Baculovirus AcMNPV modulates the host insect DNA damage response but does not require the apical signaling kinase Ataxia-telangiectasia mutated

By

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Microbiology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2022

Date of final oral examination: 8/25/2022

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Abstract

The DNA damage response (DDR) functions to maintain the integrity of the cellular genome. In addition to responding to DNA damage, the DDR has an additional non-canonical function as an intrinsic defense against invading viral pathogens. In response, viruses modulate the DDR to avoid anti-viral effects and often usurp host DDR factors to promote viral multiplication. Through my research, I sought to better define molecular interactions between viruses and the host DDR. To accomplish this objective, I used as a model the prototypic baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) when propagated in *Spodoptera frugiperda* SF21 cells, a permissive lepidopteran cell line. Previously, it was thought that AcMNPV inhibited the host ataxia-telangiectasia mutated (ATM) DDR pathway while simultaneously depending upon ATM function for multiplication. I expanded the investigation of ATM function during baculovirus infection to include multiple small-molecule inhibitors of ATM. Contrary to previous reports, I determined that ATM activity was not necessary for AcMNPV multiplication.

To more robustly investigate the role of ATM and the DDR in the baculovirus-induced DDR, I generated ATM genetic knockouts in *S. frugiperda* SF21 cells. Prior to my efforts, there were no reports of site-specific gene editing in *S. frugiperda* cell lines. CRISPR-Cas9 is a genome editing tool widely used to make genomic edits. Because we lacked a method to generate the single guide RNA (sgRNA) necessary for CRISPR-Cas9 genome editing in SF21 cells, I first developed a method utilizing the *S. frugiperda* U6 small nuclear RNA promoter to express sgRNAs. Subsequently, I identified and disabled by genome editing the *S. frugiperda atm* gene, termed *sfatm*. Consistent with known ATM function, SfATM knockout cells exhibited ablated DNA damage-induced accumulation of phosphorylated H2AX (γ-H2AX), a central marker of ATM activation. However, the loss of SfATM failed to affect AcMNPV multiplication. This finding confirmed that ATM is not required for AcMNPV multiplication. Additionally, through quantitative immunofluorescence microscopy I determined that AcMNPV triggers activation of the DDR in an ATM-independent manner. Thus, another related non-ATM kinase must contribute to virus multiplication. ATR and DNA-PKcs are two closely related DDR kinases present in *S. frugiperda*, and their functions may be required for baculovirus multiplication. My study indicated for the first time that a different DDR kinase must be responsible for baculovirus multiplication.

I extended my studies into the interaction of baculoviral proteins with the host DDR. The baculovirus protein LEF-7 is a known modulator of the host DDR. LEF-7 contributes to baculovirus DNA replication and promotes homologous recombination. LEF-7 ablates the phosphorylation of H2AX, a central component of the ATM-mediated DDR. I determined that LEF-7 interacts with SfcGAS, an innate immune sensor that also localizes to DNA damage sites to limit homologous recombination. The interaction with SfcGAS suggests that LEF-7 may promote viral multiplication by interfering with an innate immune sensor to promote homologous recombination. In summary, my work has better defined the intricate relationship between the host DDR and a large DNA virus.

Acknowledgments

First, I would like to thanks my advisors, Dr. Paul Friesen an Dr. Robert Kalejta. They have both contributed countless hours to my scientific training. I have them to thank for my development in critical thinking and scientific communication. These skills have served me well through my graduate student career and will continue to serve me well in the future.

I also owe a great deal of thanks to the past Friesen lab members. I especially want to thank Dr. Nathaniel Byers and Dr. Jonathan Mitchell, who built the foundation upon which my work was built. Dr. Byers provided much needed moral and technical support early in my career. Their notes and writings have also provided me with guidance and shaped my scientific thinking.

I also thank all the past and present Kalejta lab members. Much thanks to Dr. Emily Albright for your wisdom, near infinite patience, and support in all things science. Thank you to my former officemates in lab, Diccon Fiore, Dr. Angie Umana, and Dr. Chris Gelbmann, thank you for your friendship, support, and tolerance over the years. Additionally, thank you to Dr. Shelby Lyon, Dr. Satoko Iwahori, and Dr. Jong-Hee Lee for your advice and support.

Most of all I would like to thank my family. You have been my cheerleaders, my rock, and support through this incredible journey. Special thanks to my wife Rebecca, for her attempts at keeping me sane and reminding me what I am doing all of this for. My

children LeRoy and Anneliese have also supported me in innumerable ways. Their excitement and joy always brings me happiness. Their notes and drawings still decorate the various walls, filing cabinets, and lab benches throughout the lab. I would also like to thank my parents, Ron and Karin. You have always supported me in everything I have done, no matter the path it took me down. Thank you to the rest of my family, my grandparents, and my sisters for all of your support. I would also like to thank my in-laws, Gregory and Carol. Having your support has been invaluable for our families time in Madison.

Lastly, I want to thank everyone else I haven't specifically named, friends, teachers, advisors, and anyone else who has given me support. My journey has been a strange one, but you have helped along the way.

Contents

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Abbreviations

∆F-box F-box domain deletion.

γ-H2AX phosphorylated H2AX.

B. mori *Bombyx mori*.

D. melanogaster *Drosophila melanogaster*.

OriLyt HCMV lytic replication origin.

S. frugiperda *Spodoptera frugiperda*.

T. ni *Trichoplusia ni*.

hr homologous region.

53BP1 p53-binding protein 1.

9-1-1 Rad9-Hus1-Rad1.

AAV adeno-associated virus.

AcMNPV *Autographa californica* nucleopolyhedrovirus.

Ad Adenovirus.

AIM2 Absent In Melanoma 2.

AP apurinic/apyrimidinic.

ATM ataxia-telangiectasia mutated.

ATR ataxia-telangiectasia and Rad3 related.

ATRIP ATR interacting protein.

BARD1 BRCA1-associated RING domain protein.

BER base excision repair.

BioID proximity-dependent biotinylation.

BRCA1 breast cancer type 1 susceptibility protein.

BRCA2 breast cancer type 2 susceptibility protein.

BV budded virus.

Cas9 clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9.

cdc2 cell division cycle protein 2.

cDNA complementary DNA.

cGAMP cyclic guanosine-monophosphate adenosine-monophosphate.

cGAS cyclic guanosine-monophosphate adenosine-monophosphate (cGAMP) synthase.

Chk1 checkpoint kinase 1.

Chk2 checkpoint kinase 2.

CINP CDK2-interacting protein.

CRISPR clustered regularly interspaced short palindromic repeats.

CtIP C-terminal binding protein 1 (CtBP1) interacting protein.

D-loop displacement loop.

DDR DNA damage response.

DNA-PK DNA-dependent protein kinase.

DNA-PKcs DNA-dependent protein kinase catalytic subunit.

DSB double-strand break.

dsDNA double-stranded DNA.

E early/delayed early.

EBNA-1 EBV nuclear antigen 1.

EBNA-3C EBV nuclear antigen 3C.

EBV Epstein-Barr virus.

FAT FRAP-ATM-TRRAP.

FATC FRAP-ATM-TRRAP-C-terminal.

GAPDH glyceraldehyde-3-phosphate dehydrogenase.

H2AX variant histone H2AX.

HBc Hepatitis B virus (HBV) core protein.

HBV Hepatitis B virus.

HCMV human cytomegalovirus.

HPV Human papillomavirus.

HR homologous recombination.

HSV-1 Herpes simplex virus-1.

HSV-2 Herpes simplex virus-2.

HTLV-1 Human T-cell leukemia virus type-1.

ICL interstrand crosslink.

ICP-0 infected cell protein 0.

IE immediate early.

IE1 immediate early gene 1.

IFI16 interferon gamma inducible protein 16.

IFNB interferon-B.

IRF3 interferon regulatory factor 3.

JCV human polyomavirus JC virus.

KSHV Kaposi sarcoma herpesvirus.

Ku70 Ku70 encoded by *XRCC6*.

Ku80 Ku80 encoded by *XRCC5*.

L late.

LANA latency-associated nuclear antigen.

LEF-7 late expression factor 7.

LMP-1 latent membrane protein 1.

MCPyV Merkel cell polyomavirus.

MDC1 mediator of DNA damage checkpoint protein 1.

MMR mismatch repair.

Mre11 meiotic recombination 11.

MRN meiotic recombination 11 (Mre11), Rad50 double strand break repair protein (Rad50), and Nijmegen breakage syndrome 1 (Nbs1).

mTOR mammalian target of rapamycin.

MVM minute virus of mice.

Nbs1 Nijmegen breakage syndrome 1.

NER nucleotide excision repair.

NHEJ non-homologous end joining.

NLS nuclear localization signal.

ORF open reading frame.

OV occluded virus.

p53 cellular tumor antigen p53.

PALB2 partner and localizer of BRCA2.

PAR poly ADP-ribose.

PARP-1 Poly ADP-ribose polymerase 1.

PAXX Paralogue of XRCC4 and XLF.

PI3K phosphatidylinositol 3-kinase.

PIKK phosphatidylinositol 3-kinase-related protein kinase.

PSEA proximal sequence element A.

Rad50 Rad50 double strand break repair protein.

RAP80 receptor-associated protein 80.

RLR RIG-I-like receptor.

RNA Pol II RNA polymerase II.

RNF168 ring finger protein 168.

RNF8 ring finger protein 8.

ROS reactive oxygen species.

RPA replication protein A.

RPA32 replication protein A 32 KDa subunit.

RPA70 replication protein A 70 KDa subunit.

RT-qPCR reverse transcription quantitative real-time PCR.

SCF Skp, Cullin, F-box containing.

SfATM *Spodoptera frugiperda* (*S. frugiperda*) ortholog of ataxia-telangiectasia mutated (ATM).

SfcGAS *S. frugiperda* ortholog of cGAMP synthase (cGAS).

SfH2AX *S. frugiperda* ortholog of variant histone H2AX (H2AX).

Sfp53 *S. frugiperda* ortholog of cellular tumor antigen p53 (p53).

SfPARP-1 *S. frugiperda* ortholog of Poly ADP-ribose polymerase 1 (PARP-1).

SfSKP1 *S. frugiperda* ortholog of S-phase kinase-associated protein 1 (SKP1).

sgRNA single guide RNA.

shRNA short hairpin RNA.

siRNA small interfering RNA.

SKP1 S-phase kinase-associated protein 1.

SKP2 S-phase kinase-associated protein 2.

SMG-1 suppressor of morphogenesis in genitalia.

snRNA small nuclear RNA.

SSB single-strand break.

ssDNA single-stranded DNA.

STING Stimulator of Interferon genes.

TAN Tel1/ATM N-terminal.

TBK1 TANK-binding kinase 1.

Tip60 Tip60 histone acetyltransferase.

TLR Toll-like receptor.

TopBP1 Topoisomerase IIβ-binding protein 1.

TRRAP transformation/transcription domain-associated protein.

UBR5 ubiquitin protein ligase E3 component n-recognin 5.

vDNA viral DNA.

VL very late.

WT wild-type.

XLF X-ray repair cross-complementing protein 4 (XRCC4)-like factor.

XRCC4 X-ray repair cross-complementing protein 4.

Chapter 1

Introduction

1.1 Overview

Viruses are obligate intracellular parasites. They depend upon host cell pathways to complete the production of viral progeny. This dependence on host cell pathways makes manipulation and overcoming anti-viral responses critical for the ongoing existence of viruses. This scenario has resulted in an evolutionary arms-race between viruses and host cells. Viruses must overcome existing anti-viral responses and hosts must develop improved methods of blocking viral multiplication. The DNA damage response (DDR) consists of closely related pathways and is important during virus infection. The DDR is responsible for the faithful maintenance of the host cell genome. However, the DDR is activated during virus infection in such a way that DDR factors impair virus production or trigger apoptosis. Baculovirus infection of permissive *Spodoptera frugiperda* (*S. frugiperda*) SF21 cells provide an excellent model for study of the interactions of a large double-stranded DNA (dsDNA) virus and the host DDR. Baculoviruses replicate quickly, and robustly activate and alter the DDR [107, 180, 181]. The interactions of baculoviruses with their lepidopteran hosts could provide critical information about the role the DDR plays in the invertebrate innate immune response [42]. The goal of my research was to investigate the mechanisms by which the prototypic baculovirus

Autographa californica nucleopolyhedrovirus (AcMNPV) engaged the DDR during infection of lepidopteran hosts. There were two main goals in my research. Firstly, I wanted to characterize the role of the DDR in AcMNPV multiplication. Secondly, I wanted to define how AcMNPV blunts host DDR signaling.

1.2 Baculoviruses

Baculoviruses are members of the virus family *Baculoviridae*. Baculoviruses are rod-shaped enveloped viruses with circular dsDNA genome which ranges in size from 80 to 180 kbp [80]. The family *Baculoviridae* consists of 4 genera; *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* [99]. Baculoviruses of the genus *Alphabaculovirus* and *Betabaculovirus* infect the larval stage of insects from the order Lepidoptera (caterpillars of butterflies and moths). Baculoviruses of the genus *Gammabaculovirus* and *Deltabaculovirus* infect the larval stage of insects from the order Hymenoptera and Diptera (wasps and flies), respectively.

The prototypic baculovirus AcMNPV is an *Alphabaculovirus*. AcMNPV is an effective model for studying the mechanisms of virus gene expression, viral genome replication, and virus-induced apoptosis. AcMNPV has a broad host range, infecting the larvae of over 40 *lepidopteran* species in the wild [80]. Several permissive *lepidopteran* cell lines including *S. frugiperda* Sf21 cells and *Trichoplusia ni* (*T. ni*) High Five cells exist [80]. In addition to *lepidopteran* infections, AcMNPV infects other arthropods, including *Drosophila melanogaster* (*D. melanogaster*), although these infections do not support viral multiplication [171]. AcMNPV is able to infect and express a reporter gene from a heterologous promoter in mammalian cells [27]. The ability of AcMNPV to initiate an infection in a evolutionarily diverse group of organisms makes it an excellent model for exploring evolutionary conserved responses to viral infection. There are several recent

and thorough reviews on baculovirus biology [80, 225].

