA synthesis, or encapsidation. The proposed role of TDP2 during picornavirus infections is illustrated. **(A)** Following infection, picornavirus vRNA (VPg-RNA) is released into the cell cytoplasm. TDP2 (yellow "pac-man" symbol) hydrolyzes the phosphotyrosyl bond between VPg (red circle) and the 5' end of the viral RNA. Next, the viral RNA undergoes translation and negative-strand RNA synthesis. The negative-strand viral RNA serves as a template for positive-strand viral RNA synthesis by $3D^{pol}$ (purple oval). It should be noted that previous studies showed VPg both present and absent from the 5' end of the positive-strand RNA of the RF (Wu et al., 1978). The newly synthesized positive-strand RNA (VPg-RNA) can either undergo additional rounds of viral translation and RNA replication or become encapsidated into progeny virions. **(B)** In the absence of TDP2 (red X), VPg-RNA can still undergo viral translation and negative-strand RNA synthesis, albeit at reduced levels due to the presence of VPg on the viral RNA. Following positive-strand RNA synthesis, the newly synthesized viral RNA (VPg-RNA) is prematurely encapsidated, leading to reduced viral RNA available for additional rounds of translation and RNA replication, and thus reduced viral titers.

Picornavirus	Reduction in virus titer in TDP2$^{-/-}$	Effect on viral replication in TDP2$^{-/-}$
Poliovirus	~1-2 log$_{10}$ (transfection and infection)	Decrease in viral protein accumulation and replication possibly due to premature encapsidation
Human Rhinovirus 1a	~1 log$_{10}$	Not tested
Coxsackievirus B3	~2-2.5 log$_{10}$	Undetectable viral protein accumulation and decrease in viral replication is not solely 5' NCR dependent
Encephalomyocarditis Virus	~0.5-1 log$_{10}$	Decrease in viral protein accumulation and replication; TDP2 is relocalized and cleaved during infection

Table 4.1. Summary of how the absence of TDP2 affects picornavirus replication. This table shows a summary of the decrease in picornavirus yields observed in the absence of TDP2 and the effect that it has on viral replication.

In this dissertation it was also described how TDP2 is divergently required during picornavirus infections. These results are summarized in **Table 4.1**. Viral yields were most reduced in the absence of TDP2 during CVB3 infection, followed by poliovirus, rhinovirus, and EMCV infections. Although viral yields were reduced during rhinovirus, poliovirus, or EMCV infection in the absence of TDP2, no increase in viral yields was observed during CVB3 infection in the absence of TDP2. These results showed that TDP2 is required for CVB3 infection in our murine cell model. Why is CVB3 more dependent on TDP2 for infection than its picornavirus family counterparts? Our data showed that CVB3 protein accumulation in the absence of TDP2 was undetectable by Western blot analysis, as well as the elimination of CVB3 infectivity in the TDP2 knockout mouse cells following serial blind passaging of the virus. One might conclude that TDP2 is absolutely required for CVB3 replication; however, we also found that CVB3 RNA could be translated and replicated in the absence of TDP2 following transfection of *in vitro* transcribed RNA. These results complemented previous work showing that input VPg-linked CVB3 RNA can be translated and replicated following transfection of *in vitro* transcribed viral RNA (Langereis et al., 2014). In agreement with our hypothesis, non-VPg linked viral RNA is translated similarly in the presence or absence of TDP2. However, following initial rounds of translation and replication of this input RNA, the newly synthesized viral RNA is VPg-linked. The newly synthesized viral RNA can still be translated and replicated in the absence of TDP2, albeit at reduced levels in comparison to in the presence of TDP2. Experimental caveats that may be responsible for these contradicting data have been previously discussed in **Chapter 1 and Chapter 2**, including bypassing of receptor-mediated cell entry, a step that may initially expose different ends of the viral RNA. This viral entry may play a role in how host proteins recognize the incoming virion RNA for initiation of viral translation. This brings up a new question: how does TDP2 recognize

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the phosphotyrosyl linkages of incoming picornavirus virion RNA following receptor-mediated viral entry?