1.2.1 Baculovirus virions

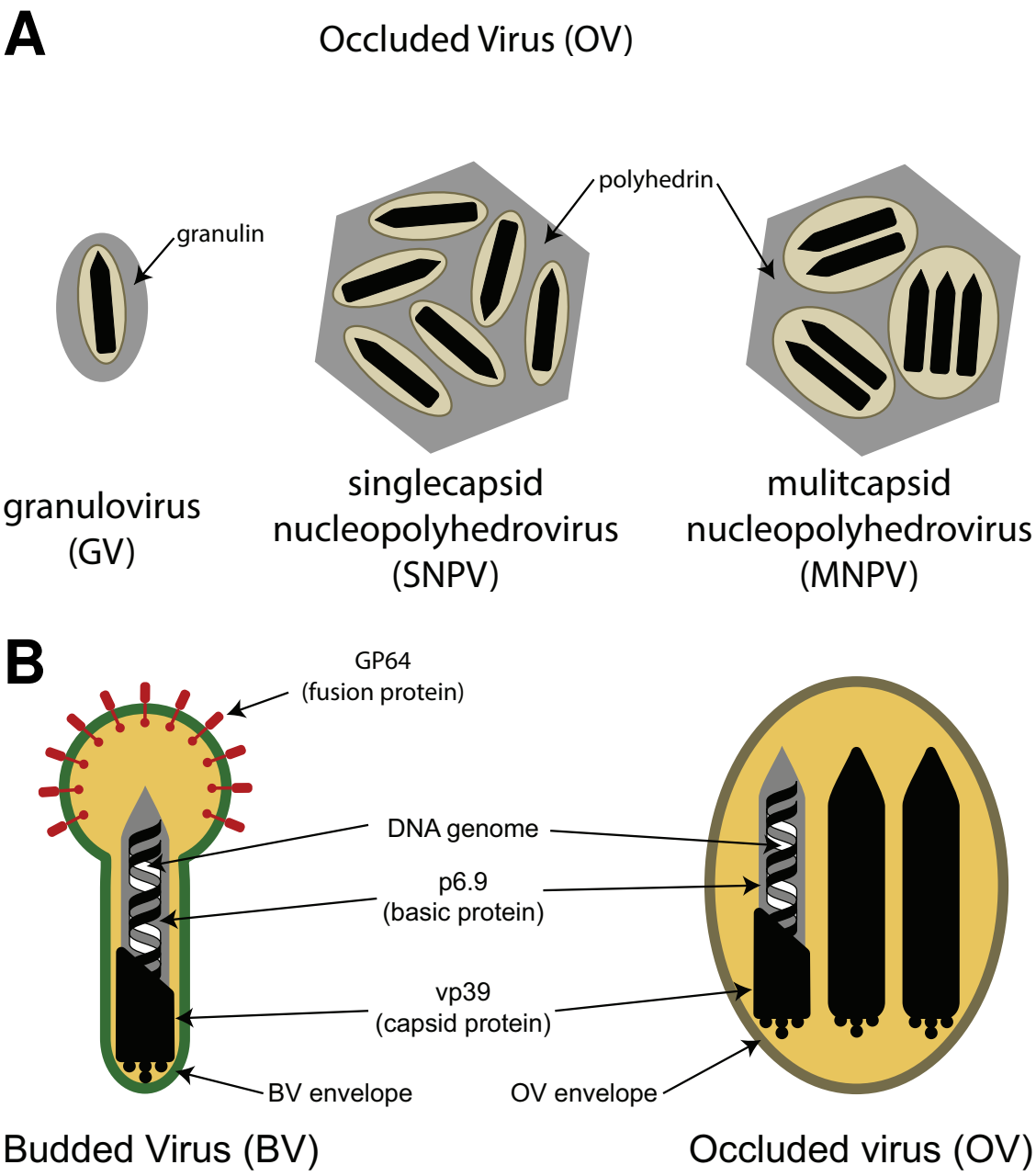
Baculoviruses are unusual in they generate two morphologically distinct forms of enveloped viruses during multiplication, occluded virus (OV), and budded virus (BV). Both forms contain cylindrical nucleocapsids 250-300 nm in length. OV consist of enveloped virions encased within a proteinaceous crystalline matrix, and form as irregular polyhedra 0.15-5 μM (FIG. 1.1A). The OV of members of the genera *Alphabaculovirus* are known as multicapsid nucleopolyhedrovirus (MNPV). MNPV contain multiple enveloped virions each containing multiple capsids (Fig. 1.1A). Members of *Betabaculovirus*, or granuloviruses (GV), generate OV known as granules. Granules are formed from the viral protein granulin, and contain a single enveloped nucleocapsid (FIG. 1.1A). The OV of members of *Gammabaculovirus* are singlecapsid nucleopolyhedroviruses, and contain multiple singly enveloped nucleocapsids (FIG. 1.1A). The OV of *deltabaculovirus* is made of a protein matrix distinct from the polyhedrin or granulin of the other baculoviruses [186]. BV are enveloped virions containing a single nucleocapsid, which buds off from the host cell plasma membrane (FIG. 1.1B).

1.2.2 Baculovirus infection cycle

AcMNPV infection begins with the larvae consuming OV contaminated foliage. Following entrance into the midgut by the OV, the alkaline environment causes the protective polyhedrin matrix to dissolve, releasing enveloped occlusion-derived nucleocapsids (FIG. 1.2). The envelope directly fuses with microvilli of the midgut epithelium and releases nucleocapsids into the cytoplasm. Inside the host cell, the nucleocapsids traffic to the nucleus via actin polymerization. Once at the nucleus, the

FIG. 1.1. Baculovirus morphology. (A) Occluded virus morphology. Baculovirus multiplication produces rod-shaped nucleocapsids that are packaged in occluded virus particles within a proteinacious matrix very-late during infection. The granuloviruses produce small OV particles 300-500 nm in length, containing a single enveloped nucleocapsid encased in the viral granulin protein. The singlecapsid nucleopolyhedroviruses (SNPV) and multicapsid nucleopolyhedroviruses produce larger OV particles 0.4 - 5.0 μM in diameter. Both SNPV and MNPV encase enveloped nucleocapsids in the viral-encoded polyhedrin protein. **(B) *Alphabaculovirus* BV and OV virions.** BV and OV have distinct roles during baculovirus infection. BV contain a single enveloped nucleocapsid and are responsible for cell-to-cell transmission within a host. The GP64 fusion proteins allows for BV entry into a diverse range of cells and hosts. OV contain multiple nucleocapsids in a single envelope and are responsible for host-to-host transmission. The nucleocapsids contained within BV and OV are identical, consisting of the vp39 capsid protein containing the viral DNA genome bound to the p6.9 basic protein. The membrane envelopes of BV and OV are distinct.

FIG. 1.1


A
Occluded Virus (OV)
granulin
polyhedrin
granulovirus
(GV)
singlecapsid
nucleopolyhedrovirus
(SNPV)
mulitcapsid
nucleopolyhedrovirus
(MNPV)
B
GP64
(fusion protein)
DNA genome
p6.9
(basic protein)
vp39
(capsid protein)
BV envelope
OV envelope
Budded Virus (BV)
Occluded virus (OV)

nucleocapsids interact head-on with the nuclear pore complex and enter the nucleoplasm through the nuclear pore complex. Once inside the nucleus, the nucleocapsid releases the viral genome.

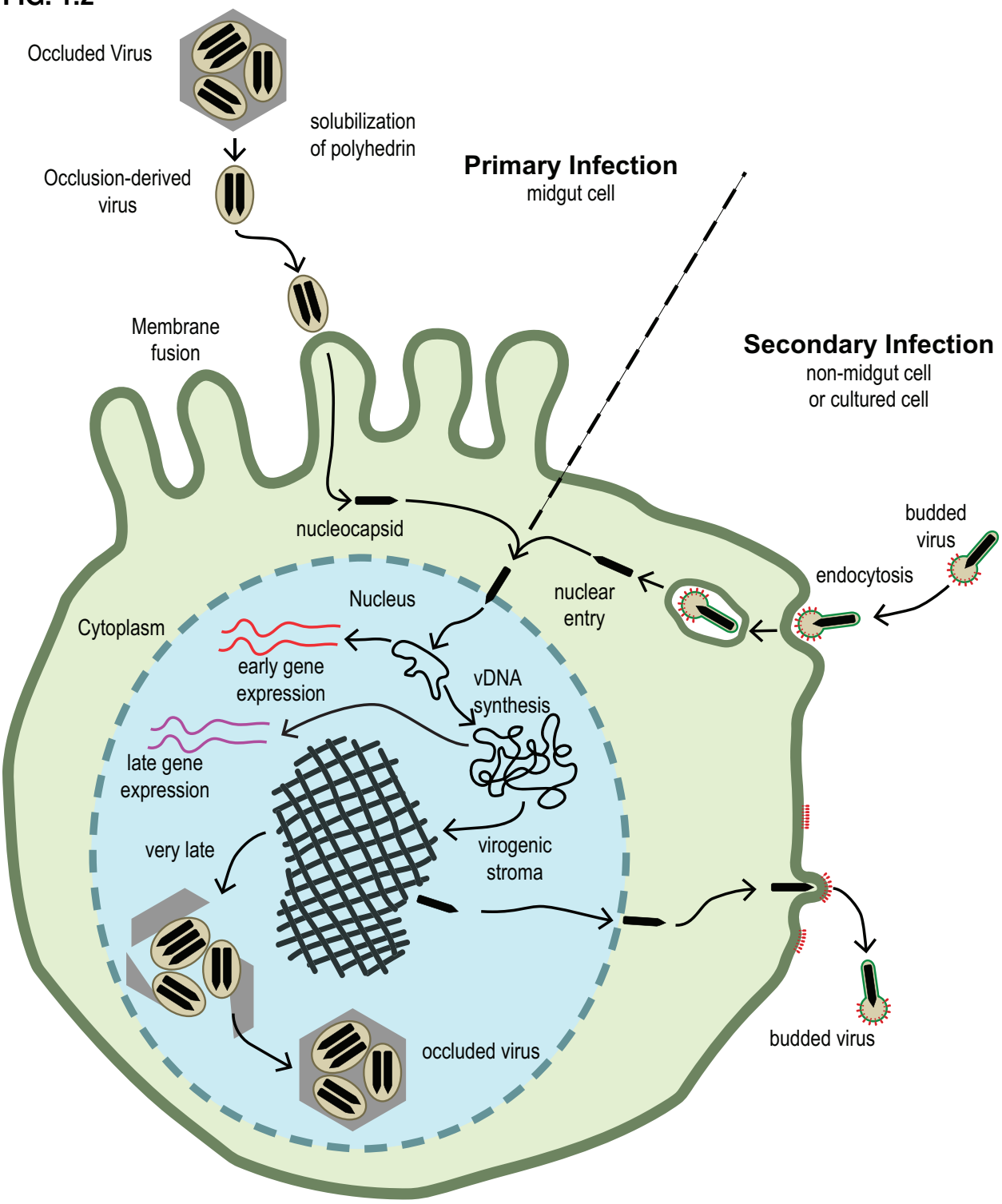
Following the release of the viral genome, the immediate early (IE) and early/delayed early (E) genes are transcribed (FIG. 1.2). These early viral genes promote viral DNA (vDNA) synthesis and the generation of the virogenic stroma (FIG. 1.2). The virogenic stroma serves as a platform for vDNA synthesis, late (L) and very late (VL) gene transcriptions, and nucleocapsid assembly. The L gene products contribute to capsid synthesis and BV production. Nascent circular viral genomes are packaged into nucleocapsids for virion assembly. Progeny BV are produced when assembled nucleocapsids are transported to the cell plasma membrane, where they acquire their GP64 fusion protein tipped envelope, bud from the cells, and contribute to cell-to-cell spread within the host (FIG. 1.2). With the expression of VL genes, the production of BV is reduced, and the nuclear production of OV begins. The nucleocapsids are retained within the nucleus, enveloped, and packaged within a proteinaceous matrix of polyhedrin. The OV remain within the nucleus until released by cell lysis, contributing to host-to-host transmission (FIG. 1.2).

1.2.3 AcMNPV genome

The AcMNPV genome (FIG. 1.3A) is a covalently closed, 134-kb dsDNA genome that encodes approximately 150 open reading frames (ORFs) [80, 225]. The ORFs are distributed throughout the AcMNPV genome on both DNA strands, showing no bias to temporal expression. Despite the large number of predicted ORFs, only 29 core genes exist in all sequenced baculovirus genomes. This low number of core genes relative to the total number of genes indicates a great amount of genetic diversity within

FIG. 1.2. Baculovirus replication cycle. Baculovirus primary infection begins with the proteinaceous polyhedrin of the OV dissolving in the midgut of the host, releasing the occlusion-derived virions. The occlusion-derived virions enter midgut epithelial cells through membrane fusion, releasing the nucleocapsids into the cytosol. Secondary infection begins with BV attaching to the cell plasma membrane and entering through endocytosis. The nucleocapsid is then released into the cytosol. Secondary infection and primary infection are identical following cytosolic release of nucleocapsids. Nucleocapsids traffic to the nucleus and release viral genomes inside the nucleoplasm. IE and E gene expression occurs, followed by vDNA synthesis and the establishment of the virogenic stroma. L gene expression begins, allowing for the formation of new nucleocapsids, which traffic to the plasma membrane and are released as BV. VL gene expression then begins, allowing for the formation of occluded virions which are packaged in polyhedrin to form OV.

FIG. 1.2


Occluded Virus
solubilization
of polyhedrin
Occlusion-derived
virus
Primary Infection
midgut cell
Membrane
fusion
Secondary Infection
non-midgut cell
or cultured cell
nucleocapsid
budded
virus
endocytosis
nuclear
entry
Nucleus
Cytoplasm
early gene
expression
vDNA
synthesis
late gene
expression
very late
virogenic
stroma
occluded virus
budded virus

baculoviruses. A key feature of baculovirus genomes is the presence of repetitive homologous regions (*hr*s) throughout the genome. The *hr*s consist of one to eight direct repeats 60 bp in length. Within each direct repeat is a highly conserved 28-bp imperfect palindrome. The AcMNPV genome contains 8 *hr*s spread through the genome: *hr1, hr1a, hr2, hr3, hr4a, hr4b, hr4c, hr5* (FIG. 1.3A). Each *hr* contains between two to eight 28-mer repeats centered around an EcoRI site (FIG. 1.3B) [54, 94]. The *hr*s serve as transcriptional enhancers and origins of viral genome replication. In AcMNPV, the *hr*s function by direct binding to the transcriptional activator IE1; and enhancement of transcription is greater than 1,000 fold [223, 224]. The *hr*s also contain cAMP response elements (CRE), and TPA response elements (TRE), that bind host transcription factors [132]. Each of the 8 AcMNPV *hr*s function as origins of viral DNA replication by binding IE1 [210]. The 8 AcMNPV *hr*s each have slight differences in nucleotide sequence; no single *hr* is critical for viral DNA multiplication [36, 223].

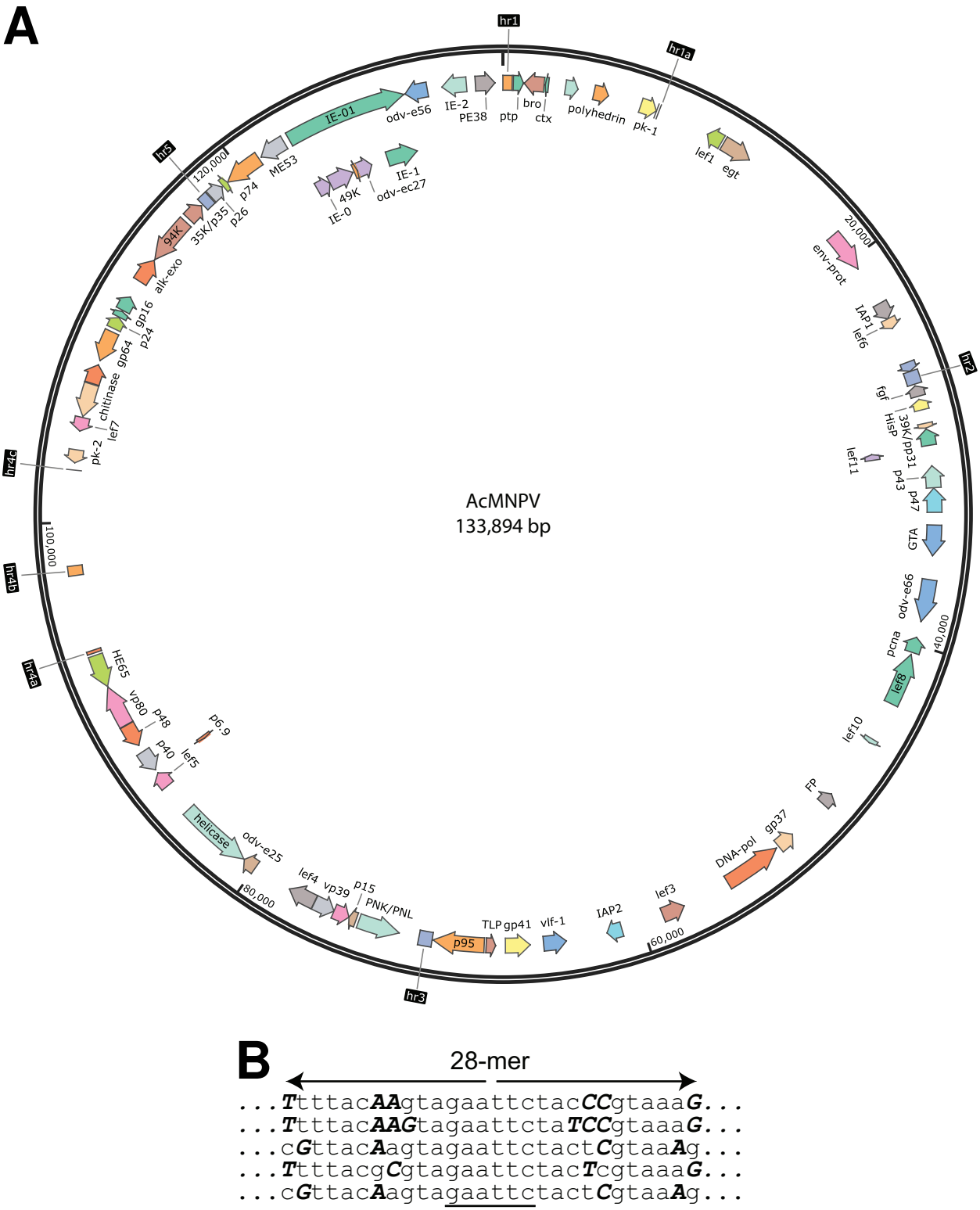
1.2.4 Baculovirus immediate early and delayed early gene expression

Baculovirus gene expression occurs in a transcriptionally regulated temporal gene cascade. The cascade is divided into 4 phases, IE, E, L, and VL. The host RNA polymerase II (RNA Pol II) transcribes the IE and E genes. In contrast, a viral-encoded RNA polymerase transcribes the L and VL genes. The transcription of viral genes of a nuclear dsDNA virus by a viral-encoded DNA-dependent RNA polymerase is unique to baculoviruses. Structural proteins are not required for IE gene expression, and transfection of permissive cells with purified baculovirus DNA results in the production of infectious progeny [32].