TDP2 contains a ubiquitin-associated protein-like domain at its N-terminus, which is suggested to be the domain responsible for its protein-protein interactions in the uninfected cell [reviewed in (Li et al., 2011)]. This sequence motif allows TDP2 to interact with ubiquitin and may play a role in TDP2 recognizing the covalent phosphotyrosyl bond in trapped TOPII-DNA cleavage complexes. These DNA adducts are processed by the ubiquitin-proteasome degradation pathway allowing TDP2 access to the phosphotyrosyl linkage (Gao et al., 2014; Mao et al., 2001). Although these data suggest how TDP2 recognizes the phosphotyrosyl linkages between TOPII and DNA, it remains unclear how TDP2 may recognize this same bond between VPg and the viral RNA. It is possible that since TDP2 is present at low concentrations in the cell cytoplasm prior to infection, TDP2 is available early on in infection to recognize this conserved picornavirus RNA linkage upon viral RNA release into the cell cytoplasm. We know that TDP2 binds DNA substrates with a much greater affinity than RNA substrates, so it may be unlikely that this VPg-RNA bond recognition requires additional factors, given the low concentration of TDP2 available in the cytoplasm (Gao et al., 2014; Pei et al., 2003). Although this mechanism could be determined, it would be difficult since the TDP2-mediated removal of VPg is predicted to occur almost immediately following infection.

Work that can be carried out to further understand how TDP2 recognizes the viral RNA includes determining how TDP2 is relocalized from the nucleus to the cell cytoplasm during picornavirus infections. As discussed in **Chapter 1**, we know that the enterovirus proteinase 2A and cardiovirus L protein are responsible for disrupting the nucleo-cytoplasmic trafficking of the host to concentrate nuclear proteins into the cell cytoplasm to promote viral replication. Since

TDP2 is predominantly a nuclear protein in uninfected cells, we would predict that either the enterovirus 2A proteinase or cardiovirus L protein is responsible for TDP2 relocalization into the cell cytoplasm (Pei et al., 2003). However, this viral-mediated alteration of the cellular environment does not explain relocalization of TDP2 to the cell periphery during peak times of poliovirus-infection. As discussed in **Chapter 1**, TDP2 has multiple binding partners in the uninfected cell. It is not known if these canonical TDP2-cellular protein interactions are disrupted or if new protein interactions are created during picornavirus infections. It would be interesting to identify such interactions throughout picornavirus infections by TDP2 co-immunoprecipitation and mass spectrometry analysis. Results from such experiments would provide insight into novel or picornavirus-specific TDP2-protein interactions important for the viral replication cycle. These findings could help elucidate a possible mechanism for how TDP2 is being excluded from putative viral replication and encapsidation sites and possible insight into how TDP2 recognizes the VPg-RNA linkage.

We further explored why TDP2 is so critical during CVB3 infection. As discussed in previous chapters, the same host proteins have been documented to differentially interact with different picornavirus 5' NCRs. We examined if the CVB3 5' NCR was responsible for the phenotype observed during CVB3 infection in the absence of TDP2. We observed viral replication occurring in the absence of TDP2 when using a chimeric poliovirus encoding the CVB3 5' NCR. The data showed that CVB3 dependence on TDP2 activity is not solely a result of differences in the 5' NCRs of the two viruses. It is possible that poliovirus proteins are more efficient at partitioning host proteins necessary for viral RNA synthesis and encapsidation than CVB3 proteins, which lead to enhanced detection of CVB3 5' NCR-mediated viral translation and replication. Alternatively, this phenotype may be due to an overall reduction in viral yields, protein