IE and E gene promoters are similar to host RNA Pol II promoters, containing a

FIG. 1.3. AcMNPV genome map. (A) Genome map of the AcMNPV stain C6. Arrows indicate the position and orientation of selected structural and viral replication genes. The homologous regions(*hrs*) are labeled outside the circular genome and highlighted in black. Tick marks indicate 20,000 bp. **(B)** Example of the 28-mers located within baculovirus *hr*s. Shown are the 28-mers from AcMNPV hr1. Mismatches are italicized, bolded, and upper-case. Underlined is the EcoRI site at the center of each palindrome.

FIG. 1.3


A
AcMNPV
133,894 bp
hr1
hr1a
hr2
hr3
hr4
hr4a
hr4b
hr4c
hr5
IE-01
odv-e56
IE-2
PE38
ptp
bro
ctx
polyhedrin
pk-1
lef1
egt
20,000
env-prot
IAP1
lef6
fgf
HisP
39K/pp31
lef11
p43
p47
GTA
odv-e66
pcna
lef8
lef10
FP
DNA-pol
gp37
lef3
IAP2
TLP
gp41
vlf-1
p95
PNK/PNL
vp39
lef4
odv-e25
helicase
p6.9
lef5
p40
p48
vp80
HE65
pk-2
lef7
chitinase
gp64
p24
gp16
alk-exo
94K
35K/p35
p26
p74
ME53
IE-0
49K
IE-1
odv-ec27
120,000
100,000
80,000
60,000
40,000
B
28-mer
...Tttacaagtagaattctaccgtaaag...
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...cgttacaagtagaattctactcgtaaag...
...Tttacgcgtagaattctactcgtaaag...
...cgttacaagtagaattctactcgtaaag...

TATA element 30 bp upstream of the transcription start site, and a CAGT motif near the transcription start site [19, 65, 93, 215, 216]. These core promoters coordinate with cis activating elements and the *hr*s to enhance transcription. Additionally, transcription is enhanced from early promoters by both viral and cellular trans acting elements, including host factors [124, 220] and baculovirus transactivators, including IE1. At least four IE genes are transcribed in the absence of viral proteins: *ie-0, ie-1, ie-2*, and *pe38*. A principal function of the IE genes is the expression of E genes. IE1 is the key AcMNPV transactivator, which activates transcription of itself and E genes [225].

1.2.5 Baculovirus genome replication

Baculovirus genome replication occurs in the nuclei of infected host cells within distinct, sub-nuclear compartments, known as viral replication centers within the virogenic stroma (FIG. 1.2). The exact mechanism by which baculoviruses replicate their DNA is not well understood. It is thought that genome replication occurs through rolling circle replication, through a recombination mechanism, or through some combination thereof. Rolling circle replication is a mechanism of genome replication utilized by other large DNA viruses, including the herpesviruses. Rolling circle replication involves the formation of large DNA concatemers, which contain copies of the viral genome linked end to end. These concatemers are processed into single genomes as part of the assembly of progeny virions. DNA fragments containing multiple copies of the AcMNPV genome have been detected during AcMNPV vDNA replication, which supports a rolling circle mechanism [204]. However, AcMNPV genome replication promotes high-frequency homologous recombination during infection [167]. It is possible that DNA fragments containing multiple genomes arose from recombination between individual genomes. Recombination could also be the mechanism through which AcMNPV resolves concatemers; homologous recombination between viral *hr*s could resolve

concatemers into single circular genomes. It has also been proposed that AcMNPV genome replication occurs through a recombination strategy without the production of concatemers [264].

Six AcMNPV genes are required for vDNA replication [126, 154]. Eleven more genes stimulate vDNA replication (FIG. 1.4). They are split between IE and E genes. The required genes include the origin binding proteins *ie-0/ie-1*, the DNA polymerase *dnapol*, the helicase *p143*, the primase and primase accessory factor *lef-1* and *lef-2*, and the single-strand binding protein *lef-3*. IE-0 and IE-1 are splice variants of each other, with IE-0 containing an additional 52 N-terminal amino acids. IE-1/IE-0 forms dimers an either can support vDNA replication. However, inactivation of both *ie-0* and *ie-1* eliminates virus production [249]. The AcMNPV DNA polymerase is essential since a *dnapol*-null bacmid fails to produce vDNA following transfection into cultured *Spodoptera* cells [265]. The primase and primase accessory factor LEF-1 and LEF-2 interact during vDNA replication, and both are required [126, 154, 282]. The single-stranded DNA binding protein LEF-3 is also involved in strand exchange and may contribute to the production of recombination intermediates [98, 176]. The helicase P143 unwinds DNA primers from longer DNA strands and is dependent upon interaction with LEF-3 for nuclear localization [170, 284]. Several other AcMNPV genes including transcriptional activators *ie-2* [126, 154] and *pe38* [177], the anti-apoptotic factor *p35* [154], DDR regulator *lef-7* [43], and the host cell specific factor *hcf-1* [155], stimulate, but are not absolutely required, for AcMNPV vDNA replication (FIG. 1.4B).

1.2.6 Baculovirus late and very late gene expression

Similar to other large DNA viruses, the demarcation between E and L gene expression is vDNA replication (FIG. 1.4A). Baculovirus L and VL genes are located

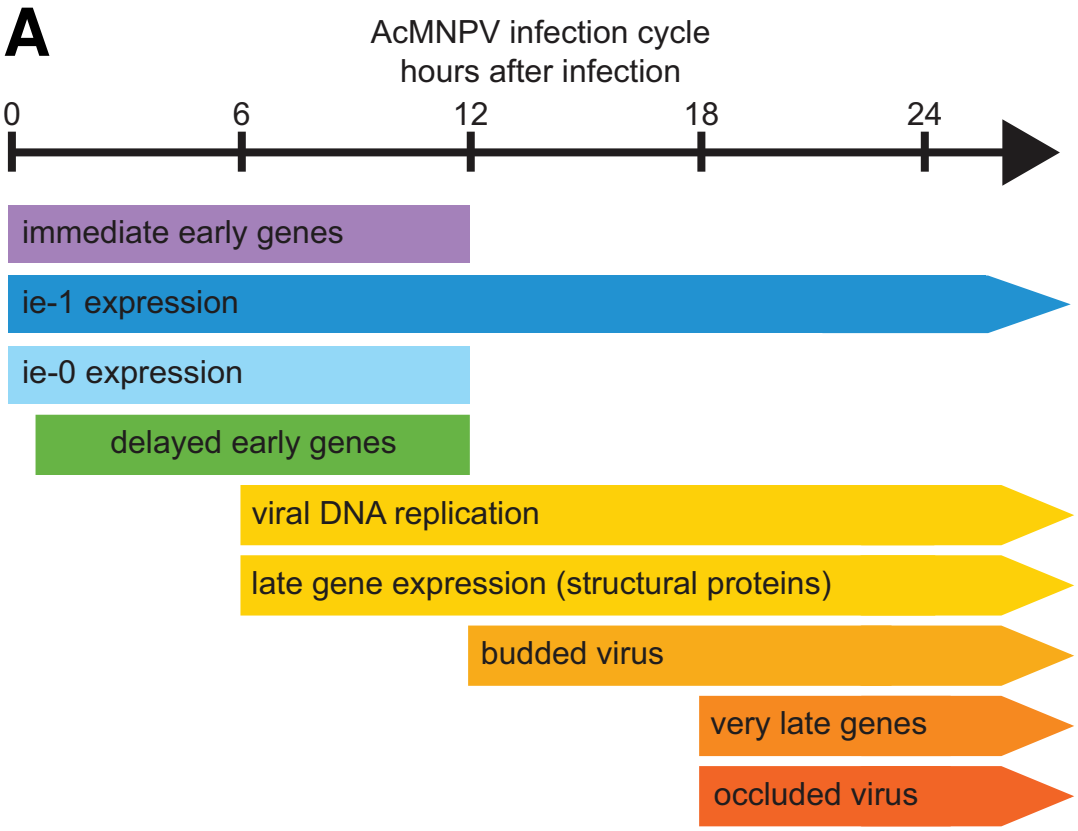
along both DNA strands and are dispersed throughout the genome with no enrichment in a specific genomic region. Unlike IE and E genes, L and VL genes are transcribed by a viral DNA-dependent RNA polymerase. L and VL genes include structural proteins, such as the GP64 fusion protein, the polyhedrin protein of OV, the VP39 capsid protein, and the alkaline nuclease packaging factor. The L and VL promoters are highly active late in infection; at 24 hpi, polyhedrin mRNA comprises 24% of total RNA within the cell [49]. L protein expression primes the infected cell for nascent virus production, and BV begin to be produced by 12 hpi (FIG. 1.4A). Nucleocapsids form near the edges of the virogenic stroma and are trafficked to the plasma membrane where they bud, acquiring their envelope and GP64 fusion protein, generating BV (FIG. 1.2). At approximately 18 hpi, the expression of VL genes begins (FIG. 1.4A). VL gene expression allows for the formation of the OV, which are retained in the nucleus until cell lysis, whereupon they are distributed in the environment following the liquefaction of the host caterpillar by the virus.

1.3 The DNA damage response

The preservation of genomic integrity and sequence fidelity is important for the preservation of cellular life. Simultaneously, mutagenesis plays a key role in evolution while contributing to cellular aging and the development of disease states such as cancer. This role is regulated by the cellular DDR. The DDR detects damage to the DNA, responds by signaling to the cell that the damage has occurred, and encourages repair of the damaged DNA or promotes apoptosis if the damage is irreparable. Importantly, the DDR pathways allow cells sufficient time to execute specific DNA repair pathways and rectify DNA lesions. DNA damage can be classified into different areas, including single-strand breaks (SSBs), double-strand breaks (DSBs), mismatched bases, apurinic/apyrimidinic (AP) sites, and interstrand crosslinks (ICLs) (reviewd in [37]). At least five major DNA repair pathways pathways can be activated by the DDR following

FIG. 1.4. AcMNPV gene expression and genome replication (A) Temporal gene expression. Viral immediate early and early genes are transcribed by host RNA Pol II following the uncoating of the genome in the nucleus and promote transcription of the E genes. E genes include genes required for vDNA replication and transcription of L and VL genes. Following the initation of vDNA replication transcription of L begins as transcription of E genes wanes. The L genes include structural genes and genes required for VL gene expression. The VL genes are involved in the production and release of the OV. Genes are shown relative to the approximate hours following infection the genes are expressed. Figure modified from [258]. (B) **AcMNPV genes that contribute to vDNA replication.** The function, AcMNPV gene name, and involvement in vDNA are listed for select IE and E genes [126, 154]. IE genes are highlighted in purple. E genes are highlighted in green.

FIG. 1.4



B AcMNPV gene function and contribution to vDNA replication

Function	AcMNPV gene	vDNA replication
Origin binding	*ie-0/ie-1*	required
Transcriptional activator	*ie-2*	stimulatory
Transcriptional activator	*pe38*	stimulatory
Helicase	*p143*	required
Primase	*lef-1*	required
Primase accessory factor	*lef-2*	required
DNA single-strand binding	*lef-3*	required
DNA polymerase	*dnapol*	required
Anti-apoptotic	*p35*	stimulatory
DDR regulation	*lef-7*	stimulatory
Host cell specific factor	*hcf-1*	stimulatory

damage: base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end joining (NHEJ). These pathways allow cells to repair DNA and maintain fidelity in their genomes. DNA repair is not completely efficient, and repair can lead to the deletion of bases of substitutions of incorrect bases, thereby fixing mutations into the genome.

The DDR can be separated into distinct steps, which all pathways follow. DNA damage can occur through endogenous sources, such as errors in DNA replication and exposure to reactive oxygen species (ROS) which arise through normal cellular function, or exogenous sources such as ultraviolet or ionizing radiation. DNA sensors next recognize the damage by the DDR sensors. The recognition allows for the recruitment of mediators to the site of the DNA lesion. Following mediator recruitment, the DNA damage signal is amplified via transducers. These factors allow for signaling to effectors, which results in chromatin remodeling, transcriptional changes, the onset of DNA repair, the triggering of cell cycle checkpoints, and if necessary, apoptosis. There are several recent and thorough reviews on the DDR [37, 41, 52, 239].

1.3.1 DDR kinases

The principal DDR kinases consist of 1) ataxia-telangiectasia and Rad3 related (ATR), 2) ataxia-telangiectasia mutated (ATM), and 3) DNA-dependent protein kinase catalytic subunit (DNA-PKcs). ATR, ATM, and DNA-PKcs are all members of the phosphatidylinositol 3-kinase-related protein kinases (PIKKs) family. All three DDR kinases share both sequence and functional homology. These DDR kinases also share homology with other PIKKs, such as mammalian target of rapamycin (mTOR). The PIKKs all contain a C terminal kinase domain, which shares homology with phosphatidylinositol 3-kinase (PI3K) [23]. While similar, the kinase domains of PIKKs are

atypical and form a distinct domain subfamily from the PI3K kinase domain [164]. There are two additional conserved domains within the PIKKs, including a FRAP-ATM-TRRAP (FAT) domain located N-terminal to the kinase domain, and a FRAP-ATM-TRRAP-C-terminal (FATC) domain, located C-terminal to the kinase domain [138]. It is also relevant that ATM contains a Tel1/ATM N-terminal (TAN) motif, which is unique to ATM and found in no other PIKK and is critical for a proper ATM-mediated DDR [236].

The non-DDR members of the PIKKs family are mTOR, suppressor of morphogenesis in genitalia (SMG-1), and transformation/transcription domain-associated protein (TRRAP). mTOR is a nutrient-regulated kinase that is involved in a diverse array of pathways linked to cellular metabolism and growth [285]. SMG-1 coordinates and mediates nonsense-mediated mRNA decay [286]. TRRAP functions as a protein complex and possesses histone acetyltransferase activity but lacks kinase functions due to a non-functional kinase domain [172, 173]. While the PIKKs are closely related to PI3Ks, they have unique functions and do not phosphorylate phosphatidylinositol [115].

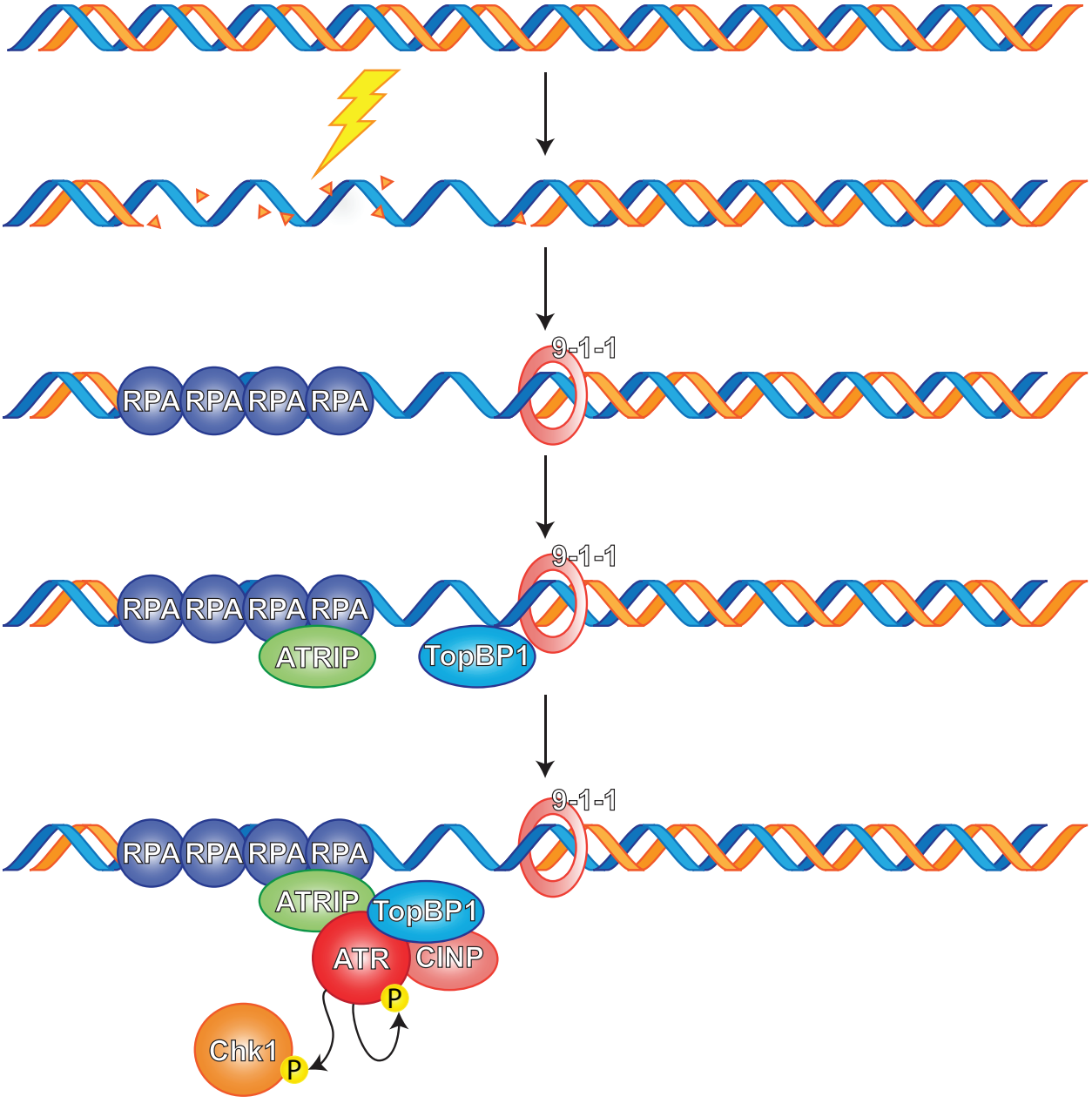
The DDR kinases are activated in response to DNA damage. DNA damage can occur from a variety of sources, including ionizing radiation, exposure to genotoxic compounds, oxidative stress, transcriptional stress, and replication stress (Reviewed in [41, 226]). The activation of the DDR kinases requires detection of damaged DNA by protein sensor complexes which then activate ATR, ATM, and/or DNA-PKcs. The ATR pathway is primarily activated by SSBs and single-stranded DNA (ssDNA), while ATM and DNA-PK pathways are activated by DNA DSBs.

1.3.2 ATR

ATR is a serine/threonine-directed member of the PIKK family. Unlike ATM and DNA-dependent protein kinase (DNA-PK) which primarily respond only to DSBs, ATR is activated in response to a variety of genotoxic stresses, all which result in the exposure of ssDNA or a SSB [169]. The interaction between endogenously generated ROS (including superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide) and the DNA backbone is a common source of SSB DNA lesions [213]. Another source of SSB is AP sites which lack either a purine or pyrimidine. AP sites in DNA are unstable and the lesion can easily be expanded into a SSB or a DSB (Reviewed in [1]). SSB are detected as stretches of ssDNA by the RPA sensor complex [17]. RPA coats the ssDNA and prevents degradation by nucleases, prevents hairpin formation, and assists in recruiting and loading the Rad9-Hus1-Rad1 (9-1-1) checkpoint clamp complex onto ssDNA:dsDNA junctions (FIG. 1.5) [15, 73, 76, 162, 295]. Following the loading of 9-1-1 it recruits the ATR co-activator Topoisomerase IIβ-binding protein 1 (TopBP1) [63, 81, 136]. ATR is then recruited to long stretches of replication protein A (RPA) coated ssDNA through interactions with ATR's partner protein ATR interacting protein (ATRIP) and a direct interaction between ATRIP and RPA [294]. The ATR/ATRIP complex also interacts with CDK2-interacting protein (CINP) at SSBs, which increases ATR signaling and promotes cell cycle regulation (FIG. 1.5) [153]. Interactions between ATR/ATRIP and TopBP1 increases ATR kinase activity [131]. ATR has a large number of substrates, which are phosphorylated preferentially on serines/threonines found within SQ or TQ motifs [122]. A key step in the ATR-mediated DDR is the direct phosphorylation of checkpoint kinase 1 (Chk1) by ATR [95, 100, 150] (FIG. 1.5). Chk1 is important because it potentiates the DDR signaling away from the site of damage, and helps to activate cell cycle checkpoints, allowing time for DNA repair to occur or promoting apoptosis in the case of extensive DNA damage (Reviewed in [190]).

FIG. 1.5. ATR DDR pathway. SSB in the DNA result in stretches of single-stranded DNA, which detected and subsequently coated by RPA. The 9-1-1 complex also forms around the damaged DNA adjacent to RPA coated ssDNA. ATR and its partner ATRIP are recruited to damage sites through ATRIP's interaction with RPA. ATR is activated through its interaction with TopBP1, and ATR is subsequently autophosphorylated. ATR also phosphorylates Chk1, which propagates the DDR signal away from the lesion. CINP interacts with ATR/ATRIP and regulates ATR signaling and G2 checkpoint maintenance.

FIG. 1.5


9-1-1
RPA RPA RPA RPA
9-1-1
RPA RPA RPA RPA
ATRIP
TopBP1
9-1-1
RPA RPA RPA RPA
ATRIP
TopBP1
ATR
CINP
P
Chk1
P

1.3.3 ATM

ATM is a serine/threonine-directed member of the PIKK family. ATM is an apical kinase responsible for the cellular response to DSB, leading to HR repair. DSBs are especially dangerous for the maintenance of the genome since the sequence information that is required to repair the lesion is no longer located within a contiguous DNA strand. Like ATR, ATM has a preference for phosphorylating serines/threonines which are found in a SQ or TQ motif [122]. When ATM is found in a resting state, it is autoinhibited through formation of a noncovalent homodimer located in the nucleus. For ATM to function, it needs to monomerize and relocalize to become fully active. When DNA damage occurs, ATM is activated by in part by Tip60 histone acetyltransferase (Tip60). Tip60 acetylates a lysine in the FATC domain of ATM, which promotes the monomerization and autophosphorylation required for ATM activation activity [254, 255].

The Mre11, Rad50, and Nbs1 (MRN) complex functions as both a DNA sensor and an ATM activator. MRN is critical to the activation and localization of ATM. MRN plays a key role in the DDR functioning as a sensor of DSBs [212]. Following detection and localization of MRN to a DSB, ATM is directly recruited to MRN through interaction between ATM and a C-terminal motif of Nijmegen breakage syndrome 1 (Nbs1) [75, 290]. Interaction between ATM and MRN also promotes autophosphorylation and monomerization of ATM [13, 135].

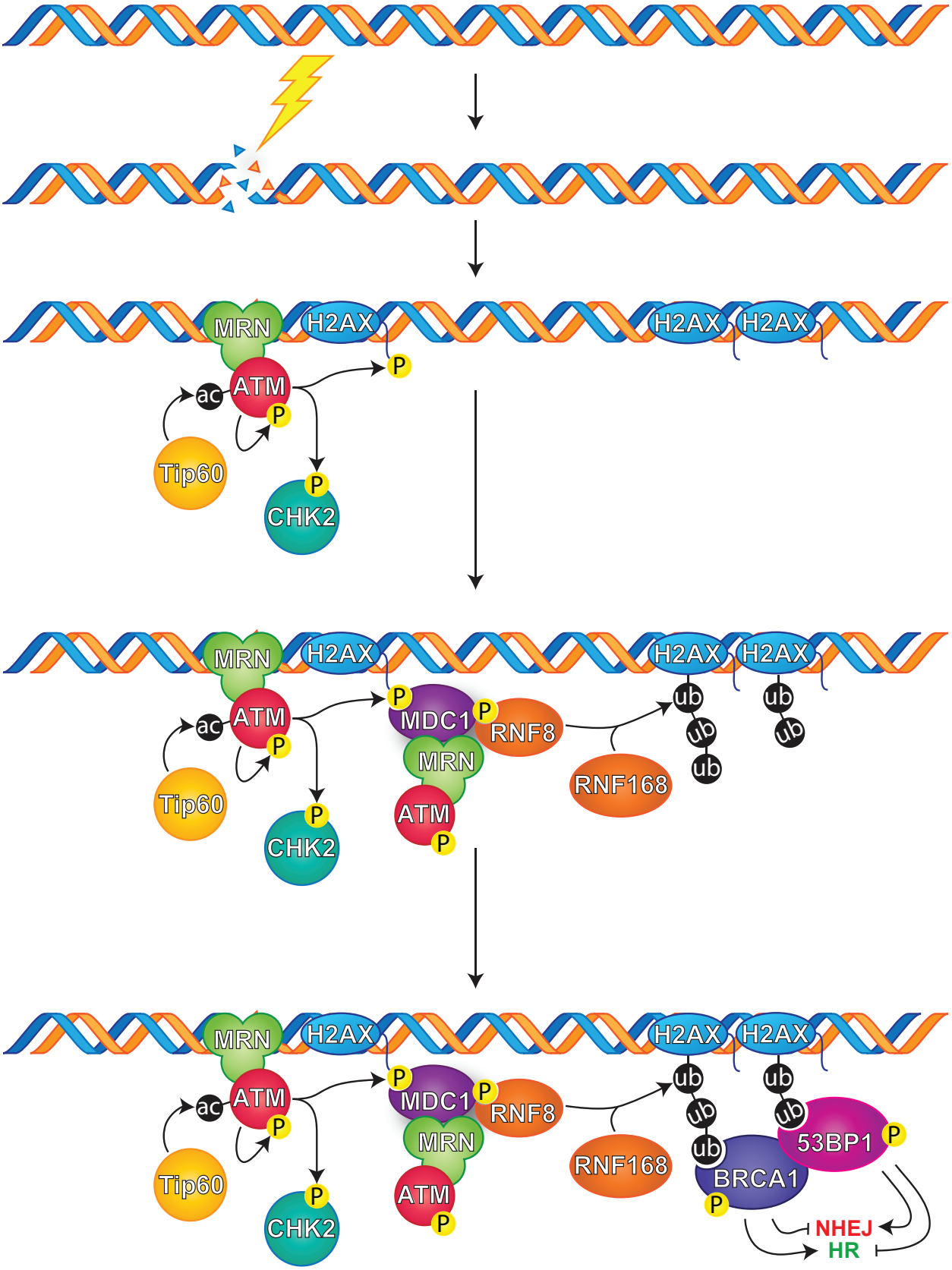
Activated ATM at DSB sites directly phosphorylates variant histone H2AX (H2AX), generating phosphorylated H2AX (γ-H2AX), a central indicator of ATM activation in cells. The presence of γ-H2AX helps recruit mediator of DNA damage checkpoint protein 1 (MDC1) which binds directly to the phosphorylated tail of γ-H2AX [152, 252]. MDC1 is also phosphorylated on its N-terminal forkhead associated domain by ATM [149]. MDC1 bound to γ-H2AX recruits and maintains phosphorylated ATM at DSB sites, promoting

additional ATM driven γ-H2AX accumulation on chromatin in a feedback loop. Phosphorylated MDC1 also recruits additional MRN through interactions with Nbs1 and a N-terminal SDT repeat motif in MDC1 [39, 174, 248]. The E3 ubiquitin ligase ring finger protein 8 (RNF8) is also recruited to DSB sites in a MDC1- and H2AX-dependent manner [108, 125, 161, 271]. When recruited to chromatin, RNF8 ubiquitinates H2A/H2AX histones and recruits the p53-binding protein 1 (53BP1) and breast cancer type 1 susceptibility protein (BRCA1) repair proteins to DSB sites [108, 161]. An additional ubiquitin ligase, ring finger protein 168 (RNF168), is recruited to ubiquitinated H2A/H2AX in a RNF8-dependent manner and promotes ubiquitination of additional H2A/H2AX which the recruits and retains 53BP1 and BRCA1 [67].

The BRCA1 protein complex, including BRCA1 along with receptor-associated protein 80 (RAP80), BRCA1-associated RING domain protein (BARD1) and Abraxas is recruited to DSB sites through the interaction between RAP80 and ubiquitinated H2A/H2AX [245, 271]. Following 53BP1 recruit, 53BP1 end protection and end joining functions are dependent upon ATM mediated phosphorylation of the N-terminus of 53BP1 [24]. The balance between 53BP1 and BRCA1 activity determines the fate of DSB repair. 53BP1 promotes end joining through NHEJ, while BRCA1 promotes HR (FIG. 1.6 (Reviewed in [60]). The balance is cell cycle dependent, with 53BP1 negatively regulating resection and promoting NHEJ during G_1 , while BRCA1 promoting end resection and HR during S/G_2 (Reviewed in [214]). Additionally, both 53BP1 and BRCA1 function to antagonize each others functions [26, 31]. Following end resection, BRCA1 also functions to promote HR through the recruitment of the breast cancer type 2 susceptibility protein (BRCA2) mediator protein [44].

FIG. 1.6. ATM DDR pathway. DSBs are sensed by the MRN complex. MRN complex then helps recruit the ATM kinase. ATM is acetylated by Tip60 and autophosphorylated to become fully active. ATM phosphorylates the variant histone H2AX propagating the DNA damage signal along the damaged chromatin away from the injury. ATM also phosphrylates checkpoint kinase 2 (Chk2), disseminating the DNA damage signal away from the chromatin. MDC1 binds to γ-H2AX, and recruits additional MRN, which in turn recruits additional ATM. MDC1 is phosphorylated by ATM. Phosphorylated MDC1 recruits the RNF8 and RNF168 ubiquitin ligases, which build K63-linked polyubiquitin scaffolds on H2A and H2AX histones. These polyubiquitin scaffolds serve to recruit the antagonists BRCA1 and 53BP1. BRCA1 promotes HR, which 53BP1 promotes NHEJ.

FIG. 1.6


MRN
H2AX
H2AX
H2AX
ac
ATM
P
P
Tip60
P
CHK2
MRN
H2AX
H2AX
H2AX
ac
ATM
P
P
MDC1
P
RNF8
MRN
ATM
P
RNF168
ub
ub
ub
ub
ub
Tip60
P
CHK2
MRN
H2AX
H2AX
H2AX
ac
ATM
P
P
MDC1
P
RNF8
MRN
ATM
P
RNF168
ub
ub
ub
ub
ub
ub
53BP1
P
BRCA1
P
NHEJ
HR
Tip60
P
CHK2

1.3.4 DNA-PK

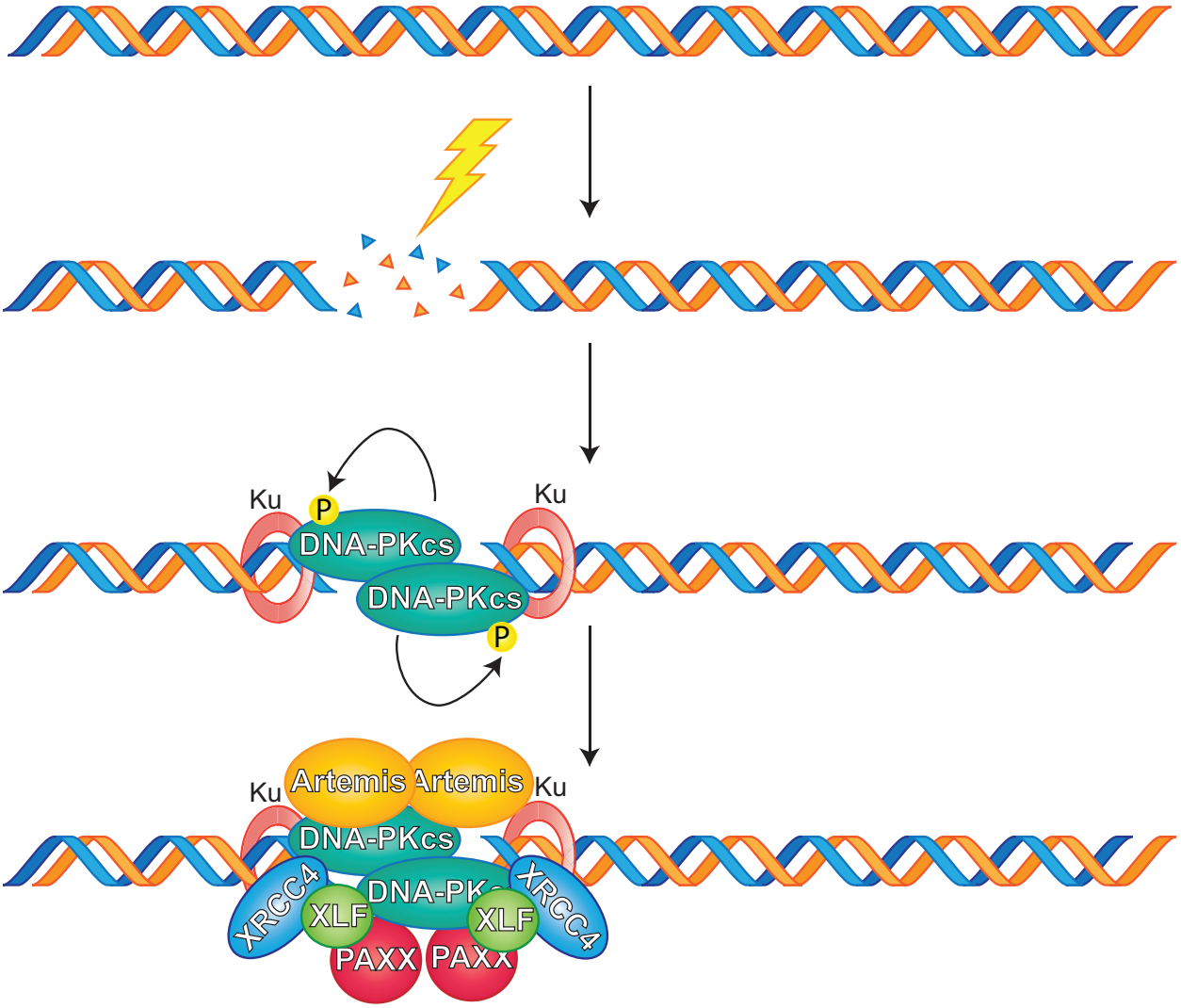
In the DNA-PK pathway the DSB is initially detected by the Ku heterodimer consisting of Ku70 encoded by *XRCC6* (Ku70) and Ku80 encoded by *XRCC5* (Ku80) (FIG. 1.7) [288]. DNA-PKcs is then recruited by Ku forming the DNA-PK holoenzyme tethered to the DSB [89]. DNA-PKcs is activated through autophosphorylation [66]. DNA-PK then recruits NHEJ repair factors to the site of the DSB, including the recruitment of X-ray repair cross-complementing protein 4 (XRCC4) [143] and XRCC4-like factor (XLF) [4]. The NHEJ factor Paralogue of XRCC4 and XLF (PAXX) helps to stabilize the complex at the DSB [199]. The endonuclease Artemis interacts directly with DNA-PKcs to promote DSB repair through NHEJ [158, 187].