accumulation, and luciferase expression observed for CVB3 replication in comparison to poliovirus replication in wild type mouse cells. Previous unpublished work carried out in our laboratory showed that cap-dependent translation is not shut down during poliovirus infection in mouse embryonic fibroblasts but is shut down during poliovirus infection in HeLa cells. Although mouse cells are naturally susceptible to CVB3 infection, other mouse-specific anomalies may exist in these cells that could lead to an overall decrease in CVB3-infection. For example, we infected HeLa cells, wild type, and TDP2 knockout mouse embryonic fibroblasts with a CVB3 virus encoding a green fluorescent protein (GFP) upstream of its structural proteins and assessed viral translation and replication by visualization of GFP expression. We found that the HeLa cells express high levels of GFP, while our wild type mouse cells weakly expressed GFP (data not shown). No GFP expression was detected in our knockout TDP2 mouse cells, as expected (data not shown). These experiments showed that overall CVB3 replication is reduced in our mouse cells in comparison to a human cell line, and thus, CVB3 replication may be more sensitive to TDP2 being absent in mouse cells. We hypothesize that if these same experiments were carried out using a wild type and knockout TDP2 human cell line, it may be possible for CVB3 to replicate at low levels in the absence of TDP2. This difference in expected results would be due to an overall increase in infection efficiency using human cells. Although previous attempts to knockdown TDP2 by shRNA in HeLa cells in our laboratory resulted in a loss of cell viability, other laboratories have successfully knocked down or knocked out TDP2 in human cell lines (Cui et al., 2015; Li et al., 2011).

Having a human TDP2 knockout or knockdown cell line would allow us to carry out many experiments that could help determine the exact role of TDP2 during the picornavirus replication cycle that were not technically feasible in our mouse embryonic fibroblast cell lines. One

experiment includes polysome fractionation of picornavirus-infected mouse cells to determine the ratio of VPg-linked versus unlinked viral RNA in the polysomes of our wild type or knockout TDP2 mouse embryonic fibroblasts. Due to a low concentration of ribosomes in the fractionated wild type mouse embryonic fibroblasts compared to HeLa cells we were not able to perform polysome analysis. This analysis would allow us to determine the impact of VPg on viral translation initiation and further test the hypothesis that TDP2 distinguishes the viral RNA for translation to efficiently occur. Based on our results, we would predict that VPg-linked RNA would be detected at low levels in the absence of TDP2, but no unlinked viral RNA would be associated with the polysomes in the absence of TDP2. We would also expect low levels of VPg-linked CVB3 viral RNA associated with polysomes in the absence of TDP2 in comparison to the other picornavirus RNAs. In the presence of TDP2, we would expect unlinked viral RNA associated with the polysomes. However, it is possible that removal of VPg removal from the viral RNA is not necessary for efficient viral translation to occur and that TDP2 is playing a role in the downstream steps of the viral replication cycle.

Another technically challenging experiment using our mouse embryonic fibroblast cell lines involved quantifying the viral yields produced from transfected *in vitro* transcribed poliovirus RNA (non-VPg-linked) in the presence or absence of TDP2. Viral yields were significantly reduced when we transfected purified poliovirus virion RNA into our wild type and TDP2 knockout mouse embryonic fibroblasts in comparison to HeLa cells as shown in **Figure 2.4** in **Chapter 2**. Although previous reports showed that *in vitro* transcribed poliovirus RNA is infectious, we have observed a decrease in overall viral yields following transfection of transcript versus purified virion RNA (Cathcart et al., 2013; Kaplan et al., 1985). This could explain why we were not able to detect virus produced by our mouse cells following transfection of *in vitro*

transcribed reporter RNA. If this experiment could be carried out in a human cell line, we would expect virus to be detected in the absence of TDP2 following transfection of *in vitro* transcribed poliovirus RNA (non-VPg linked). To directly compare the effect of VPg-linked versus unlinked viral RNA on virus production in the absence of TDP2, we could use recombinant TDP2 to cleave VPg from purified virion RNA or mock-treat the virion RNA. Next we could transfect these VPg-linked or unlinked viral RNAs in parallel into human cells with or without TDP2 and quantify viral yields by plaque assay. We would expect viral yields to be increased in the absence of TDP2 from the cells transfected with the TDP2-treated virion RNA, which would suggest that TDP2 plays a role early in viral replication.