1.3.5 Homologous recombination

Homologous recombination is a DNA repair pathway dedicated to the faithful repair of DNA DSBs using homologous DNA sequences, typically found on sister chromatids. Compared to NHEJ, HR results in accurate repair as sequence lost due to the DSB end processing is replaced from the template. HR proceeds through resection and filament formation, homology search, strand invasion, DNA syntheses, and resolution. HR begins with the resection of the DSB ends 5' to 3' producing 3'-OH ssDNA ends. This end resection is dependent upon MRN binding to the free DNA end and promoting long range resection of the DNA by exonucleases (FIG. 1.8) [188]. MRN is also involved in recruiting ATM to the lesion and activating the ATM DDR. The ssDNA generated by end resection is rapidly coated by the RPA heterotrimer, which consists of RPA70, RPA32, and RPA14 subunits [6]. BRCA1 is also recruited to the lesion through interactions with ubiquitin conjugated DDR proteins [214]. BRCA2 localizes to the DSB lesion in part through interactions with BRCA1 and BRCA1 recruitment of partner and localizer of

FIG. 1.7. DNA-PK DDR pathway. Following the induction of a DSB, Ku70 and Ku80 form a Ku heterodimer around the broken DNA end. DNA-PKcs is recruited to the break where it assists in synapsis through interactions with Ku, forming the DNA-PK holoenzyme. DNA-PKcs autophosphorylation and Artemis recruitment promote end processing and end protection. Recruitment of XRCC4, XLF, and PAXX allows for the religation of the broken DNA strands.

FIG. 1.7


Ku
P
DNA-PKcs
DNA-PKcs
P
Ku
Ku
Artemis
Artemis
Ku
DNA-PKcs
XRCC4
XLF
DNA-PK
XLF
XRCC4
PAXX
PAXX

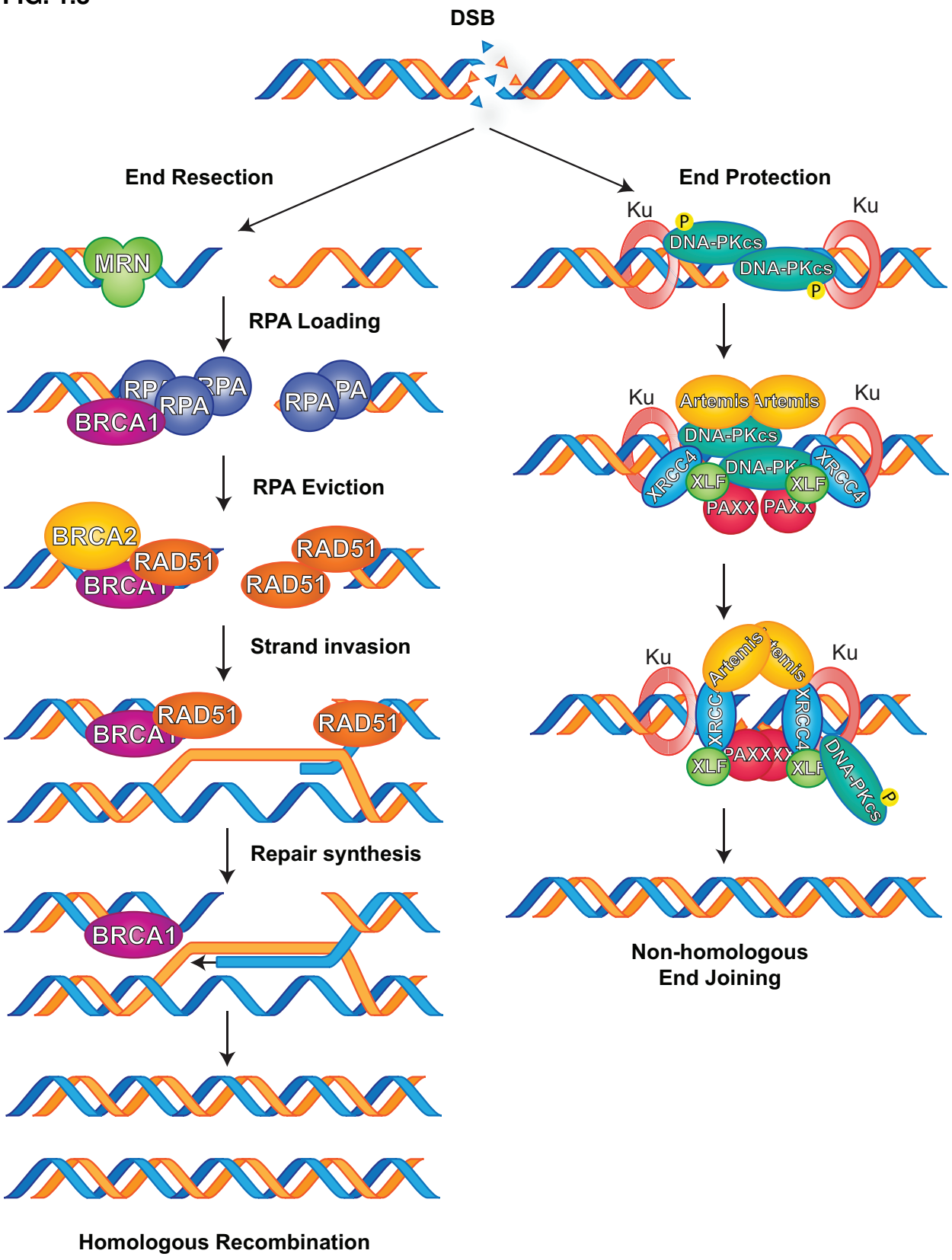
BRCA2 (PALB2) [148, 293]. BRCA2 then assists with the loading of RAD51 onto DNA, which is concomitant with RPA eviction forming RAD51 coated DNA filaments [292]. RAD51 when bound to DNA performs a homology search of dsDNA followed by strand invasion of an identified homologous template [90, 281]. This forms the displacement loop (D-loop) of the heteroduplex DNA of the invading strand and the donor strand of DNA. DNA polymerase then cooperates with other HR factors including RAD51 to extend the D-loop through 3' end extension [281]. The D-loop is then disrupted, and DNA ligases and resolvases are able to resolve Holliday junctions restore any remaining nicks and restore the integrity of the DNA strand.

1.3.6 Non-homologous end-joining

The NHEJ repair pathway is responsible for repair of the majority of DSB that occur in a cell, responsible for the repair of up to 80% of all breaks [117]. NHEJ can be split into three major steps, recognition of the break, end processing, and ligation. Break recognition is carried out by the Ku heterodimers of the DNA-PK pathway (FIG. 1.7) [269]. End processing occurs through proper synapsis which aligns the correct DNA strands, followed by processing of the DNA ends. Synapsis is critical to ensure DNA ends connected through NHEJ are from the same DSB, preventing possible chromosome translocations. This rapidly occurs through the recruitment of DNA-PKcs by the Ku heterodimer, forming the DNA-PK holoenzyme (FIG. 1.8) [89]. The formation of the DNA-PK holoenzyme and stabilization of DNA ends occurs along with the recruitment of end processing factors, including the exonuclease Artemis, which can assist in processing DNA ends for ligation [38]. DNA-PK kinase activity along with the recruitment of XLF and XRCC4 assist in bringing the DNA ends into closer proximity [89]. XRCC4 along with DNA ligase IV are able to process the nick held between the Ku and XLF-anchored protein scaffold religating the DNA ends [47].

FIG. 1.8. HR and NHEJ. Homologous recombination. HR begins following end resection and ATM activation promoted by the MRN complex. The ssDNA is bound by RPA, and BRCA1. RPA and BRCA1 both recruit BRCA2 through PALB2. RAD51 is subsequently recruited to the DNA which displaces RPA. RAD51 aids in strand invasion of a homologous template from a donor strand of DNA. The broken DNA is then extended by repair synthesis using the homologous template, resulting in strand annealing of the broken strands and resolution of the DSB. **Non-homologous End Joining**. Following the Ku binding of DNA ends and activation of the DNA-PK DDR response additional core NHEJ factors are recruited. These core factors include XRCC4-LigIV, XLF, Artemis, and PAXX. These factors coordinate to bring DNA ends together, where they can be ligated together.

FIG. 1.8


DSB
End Resection
End Protection
MRN
RPA Loading
RPA
BRCA1
RPA Eviction
BRCA2
RAD51
Strand invasion
Repair synthesis
Homologous Recombination
Ku
DNA-PKcs
P
Artemis
XRCC4
XLF
PAXX
Non-homologous
End Joining

1.4 Viruses and the DNA damage response

The host DDR also responds to viral infection and functions as an innate defense to limit viral multiplication. Viral multiplication can jeopardize host genome fidelity through competition with the cellular genome for critical DNA synthesis and DNA repair factors. Additionally, viral dysregulation of the cell cycle can cause genomic distress in infected cells (Reviewed in [206, 263, 276]). To counter the antiviral effects of the DDR and to manipulate the cell into a pro-viral environment, viruses have evolved strategies to suppress and appropriate DDR pathways. The connection between the cellular DDR and infecting viruses is rational because the DDR is focused on aberrant nucleic acids, and in many ways viral nucleic acids appear to the cell as aberrant nucleic acids. The interactions between viral factors and the DDR represents an important interaction that has implications in viral replication and disease.

1.4.1 Viral activation of DDR

A large variety of viruses activate DDR pathways during infection [206]. The DDR is activated following the detection of damaged DNA, following which host DDR kinases phosphorylate their downstream targets. These phosphorylations promote DNA repair, cell cycle arrest, and in cases where these fail, apoptosis. The triggers for viral-induced DDR aren't well understood, but is thought to include incoming viral genomes, the onset of viral genome synthesis, viral-induced changes to cellular metabolism, and direct activation of the DDR by viral proteins [276].

The detection of foreign nucleic acids is a major contributor to the innate immune response against viruses. Incoming viral genomes can be sensed by cellular nucleic acid sensors including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), cGAMP

synthase (cGAS), interferon gamma inducible protein 16 (IFI16), and Absent In Melanoma 2 (AIM2) (Reviewed in [160]). Additionally, there is evidence of crosstalk and regulation of the DDR by innate immune sensing pathways suggesting both that innate immune sensing is involved in the DDR and the involvement of the DDR in innate immunity detecting incoming viral genomes during infection (Reviewed in [256]). Herpes simplex virus-1 (HSV-1) infection causes stimulation of the DDR as indicated by phosphorylation of Chk1, ATM, and ATR [72]. During early HSV-1 infection, incoming genomes associate with γ-H2AX and MDC1 in distinct arcs in the nucleus. A number of DDR proteins associate with HSV-1 genomes during infection, including DNA-PKcs, Ku70, Ku80, the MRN complex, Poly ADP-ribose polymerase 1 (PARP-1), and ATM [64]. It is believed that nicks and gaps that normally occur in HSV-1 genomes trigger Ku70, Ku80, and MRN complexes which normally sense damaged host DNA. In a ICP0-null HSV-1 infection 53BP1 and BRCA1 are also localized near incoming genomes [145]. HSV-1 virion DNA contains nicks and gaps, and these viral genomes stimulate DNA-PKcs and the NHEJ pathway [244]. The activation of DNA-PKcs by incoming viral genomes is blocked during infection by viral ICP0 [244]. Incoming adenovirus genomes also activate the DDR, as the adenovirus core protein VII prevents the detection of incoming viral genomes by the MRN complex and subsequent activation of the DDR [118]. During Kaposi sarcoma herpesvirus (KSHV) infection viral infection activates ATM and γ-H2AX colocalizes with viral genomes shortly after entering the nucleus [242].

In addition to the activation of the DDR by incoming viral genomes, the generation of new viral genomes during infection can create structures that are recognized as DNA damage by host cells. During Adenovirus (Ad)/adeno-associated virus (AAV) co-infection, there is an activation of DNA-PK and ATM pathways that is distinct from the DDR response of Ad infection alone. This DDR response corresponds with the accumulation of AAV genomes, suggesting that AAV genome synthesis activates the DDR [55, 235]. For HSV-1/AAV co-infection, ATR and DNA-PKcs accumulate in

co-infected cells, despite DNA-PKcs being degraded by infected cell protein 0 (ICP-0) in cells infected by HSV-1 alone. However, Chk1 is not phosphorylated, suggesting that while phosphorylated ATR may accumulate in response to HSV-1/AAV co-infection, down-stream activation of Chk1 by ATR is blocked [267]. During AAV replication, activated DDR proteins including DNA-PKcs, Ku70, Ku80, and RPA32 accumulate at replication centers [235]. The infection of the minute virus of mice (MVM) triggers the activation of the DDR, characterized by the accumulation of phosphorylated H2AX, Nbs1, RPA32, Chk2, and p53 at sites of viral DNA synthesis [2]. Additionally, MVM infection resulted in the localization of ATR to viral replication compartments [2].

While detecting viral nucleic acids as damaged DNA is one method through which viruses activated the DDR, viruses are also able to activate the DDR through directly causing damage to host DNA or directly activating DDR components. During KSHV infection, the KSHV latency protein v-cyclin induces and ATM-mediated DDR through the induction of replicative stress on the host genome [127]. The Human papillomavirus (HPV) protein E7 interacts with ATM and is able to induce phosphorylation of the ATM substrate Chk2 [184]. The HPV E1 helicase induces DNA damage in cellular DNA when over-expressed through its helicase activity, activating the ATM pathway, and resulting in phosphorylation of ATM, ATR, Chk2, and p53 [78, 222, 229]. Additionally, the HPV E7 and E6 oncogenes result in increased activation of the DDR, and results in an increased accumulation of DDR factors, including ATM, Chk2, MRN, BRCA1, and Rad51 [114, 270]. The increases due to E7 are dependent on E7 binding Rb, and with both E6 and E7 the increases occur due to increases in DDR factor half-life [114]. However, in cells expressing E6 and E7, HR is reduced and there is a reduction in RAD51 localization away from DSBs [270]. Merkel cell polyomavirus (MCPyV) infection causes an activation of the DDR, an increase in p53 phosphorylation, and a corresponding up-regulation of p53 target genes [139]. The MCPyV induced DDR activation is dependent upon the helicase activity of MCPyV Large T antigen acting on host DNA [139]. The expression of

Epstein-Barr virus (EBV) latency genes, but not EBV lytic replication, induces a DDR response during infection [195]. The EBV latency associated protein EBV nuclear antigen 1 (EBNA-1) induces DNA damage through ROS induction by up-regulating Nox2 [92]. Lytic reactivation of cells latently infected with KSHV triggers widespread DDR activation, including phosphorylation of ATM, DNA-PKcs, Chk2, Nbs1, replication protein A 32 KDa subunit (RPA32), and H2AX [103]. This reactivation is dependent upon early gene expression and doesn't require viral genome replication [103]. The KSHV early gene product ORF57 also induces the DDR by promoting genome instability and DSBs [110].