As noted in previous sections of this dissertation, it is difficult to assess the role of TDP2 during viral translation, RNA synthesis, and encapsidation since all these steps during the viral replication cycle are closely coupled. If a decrease in viral translation is observed from the input viral RNA, then we expect to see a decrease in viral RNA synthesis and encapsidation due to a decrease in viral proteins available for carrying out subsequent viral replication steps. Additionally, previous studies have shown that the viral replication and encapsidation steps are physically coupled (Caliguiri and Compans, 1973; Caliguiri and Mosser, 1971; Pfister et al., 1995; Pfister et al., 1992). This has made it challenging to dissect the role of TDP2 during viral replication. We initially generated S10 extract from our wild type and knockout TDP2 mouse cells to look at the impact of the absence of TDP2 on viral translation and replication using our well-established *in vitro* viral translation and replication assays. Our attempts to carry these assays out in mouse S10 extracts proved unsuccessful. We next analyzed viral translation and replication by transfection of our infectious reporter RNA replicons. Although we were successful at measuring viral translation and RNA replication by this method, we still encountered experimental caveats

due to transfection. An alternative approach to circumvent these caveats would be to generate virus (VPg-linked) from these infectious reporter RNA replicons and infect our wild type and TDP2 knockout cells. Next we could measure luciferase expression in the presence or absence of viral RNA synthesis inhibitors (GuHCl or dipyridamole) and quantify viral yields. This experiment would provide a more sensitive method for measuring viral translation and RNA replication in the presence or absence of TDP2 following a viral infection. We would predict that viral translation, and thus viral RNA replication, is reduced in the absence of TDP2. However, if TDP2 has a greater impact on encapsidation, as observed with our results using our luciferase reporter poliovirus RNA replicon without the capsid proteins sequence, we would find a minimal decrease in viral translation in the absence of TDP2 (with GuHCl) and a greater decrease in viral RNA replication (without GuHCl).

Although the experiments described above using a TDP2 knockout or knockdown human cell line could help in determining the direct role TDP2 has on virus replication, it is still probable that each picornavirus would be divergently dependent on TDP2. As mentioned above, these differences can be attributed to how the individual picornavirus modifies its host environment to help carry out their replication cycle. One process that can dampen viral replication is the antiviral response from the host. Picornaviruses are known to differentially modify cellular proteins involved in antiviral signaling pathways, specifically in the interferon response. For example, a host protein belonging to the viral RNA sensing RIG-like receptors (RLR) family, LGP2, has been shown to interact with EMCV RNA but not enterovirus RNAs (Deddouche et al., 2014). Enterovirus RNAs are recognized by another member of the RLR family, MDA5, which is a host protein that recognizes long double-stranded RNA, and the MDA5 adaptor molecule MAVS (Abe et al., 2012; Feng et al., 2012; Wang et al., 2010). Interestingly, previous work has found

differences in mortality in picornavirus-infected mice when MDA5 and MAVS are knocked out (Abe et al., 2012; Wang et al., 2010). It appears that MDA5 and MAVs play a more significant role in suppressing CVB3 infection than poliovirus infection in mice (Abe et al., 2012; Wang et al., 2010). This greater impact of the murine host's antiviral response on CVB3 versus poliovirus infection shows how CVB3 replication may be more sensitive to its cellular environment than other picornaviruses, and potentially, more sensitive to TDP2 activity.

Another explanation as to why TDP2 is divergently required during picornavirus infections is that TDP2 may interact with specific viral proteins belonging to one picornavirus and not another. A way to determine this would be to immunoprecipitate TDP2 from lysates generated from picornavirus-infected cells and assay for viral proteins by Western blot. These TDP2-viral protein interactions are probably unlikely during poliovirus infections since TDP2 does not colocalize with nonstructural viral proteins as observed by confocal microscopy. However, since TDP2 is closely localized with EMCV 3D during peak times of EMCV infection and is also cleaved to generate a TDP2 fragment matching the molecular weight of a predicted EMCV 3C cleavage product, it is possible that TDP2 interacts with viral proteins during EMCV infection. Identifying putative TDP2-viral protein interactions during picornavirus infections could provide further insight into the role of TDP2 during the viral replication cycle.