1.4.2 Viral repression of DDR

EBV targets several components of the DDR, and repression of the DDR is critical for EBV replication. The EBV protein EBV nuclear antigen 3C (EBNA-3C) interacts with the ATM pathway signaling protein Chk2 and promotes cell cycle progression by blocking the G_2/M cell cycle arrest induced by the Chk2 mediated phosphorylation of Cdc25c [50]. Additionally, EBNA-3C binds the p53 tumor suppressor, and prevents both p53-mediated transcriptional activity and p-53-dependent apoptosis [289]. The EBV protein EBNA-LP is both phosphorylated by DNA-PKcs and blocks the interaction between DNA-PKcs and Ku70/Ku80 [97]. The EBV protein LMP-1 down-regulates ATM, reduces ATM-mediated phosphorylation of downstream ATM targets, and impairs DSB repair. [92]. ATM activity is reduced during EBV infection by the latency-associated oncoprotein EBNA-3C, and inhibition of the DDR kinases ATM and Chk2 enhance EBV-mediated B cell transformation, [195]. The EBV protein latent membrane protein 1 (LMP-1) down-regulates ATM and promotes genomic instability [92].

The gammaherpesvirus KSHV also inactivates portions of the DDR during infection.

The KSHV protein latency-associated nuclear antigen (LANA) interacts with cellular p53 and inactivates it, preventing p53 transcriptional activity [48, 79]. This LANA-mediated inactivation of p53 contributes to the oncogenic properties of KSHV, allowing for rapid growth of cells. Interestingly, the activation of the DDR disrupts LANA-p53 complexes and allows for p53 to become activated and functional [48]. Inhibition of DNA-PK or knockdown of Ku80 enhances KSHV viral DNA synthesis, suggesting that members of the NHEJ repair may have anti-KSHV activity during lytic replication [102].

Alphaherpesviruses also modulate the DDR during infection. The HSV-1 immediate early protein ICP0 degrades the histone ubiquitin ligases RNF8 and RNF168, preventing the recruitment of DDR components including MDC1 and 53BP1 to sites viral genomes [145, 146]. During HSV-1 infection, the NHEJ protein PAXX is excluded from viral replication centers, and while *PAXX*$^{-/-}$ cells have lower levels of viral DNA synthesis, they produce higher titres of infections virions compared to WT cells [261].

HPV regulates and represses the DDR during infection. The HPV protein E6 interacts with p53, and E6 is able to induce p53 ubiquitination and subsequent degradation [232]. The HPV protein E6 destabilizes the DNA damage-induced histone acetyltransferase Tip60, relieving the Tip60 mediated repression of HPV promoters [112]. Yet in HPV infected cells Tip60 activity contributes to the activation of ATM and is necessary for HPV differentiation dependent genome amplification [104].

During Human T-cell leukemia virus type-1 (HTLV-1) infection, the HTLV-1 protein Tax is a master regulator of the DDR. Tax binds DNA-PKcs, activates it, and re-localizes DNA-PKcs and Ku70 preventing the normal DNA-PK-mediated response to DNA damage [69]. HTLV-1 Tax also interacts with Chk1, a kinase downstream of ATR, and blocks Chk1 kinase activity, thereby preventing the phosphorylation-dependent degradation of Cdc25A, thereby inhibiting DNA damage-induced G_2 arrest [208]. Additionally, HTLV-1 Tax interacts with the ATM phosphorylation target Chk2 and inhibits

Chk2 kinase activity, blocking Chk2-meditated apoptosis in response to DNA damage [207].

Adenovirus infection triggers an anti-viral DDR response, and correspondingly has a number of proteins which alter and repress the DDR. During Adenovirus infections, the viral proteins E4orf3 and E1b55K target and degrade the cellular MRN proteins to prevent localization of MRN complexes to viral replication centers [168, 251]. Adenovirus E4 mutants are unable to prevent MRN complexes from localizing to viral replication centers, and ATM kinase activity interferes with E4 mutant Adenovirus DNA replication and late gene expression [84]. Adenovirus utilizes E4orf6 to target TopBP1 for proteasomal-mediated degradation and prevent the activation of an ATR dependent DDR in response to infection [18]. Additionally, Adenovirus proteins E1B55k and E4orf6 target Tip60 for degradation, relieving the Tip60-mediated repression of the immediate early adenovirus promoter and preventing ATM activation [96]. Together, Adenovirus uses several viral proteins to target important components of DDR activation in order to promote viral replication.

1.4.3 Viral modulation of the DDR

The herpesviruses encode several conserved proteins, and some of these proteins have conserved functions of targeting the DDR for modulation during infection. The herpesvirus conserved nuclear egress protein, UL31 in HSV-1, which binds to damaged DNA through a poly ADP-ribose (PAR) binding motif and contributes to viral DNA packaging and nuclear egress in a cellular PAR polymerase dependent manner [238]. Additionally, Tip60 represents a conserved target of herpesvirus kinases, with KSHV ORF36, human cytomegalovirus (HCMV) UL97, EBV BGLF4, and HSV-1 UL13 increasing the phosphorylation of Tip60.

It is clear that the DDR plays an important role during infection of alphaherpesviruses as several viral factors target and modulate the DDR. However, the exact role or roles of the DDR during infection remain to be elucidated. HSV-1 infection induces H2AX phosphorylation [142, 278]. HSV-1 gene expression, but not viral DNA synthesis, is required to stimulate γ-H2AX accumulation [25]. Interestingly, during Herpes simplex virus-2 (HSV-2) infection viral DNA synthesis is required to stimulate γ-H2AX accumulation [25]. ATM, but not ATR, is required for Herpes simplex induced γ-H2AX [25]. Pharmacological inhibition and siRNA knockdown of ATM or ATR had no impact on HSV-1 or HSV-2 vDNA synthesis or lytic virus production [25]. This directly contradicts the results that both $ATM^{-/-}$ and ATR-deficient cell lines show reductions in HSV-1 and HSV-2 lytic viral replication [25, 72, 144]. Other reports show pharmacological inhibition of both ATR and the ATR substrate Chk1 inhibited HSV-1 lytic replication [72]. While ATR is inactivated during HSV-1 viral infection, ATR and several ATR-mediated DDR pathway proteins are recruited to viral replication centers [182, 183]. Knockdown of several of these ATR-pathway members, including ATRIP, replication protein A 70 KDa subunit (RPA70), TopBP1, and CINP reduce viral gene expression and virus production [182, 183]. Additionally, activation of ATR prior to infection with HSV-1 reduces recombination between viral genomes but has no effect on virus production [182]. Both pATR and pChk1 accumulated at HSV-1 replication centers in infected nuclei [72]. Additionally, there is an accumulation of ATM/ATR pS/TQ substrates at HSV-1 replication centers [72].

The DDR is important for HCMV replication, as pharmacological inhibition of ATM reduces HCMV DNA synthesis in a dose dependent manner [141]. During HCMV infection, the MRN complex, along with ATM, ATRIP, Chk2 and Chk1 are localized to viral replication centers [83, 157]. Additionally, Ku70, Ku80, DNA-PKcs, and ATR are excluded from HCMV viral replication centers [157]. HCMV recruits Tip60 to HCMV lytic replication origin (*OriLyt*) and knockdown of Tip60 reduces HCMV DNA synthesis [141].

EBV also interacts with DDR components and uses the DDR to promote viral replication. The EBV protein Zta binds to 53BP1 and promotes the viral lytic cycle, as both the knockdown of 53BP1 and the perturbation of the Zta 53BP1 interaction reduced productive viral replication [11]. The EBV viral kinase BGLF4 phosphorylates Tip60 and recruits Tip60 to EBV lytic promoters and Tip60 knockdown reduces synthesis of EBV viral genomes [141].

The DDR is extensively involved with KSHV replication, and it is involved in both lytic replication and latency. During KSHV lytic reactivation RPA32 and meiotic recombination 11 (Mre11) localize to viral replication centers, despite other DDR members being marginalized to the periphery of the nucleus [103]. ATM and H2AX both promote the entering of KSHV into latency, as pharmacological inhibition of ATM and small interfering RNA (siRNA) mediated knockdown of H2AX reduced expression of LANA and reduced vDNA genome synthesis [242]. During KSHV lytic replication, DDR DNA sensors including the MRN complex, Ku70 and Ku80 localize to viral replication centers [102]. Depletion of MRN complex member Mre11 or reduction in its exonuclease activity reduces KSHV lytic virus replication [102]. During KSHV latency, cytoplasmic LANA interacts with cytoplasmic MRN complexes, and is thought to aid in KSHV lytic reactivation by interfering with MRN mediated NF-κB activation [166]. Nuclear LANA interacts with host γ-H2AX and promotes interaction between γ-H2AX and KSHV terminal repeats, and knockdown of H2AX reduces KSHV episome copy number [111].

The AAV-mediated modulation of the DDR plays an important role in the outcome of AAV co-infection with Ad. The AAV protein Rep localizes to sites of the DNA damage factor γ-H2AX in an ATM dependent manner [21]. Rep interacts with several additional DDR proteins during infection, including DNA-PKcs, Ku70, Ku80, and the MRN complex [189, 192]. AAV site specific integration into AAVS1 on human chromosome 19 is dependent on MRN function, as both knockdown of MRN and degradation of MRN

during Ad co-infection ablates AAV integration [179].

MVM infection results in phosphorylation of the RPA32 subunit of RPA [228]. Phosphorylation of RP32 is a potent trigger of Chk1 and ATR phosphorylation. Despite this, MVM blocks the phosphorylation of ATR and the ATR substrate Chk1 in response to DNA damage, suggesting that despite triggering the DDR, MVM infection inactivates the ATR pathway in whole or in part [3].

Polyomaviruses also modulate the host DDR. During MCPyV infection DDR components, including γ-H2AX, pChk2, pATM, ATR, and pRPA32, localize to sites of viral DNA synthesis. Additionally, pharmacological inhibition and RNA mediated knock-down of the DDR reduced MCPyV DNA synthesis [262]. human polyomavirus JC virus (JCV) virus multiplication is dependent on the DDR-mediated induction of G_2 arrest, and inhibition of the DDR by caffeine suppresses JCV production [205].

During HPV infection a number of DDR and HR components, including 53BP1, pATM, and pChk2, localize to HPV replication compartments in both undifferentiated and differentiate cells [85]. HPV also requires the MRN complex to direct cellular HR factors, such as Rad51 and BRCA1, to viral genomes to ensure viral replication [9, 40]. HPV induces localization of ATR pathway members ATRIP and TopBP1 to viral replication centers [222].

1.4.4 Baculoviruses and the DDR

Baculoviruses, including the prototypic baculovirus AcMNPV, have a complicated interaction with the host DDR during infection. AcMNPV both activates portions of the DDR [107], represses other portions of the DDR [180, 181], and is dependent upon the DDR for its own multiplication (FIG. 1.10) [107, 180, 181]. During AcMNPV

multiplication, the replication of vDNA triggers the DDR-mediated phosphorylation of *S. frugiperda* ortholog of p53 (Sfp53), and at later times phosphorylation of *S. frugiperda* ortholog of H2AX (SfH2AX) [107]. However, at earlier times baculovirus late expression factor 7 (LEF-7) limits the accumulation of γ-H2AX in response to viral infection or pharmacological induction of DNA damage [180]. LEF-7 expression in *S. frugiperda* SF21 cells is sufficient to limit DNA damage induced DDR activation [180]. Additionally, a AcMNPV virus which lacks LEF-7 (AcMNPVΔLEF-7) has reduced budded virus production and allows γ-H2AX accumulation [180].

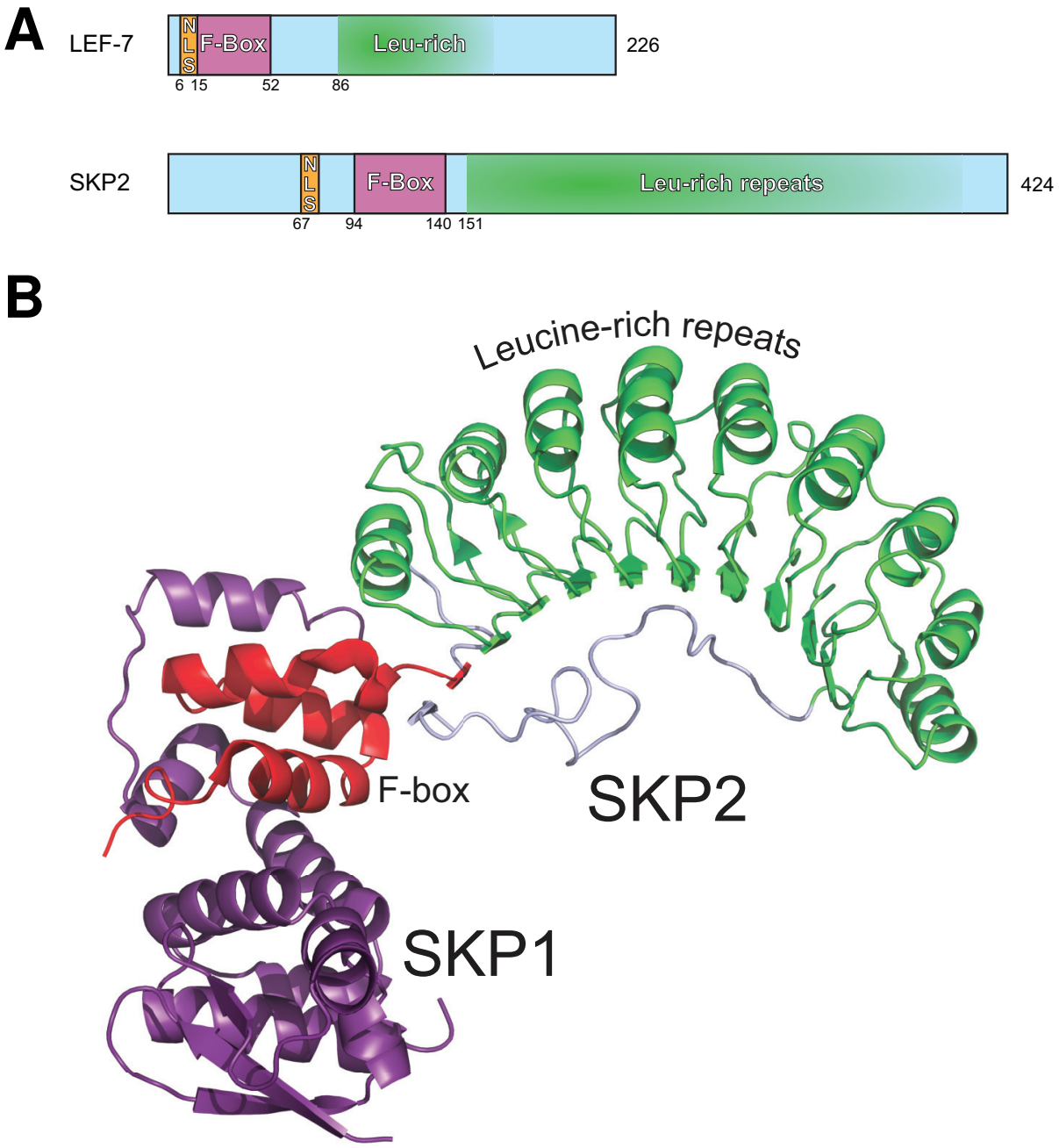
Importantly, the DDR facilitates baculovirus multiplication. Pharmacological inhibition of the DDR results in a reduction of the AcMNPV budded virus production, as demonstrated using the pan-DDR inhibitor caffeine or the ATM specific inhibitor KU55933 [107, 181]. Since ATM inhibition dramatically decreases budded virus production, it is surprising that LEF-7 inhibits γ-H2AX, as γ-H2AX amplifies ATM-mediated DDR signaling. This apparent paradox suggests that baculoviruses modulate the DDR, allowing ATM activation and phosphorylation of certain substrates but preventing ATM access to SfH2AX. Importantly, wild type AcMNPV induces γ-H2AX accumulation beginning at 24 hpi [107], while an AcMNPV ΔLEF-7 deletion mutant induces γ-H2AX at 6 hpi [180], at the same time as the onset of vDNA synthesis. Thus, baculoviruses utilize the DDR to their advantage, using activated ATM while depending on LEF-7 to prevent H2AX phosphorylation.