Elucidating the mechanism of TDP2 during picornavirus infections using a relevant human cell line, such a cardiomyocytes for CVB3, would further validate TDP2 as a potential antiviral therapeutic target. Other laboratories have identified small molecule inhibitors that target TDP2 catalytic activity. These inhibitors include toxoflavins, deazaflavins, NSC375976, NSC114532 NSC3198, and isoquinoline-1,3-dione (Kankanala et al., 2016; Kossmann et al., 2016; Raoof et al., 2013). The toxoflavins and deazaflavins were identified using a high-throughput screen against

over 100,000 compounds and shown to inhibit TDP2 at sub-micromolar concentration (Raoof et al., 2013). The deazaflavin analogue 163, which was the best TDP2 inhibitor candidate from this study, was confirmed to occupy a position similar to the second nucleotide of the DNA in the catalytic domain of TDP2 (Hornyak et al., 2016). Unfortunately, these compounds are not able to serve as potential therapeutics due to toxoflavins susceptibility to redox activity and the poor cell permeability of deazaflavins (Raoof et al., 2013). Additionally the deazaflavins were shown to inhibit TDP1 at higher concentrations. The NSC375976, NSC114532 and NSC3198 compounds were identified from a series of virtual screens and *in vitro* biochemical assays to inhibit TDP2 catalytic activity at a low micromolar concentration by interacting with the DNA-binding cleft (Kossmann et al., 2016). Isoquinoline-1,3-dione was identified by a high-throughput screen and also showed substantial inhibition of TDP2 activity at a low micromolar concentration (Kankanala et al., 2016). In an effort to determine if a TDP2 small molecule inhibitor would decrease viral yields during picornavirus infections, we have obtained the isoquinoline-1,3-dione small molecule to test using our *in vitro* VPg unlinkase assay and single-cycle growth experiments. Additionally, with the development of various TDP2 assays using fluorescence-conjugated or chromogenic substrates available for purchase, it is much more plausible for us to perform our own high throughput assay to identify novel TDP2 small molecule inhibitors (Adhikari et al., 2011; Hornyak et al., 2016).

The small molecules described above are all aimed at targeting TDP2 catalytic activity to improve cancer cells sensitivity to topoisomerase II poisons such as etoposide. It would be ideal to identify a small molecule inhibitor targeting the VPg unlinkase activity independent of DNA repair activity (Cortes Ledesma et al., 2009; Zeng et al., 2011). Since we propose that TDP2 catalytic activity is responsible for marking the viral RNA for its use in the different steps of the

viral replication cycle, this may be difficult to achieve. If we can further elucidate the role of TDP2 during infection, we could possibly target other host proteins involved in promoting the VPg unlinkase activity, such as recognition of the VPg-RNA linkage or its recruitment into the cell cytoplasm. Alternative to targeting TDP2 catalytic activity, we could test if a previously published antibody that specifically recognizes the VPg-RNA linkage can outcompete TDP2. Previous work done in our lab showed that an antibody against VPg alone did not affect VPg unlinkase activity (Rozovics et al., 2011). Since we know that the phosphotyrosyl bond between VPg and the viral RNA is recognized during infection, it is possible that the antibody binding this linkage may be more specific and successful in inhibiting the VPg unlinkase activity than a TDP2 inhibitor targeting its catalytic activity.

We will need to further explore various approaches and multiple inhibitors to obtain an antiviral therapeutic that broadly inhibits picornavirus infections. First, the role of TDP2 during picornavirus infections will need to be elucidated in a human cell line. After characterizing the phenotype during picornavirus infections in a TDP2 knockout human cell line, we can then proceed with identifying small molecule inhibitors targeting TDP2. In the future, it is possible that these small molecules can then be used as inhibitors of CVB3 infection or in combination with other antiviral therapeutics for inhibiting other members of the picornavirus family.

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