1.4.5 Baculovirus LEF-7

LEF-7 is a widely conserved baculovirus protein, with homologs present in all Group I NPVs, a few Group II NPVs, and a few GVs. LEF-7 has been shown to contribute to baculovirus DNA synthesis, as deletion of *lef-7* in AcMNPV and BmNPV showed

FIG. 1.9. Comparison of F-box proteins LEF-7 and SKP2. A. While LEF-7 and S-phase kinase-associated protein 2 (SKP2) don't share sequence homology, they do have a similar domain structure. Both contain a N-terminal nuclear localization signal (NLS), followed by a F-box domain. The LEF-7 and SKP2 C-terminus both contain leucine rich repeats, with SKP2 having 10 repeats, and LEF-7 likely containing 2-3 repeats. **B. Ribbon model of SKP2 bound to SKP1.** The SKP2 F-box domain (red) interacts with SKP1 (purple). The SKP2 C-terminal leucine-rich repeats (green) bind SKP2 substrates for polyubiquitination by the SKP2/Skp, Cullin, F-box containing (SCF) complex. Image courtesy of Dr. Nathaniel Byers and adapted from Protein Data Bank: 1FQV [234]

FIG. 1.9


A
LEF-7
NLS
F-Box
Leu-rich
226
6 15
52
86
SKP2
NLS
F-Box
Leu-rich repeats
424
67
94
140 151
B
Leucine-rich repeats
F-box
SKP2
SKP1

reductions in viral DNA synthesis [87, 180]. Interestingly, AcMNPVΔLEF-7 has impaired virus production in *S. frugiperda* cells, but wild type levels of virus production in *Tricopulsia ni* cells [43, 180].

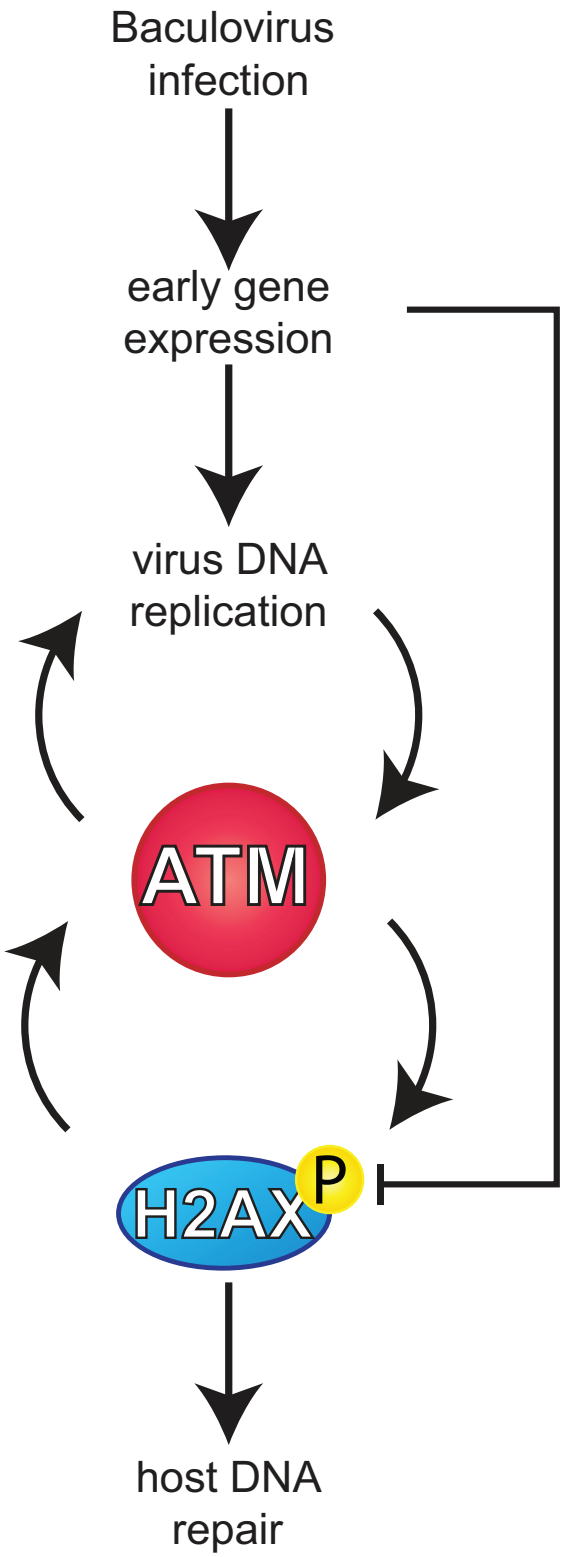
AcMNPV LEF-7 contains 226 amino acids, is 26.6 kDa in size, and is expressed from *lef-7* near the hr4c *hr* (FIG. 1.3A). LEF-7 contains a nuclear localization signal near its N-terminus (FIG. 1.9A), and immunofluorescence studies show that LEF-7 is predominately localized to the nucleus [180]. Positioned adjacent to the nuclear localization signal is a F-box domain signifying that LEF-7 is an F-box protein (FIG. 1.9A). The domain structure of LEF-7 is similar to that of the F-box protein SKP2 (FIG. 1.9). F-box proteins interact with S-phase kinase-associated protein 1 (SKP1) of the SCF complex, and mediate selective protein substrate ubiquitination. Substrates polyubiquitinated in this manner are commonly subjected to proteasomal degradation, although non-degradative polyubiquitination can also occur. Indeed, LEF-7 functions as an F-box protein in *S. frugiperda* cells, interacting with *S. frugiperda* ortholog of SKP1 (SfSKP1) of the *S. frugiperda* SCF complex [180]. LEF-7, in the presence of all other AcMNPV DNA replicative genes, contributed to homologous recombination between two GFP reporter plasmids [59]. LEF-7 increases homologous recombination between GFP reporter plasmids even in the absence of AcMNPV *hr*s, suggesting a broad promotion of homologous recombination [59].

1.5 Significance and goals of my research

Viruses, as obligate intracellular parasites, are required to re-purpose cellular factors to promote viral multiplication. Cells in return have evolved complex mechanisms in an attempt to thwart viral invaders. One area that both serves as a viral target for exploitation and a cellular anti-viral response is the DDR. Numerous recent studies have

FIG. 1.10. AcMNPV and the DDR. Following viral entry and the initiation of baculovirus infection, early gene expression occurs. Early gene expression is necessary for the onset of baculovirus DNA replication. Baculovirus DNA replication has been shown to activate the ATM-mediated DDR. A central step of the ATM-mediated DDR is the phosphorylation of the variant histone H2AX. γ-H2AX aids in the recruitment and retention of DDR and DNA repair factors, including the recruitment and activation of additional ATM creating a positive feedback loop. Activated ATM is required for baculovirus DNA replication. In an apparent contradiction, the baculovirus early protein LEF-7 reduces the accumulation of γ-H2AX, which in turn should reduce further ATM activation and reduce virus DNA multiplication.

FIG. 1.10


Baculovirus infection
early gene expression
virus DNA replication
ATM
H2AX
P
host DNA repair

illuminated areas of this virus-host interface. However, many important answers and questions remain undiscovered. The broad goal of my research is to better define how viruses and the DDR interact during infection. More specifically, my major aims in this thesis were to i) investigate how the DDR is activated following virus infection, ii) define the role of the DDR kinase ATM in response to viral infection, iii) characterize viral factors that modulate the host DDR. In my attempts at completing these goals, I utilized infection of *S. frugiperda* SF21 cells by the prototypic baculovirus AcMNPV as a model (FIG. 1.10).

Prior to my thesis work, investigation into baculovirus interactions with the DDR within lepidopteran cells included kinase inhibitor studies [107, 181]. However, the inhibitors were not well characterized within lepidopteran cells, and it was not clear if inhibitors that were specific for DDR kinases in mammalian cells were also specific in lepidopteran cells. To address this issue, I focused on developing a genetic knock-out of the ATM DDR kinase. CRISPR-Cas9 genome editing is a powerful tool for site specific genome editing, it had not been previously performed in *S. frugiperda* cells. The specific limitation was the inability to express small RNAs, which is needed for the single guide RNAs (sgRNAs) to target the Cas9 nuclease. Through studies in Chapter 3 I developed a clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9) system that could be successfully utilized to perform site-specific genome edits in SF21 cells. My studies, in conjunction with a concurrent report [159], identified the *S. frugiperda* U6 small nuclear RNA (snRNA) promoter. The U6 promoter was utilized to express the sgRNAs necessary for CRISPR-Cas9 editing. I identified the *sfatm* gene and using this CRISPR-Cas9 system I developed, I successfully generated genetic knockouts in *S. frugiperda* SF21 cells (Chapter 3 and Chapter 2). Additionally, the U6 promoter I identified can be successfully used to express short hairpin RNAs (shRNAs) and knockdown transcripts in *S. frugiperda* cells (3).

The viral processes through which cellular DDR is engaged are poorly understood [276]. Previous work, utilizing an ATM specific inhibitor suggested that the *S. frugiperda* ortholog of ATM (SfATM) contributes to AcMNPV multiplication [107, 181]. To better understand how ATM is involved in AcMNPV multiplication, I examined an additional ATM specific inhibitor in SF21 cells. However, I also discovered that ATM inhibitors are toxic to SF21 cells at concentrations near those used to examine AcMNPV multiplication. Non-toxic levels of ATM inhibitors reduced SfATM mediated phosphorylation of SfH2AX in response to pharmacologically induced DNA damage but failed to reduce AcMNPV budded virus production (Chapter 2). To further investigate this finding, I generated SF21 SfATM knockout cells. Consistent with known ATM function, SfATM knockout cells exhibited a reduced accumulation of γ-H2AX in response to DNA damage. These SfATM knockout cells supported AcMNPV multiplication to levels indistinguishable from the parental SF21 cells (Chapter 2). Caffeine treatment of SfATM knockout cells reduced budded virus production, indicating that caffeine-mediated inhibition of SfATM was not responsible for the inhibition of AcMNPV multiplication.

Recent work in the Friesen lab revealed during infection a baculovirus-mediated reduction in DDR activation occurred, as measured by reduced accumulation of γ-H2AX [181]. Further investigation identified the baculovirus protein LEF-7 as necessary and sufficient for reducing γ-H2AX [180]. LEF-7 was identified as a F-box protein [180] indicating that LEF-7 interacts with specific substrates to promote their polyubiquitination by a SCF complex. To further investigate LEF-7's mechanism of action, I used a biotin proximity labeling strategy to identify LEF-7 substrates (Chapter 4). I created a LEF-7 fusion protein with a BirA biotin ligase domain, allowing for biotinylation of proteins in close proximity to LEF-7. Using this BioID-LEF-7 fusion, I identified *S. frugiperda* ortholog of cGAS (SfcGAS) as a potential substrate for polyubiquitination. cGAS functions as an innate immune sensor, activating an anti-viral innate immune response to foreign nucleic acids. Additionally, cGAS influences DNA repair, translocating to the

nucleus following DDR activation and reducing homologous recombination-mediated repair [147]. My preliminary result indicates baculoviruses may be using a common viral strategy of interfering with cGAS-STING pathway, or possibly depleting cGAS to promote homologous recombination required for baculovirus DNA replication.

Collectively, my thesis studies have furthered our understanding of viral interaction with the DDR. I developed a critical tool in *S. frugiperda* specific genome editing by creating a CRISPR-Cas9 system that functions in *S. frugiperda* cell lines (Chapter 3). Using this tool, I further winnowed how the DDR functions in response to baculovirus infection, showing that a host kinase other that ATM contributes to baculovirus multiplication (Chapter 2). Additionally, my research uncovered a link between the baculovirus protein LEF-7 and SfcGAS, which is a potential mechanism to promote baculovirus stimulated homologous recombination (Chapter 4). Thus, my work has better defined the relationship between baculovirus and the host DDR.

Chapter 2

Ataxia-telangiectasia mutated is Activated but Not Required for Productive *Autographa californica* Multiple Nucleopolyhedrovirus Infection

Manuscript in submission as titled above. Erickson, JR; Kalejta, RF; Friesen, PD.

2.1 Abstract

Multiplication of the invertebrate DNA baculoviruses activates the host DNA damage response (DDR), which promotes virus DNA replication. DDR signaling is initiated by the host insect's phosphatidylinositol-3 kinase-related kinases (PIKKs), including ataxia-telangiectasia mutated kinase (ATM). Like other PIKKs, ATM phosphorylates an array of host DDR proteins at SQ and TQ (S/TQ) motifs, the result of which leads to cell cycle arrest, DNA repair, or apoptosis. To define the role of host PIKKs in baculovirus replication, we compared replication levels of the baculovirus prototype *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in permissive *Spodoptera frugiperda* (SF21) cells with and without ATM function. Caffeine, which inhibits multiple DDR kinases, and the ATM-specific inhibitors KU-55933 and KU-60019 each prevented phosphorylation of *Spodoptera* histone H2AX (SfH2AX), a recognized indicator of ATM activity. However, only caffeine reduced AcMNPV-induced bulk phosphorylation of S/TQ protein motifs. Furthermore, only caffeine, not KU-55933 nor KU-60019, reduced AcMNPV yields, suggesting a limited role for ATM. To investigate further, we identified and edited the *Spodoptera* ATM gene (*sfatm*). Consistent with ATM's known functions, CRISPR/Cas9-mediated knockout of *sfatm* eliminated DNA damage-induced phosphorylation of DDR marker SfH2AX in SF21 cells. However, loss of *sfatm* failed to affect the levels of AcMNPV multiplication. These findings suggested that in the absence of the kinase SfATM, another caffeine-sensitive host DDR kinase promotes ST/Q phosphorylation and baculovirus multiplication. Thus baculoviruses activate and utilize the host insect DDR in an ATM-independent manner.

2.2 Importance

The DDR, while necessary for the maintenance and fidelity of the host genome, represents an important cellular response to viral infection. The prolific DNA baculoviruses activate and manipulate the invertebrate DDR by using mechanisms that positively impact virus multiplication, including virus DNA replication. As the key DDR initiator kinase, ATM was suspected to play a critical role in this host response. However, we show here that baculovirus AcMNPV activates an ATM-independent DDR. By identifying the insect host ATM ortholog (*Spodoptera frugiperda* SfATM) and evaluating genetic knockouts, we show that SfATM is dispensable for AcMNPV activation of the DDR and for virus replication. Thus, another PIKK, possibly the closely related kinase ATR (ATM- and Rad3-related kinase), is responsible for efficient baculovirus multiplication. These findings better define the host pathways used by invertebrates to engage viral pathogens, including DNA viruses.

2.3 Introduction

Baculoviruses are a family of large DNA viruses, which are highly prolific in insects. Like other viruses, baculoviruses modify the host cell to promote multiplication. In turn, the host cell activates anti-viral pathways. These intrinsic pathways include the host DNA damage response (DDR). The DDR machinery detects incoming or newly synthesized DNA viral genomes and initiates a complex response involving activation of phosphatidylinositol 3-kinase-like kinases (PIKK) (reviewed in [275]). PIKKs are activated by phosphorylation as a key step in the response. The three principal DDR PIKKs are ataxia-telangiectasia mutated kinase (ATM), ATM- and Rad3-related kinase (ATR), and DNA-dependent protein kinase (DNA-PK) [70, 75]. ATM plays a key role

activating the DDR in response to deleterious double-strand breaks, which are subsequently repaired by homologous recombination [185]. ATR responds to DNA lesions that occur due to replicative stress, including that induced by DNA viruses [53]. Like ATM, DNA-PK responds to double-stranded breaks. However, DNA-PK promotes DNA-repair via non-homologous end joining (NHEJ). ATM, ATR, and DNA-PK phosphorylate multiple substrates when activated [17]. ATM, and to a lesser degree ATR and DNA-PK, phosphorylate the variant histone H2AX [33, 250, 274]. Phosphorylated H2AX (γ-H2AX) is a key indicator of ATM-mediated DDR activation. In addition to promoting DNA-repair, DDR activation induces cell cycle arrest, promotes apoptosis, and responds to viral infections.

Diverse viruses trigger the host DDR, including dsDNA viruses (Human Cytomegalovirus (HCMV), BK polyomavirus, and adenovirus) and ssDNA viruses (minute virus of mice (MVM)) [2, 83, 140, 251]. Infection often triggers a DDR response that promotes viral multiplication [2, 40, 182]. For example, BK polyomavirus requires the virus-induced activation of the ATM and ATR pathways for efficient viral DNA synthesis [113]. HCMV activates the ATM pathway and ATM-phosphorylated host DDR proteins localize to viral replication centers within the nucleus. However, ATM activity is not required for HCMV replication [157]. Adenovirus E4 ORF3 reorganizes the Mre11-Rad50-Nbs1 DDR complex to promote the establishment of viral replication centers [74]. During MVM infection, viral genomes associate with DNA damage sites on the cellular genome [163]. The DNA baculoviruses, including the prototypic *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), also trigger the host insect DDR. AcMNPV-induced DDR activation triggers apoptosis, which is blocked by viral-encoded caspase inhibitors [181].

AcMNPV both activates and manipulates the DDR to promote viral multiplication [107, 181]. This DNA virus, with its 134-kbp circular DNA genome, interferes with the

normal ATM-induced DDR response [180]. AcMNPV blocks DNA damage-induced accumulation of γ-H2AX at early times after infection [107], through the activity of its early *lef-7* gene [180]. Previous studies demonstrated that the broad-spectrum PIKK inhibitor caffeine also blocked γ-H2AX accumulation and severely reduced budded virus yields [107, 181]. These findings implicated host DDR activity as a key requirement for AcMNPV multiplication.

Here we further define the connection between AcMNPV and the induction of the invertebrate DDR by investigating the role of the PIKKs in initiating the host response and also contributing to virus multiplication. We report that AcMNPV induces the nuclear accumulation of DDR-specific protein phosphorylations early during infection of permissive *Spodoptera frugiperda* SF21 cells. Phosphorylation was specific for SQ and TQ (S/TQ) motifs, which is consistent with virus-induced PIKK activity. We confirmed that the PIKK inhibitor caffeine blocked S/TQ phosphorylation. However, other inhibitors known to be specific for ATM did not when used to treat AcMNPV-infected cells at nontoxic levels. Identification and genetic knockout of the *Spodoptera frugiperda atm* gene demonstrated that SfATM is required for DDR-mediated H2AX phosphorylation but not for AcMNPV productive infection. These findings indicate that a caffeine-sensitive PIKK, other than SfATM, is activated upon AcMNPV infection and contributes to efficient virus multiplication.

2.4 Materials & Methods

Cells and infections. *Spodoptera frugiperda* IPLB-SF21 (SF21) cells [266] were maintained at 27°C in TC100 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone). Wild-type AcMNPV (L-1 strain) [134] was previously described. The AcMNPV recombinant wt/vAcIE1$^{HA\text{-}H6}$ that encodes epitope HA-tagged immediate

early IE-1 (AcIE1$^{HA\text{-}H6}$) under control of the AcMNPV *IE1* promoter was generated by David J. Taggart using the Bac-to-Bac system (Invitrogen) [258]. In all infections, virus inoculum was allowed to adsorb for 1 h at room temp with rocking. The inoculum was then replaced with fresh medium at time zero. When indicated, the inoculum was replaced with fresh medium containing drug or vehicle. Extracellular budded virus (BV) was quantified by $TCID_{50}$ assays as previously described [197].

Plasmids and Transfections. CRISPR-Cas9 constructs were designed for genome editing of SF21 cells. In brief, the *S. frugiperda* U6 snRNA (sfU6) promoter and terminator sequence (GenBank: NJHR01000755.1) were identified using the *Drosophila melanogaster* U6 sequence to query *S. frugiperda* whole genome shotgun contigs. While our work was ongoing, a similar strategy was published [159]. To create pSfU6-neo, the sfU6 gene (accension JQCY02000000) and 400 bp upstream and 100 bp downstream, along with a Kan/neoR cassette under control of an AcMNPV IE1 promoter, was cloned into the pBluescript KS+ (Invitrogen) backbone using NEBuilder Hifi (New England Biolabs). To create pIE1-Cas9-2A-GFP-U6, the sfU6 sequence was cloned along with Cas9-2A-GFP from pSpCas9(BB)-2A-GFP (PX458) into the pBluescript KS+ backbone using NEBuilder Hifi. pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138) [218]. Transfections were carried out using *Trans*IT-Insect (Mirus Bio) with 2 µl of *Trans*IT-Insect per µg of plasmid DNA. Transfections were carried out in 6 well plates using 10 µg of plasmid DNA per well containing 10^6 SF21 cells.

Inhibitors. ATM-specific inhibitors KU-55933 and KU-60019, and the Topoisomerase II inhibitor etoposide (Selleckchem), were dissolved in DMSO, and diluted in TC100 medium supplemented with 10% FBS to the indicated concentrations. Caffeine (Alexis Biochemicals) was dissolved directly in TC100 medium supplemented with 10% FBS. When indicated SF21 cells were overlaid with TC100 medium containing

inhibitor at the indicated concentrations and incubated at 27°C. For infection of drug treated cells, all drugs were replaced immediately following inoculation.

Immunoblots and antisera. Cells were collected and lysed in 50 mM Tris pH 7.4, 500 mM NaCl, 0.4% SDS, 5 mM EDTA, 1 mM DTT, 1x PhosSTOP™(Roche) phosphatase inhibitor, and 1x cOmplete™(Roche) protease inhibitor for 10 min on ice. Lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Osmonics, Inc.). Membranes were blocked in 0.2% (w/v) I-Block™(Applied Biosystems) dissolved in PBS containing 0.1% Tween-20. The indicated primary antibodies were used followed by LiCoR IRDye 680- or 800- secondary antibodies (LI-COR) and imaged on a LI-COR Odyssey FC. The following commercial antiserum were used: monoclonal mouse anti-HA (Covance, MMS-101P), polyclonal rabbit anti-γ-H2A.X pS139 (AbCam, ab11174), mouse anti-Tubulin (Sigma, DM 1A), rabbit anti-pST/Q (Cell Signaling, 2851S).

Immunofluorescence microscopy. SF21 cells on glass coverslips were fixed with 1% formaldehyde in PBS, washed 3x with PBS containing 0.1% Triton X-100 and 0.05% Tween 20 (PBST), and then blocked with 5% bovine serum albumin and 5% goat serum in PBST. Coverslips were incubated with the indicated primary antibodies, then washed 3x in PBST, and stained with Alexa Fluor 594-conjugated goat anti-Rabbit IgG (Invitrogen, A-11017) and Alex Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, A-11020). Fixed SF21 cells were then washed, counterstained with Hoechst 33342, and mounted using Fluoromount-G (Southern Biotech, 0100-01). Coverslips were imaged on a Leica Stellaris 5 confocal microscope using a 63X objective. Images were analyzed using FIJI [233], and figures were prepared by using Adobe Illustrator.

Sequence identification. The gene (*sfatm*) encoding the *Spodoptera frugiperda* Ataxia-telangiectasia mutated (SfATM) kinase was identified by using a BLAST search of a whole cell transcriptome of SF21 cells (NCBI Accession: PRJNA271593). The human

ATM amino acid sequence (NCBI Reference Sequence: NP_000042.3) was used as the query. The BLAST search identified a transcript containing the potential SfATM coding sequence (GenBank: GCTM01013287.1). Subsequently, the SfATM transcript and predicted protein sequence was used to identify domains matching those within human ATM by using NCBI's Conserved Domain Database [165]. The putative *sfatm* sequence was used to query a *S. frugiperda* whole genome shotgun sequence (Accession: PRJNA380964) to identify the *sfatm* genomic context (GenBank: NJHR01000076.1).

CRISPR-Cas9 cell lines. Cas9-cleavage sites and corresponding single-guide RNAs (sgRNAs) were identified using crispor.tefor.net [56] targeting the *sfatm* genomic region. Oligo DNAs corresponding to the sgRNAs 5'-CACCGATAAAAGAAAACAGGCGATA-3' and 5'-GCCGACCGTTGGGATAAACAGCCAC-3' were cloned into pIE1-Cas9-2A-GFP-U6 and pSfU6-neo respectively. SF21 cells were transfected with TransIT-insect (Mirus Bio) and placed under selection in TC100 medium containing 500 µg/ml G418 (Gibco). Seven days later, the G418 was removed and cells were diluted into 96-well plates to obtain single cell clones. Colonies were expanded, and genomic DNA was screened via PCR using primer pairs that amplified 500 bp upstream and downstream of both sgRNA sites. DNA isolated from selected colonies were sequenced. The selected colonies had two distinct edited copies of SfATM from the two alleles present in each cell. ATM KO #1 had two edited sequences: a 17,613-bp deletion that removed a section from exon 1 through exon 35 leaving only the C-terminal 5 amino acids, or a 1-bp deletion in the second codon causing a premature stop codon after 6 amino acids. ATM KO #2 had two edited sequences: a 17,722-bp deletion that removed all exons, and a 360-bp deletion in exon 1 and 2 resulting in a premature stop codon after 6 amino acids and a 2-bp deletion in exon 35.

2.5 Results

2.5.1 ATM-specific inhibitors block DNA damage-induced H2AX phosphorylation in permissive *Spodoptera* cells

. Upon infection, AcMNPV triggers the host lepidopteran DDR as indicated by the phosphorylation of histone H2AX [107, 180, 181], a direct substrate of ATM during DNA repair. These previous studies also suggested a role for ATM in the virus-induced DDR as demonstrated by the reduction of γ-H2AX by treatment of cells with known ATM inhibitors during DNA damage and baculovirus infection. Two ATM inhibitors, caffeine and KU55933, blocked γ-H2AX accumulation and AcMNPV productive multiplication [107, 181]. Here, we performed side-by-side comparisons of ATM inhibitors at drug concentrations that did not affect cell viability. To this end, we first conducted dose response assays for cell viability using increasing concentrations of caffeine, KU-55933, and KU-60019, each known ATM inhibitors [86]. Using MTT to monitor SF21 cells 72 h after drug treatment, viability decreased noticeably above concentrations of 8 mM, 54 µM, and 32 µM for caffeine, KU-55933, and KU-60019, respectively (Fig. 2.1). From these data, we chose 4 mM caffeine, 32 µM KU-55933, and 16 µM KU-60019 as the highest concentrations that exhibited no obvious effects on cell viability.

Because ATM is the principal PIKK responsible for phosphorylation of histone variant H2AX after DNA damage, we tested the independent effect of the three ATM drugs on the accumulation of γ-H2AX after pharmacological induced DNA damage using the topoisomerase inhibitor etoposide [35]. γ-H2AX was monitored by using a GFP-fusion of SfH2AX that was transfected into SF21 cells prior to treatment with etoposide [181]. DDR-mediated phosphorylation of GFP-SfH2AX occurs at a C-terminal SQ motif and is a sensitive marker of the DDR in SF21 cells [181]. In vehicle treated

FIG. 2.1. SF21 cell viability in the presence of DDR inhibitors.SF21 cells were treated with caffeine, KU-55933, or KU-60019 at the indicated concentrations. Cell viability was determined via MTT assay 72 h later. Data is reported relative to values obtained from cells treated with vehicle control alone ±SD (n = 3). Arrows indicate highest drug concentration that exerted limited effects on cell viability.

FIG. 2.1

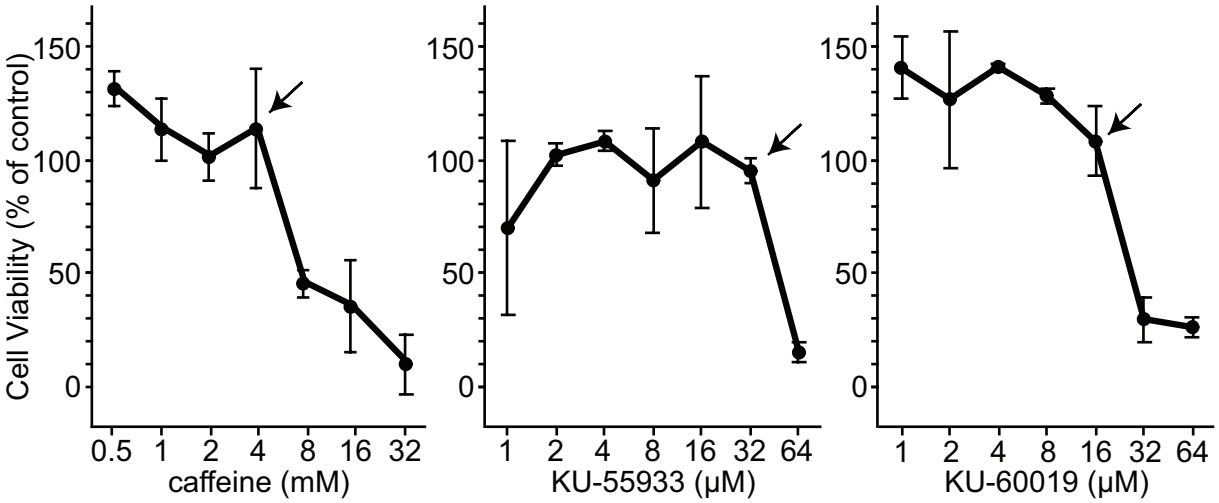
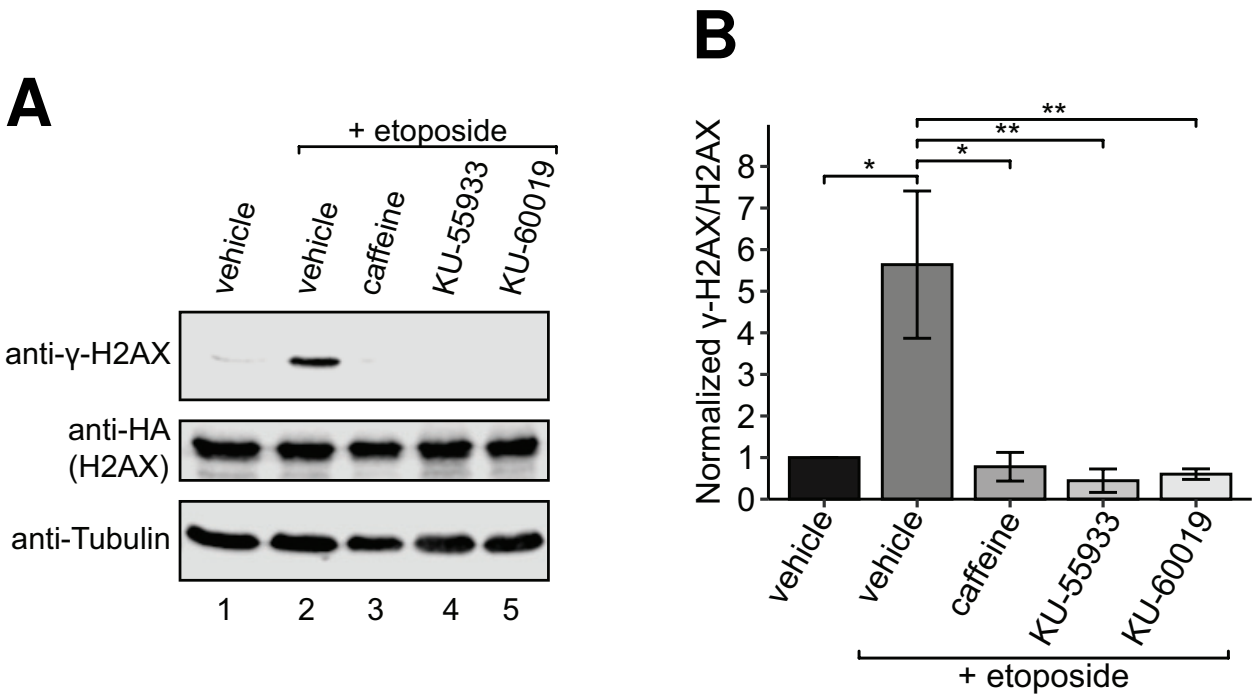

Cell Viability (% of control)
150
100
50
0
0.5 1 2 4 8 16 32
caffeine (mM)
1 2 4 8 16 32 64
KU-55933 (μM)
1 2 4 8 16 32 64
KU-60019 (μM)

FIG. 2.2. Comparison of H2AX phosphorylation in the presence of DDR kinase inhibitors. SF21 cells expressing GFP-HA-SfH2AX were treated with DMSO (vehicle), 10 mM caffeine, 32 μM KU-55933, or 16 μM KU-60019 for 24 h. The cells were then treated with DMSO or 100 μM etoposide for 1 h. **(A)** Immunoblots. Cell lysates were prepared and subjected to immunoblot analysis using phospho-specific anti-γ-H2AX (top), anti-HA to detect GFP-HA-SfH2AX (middle), or anti-Tubulin (bottom). The results are representative of three independent experiments. **(B)** Quantitation. The intensities of the anti-γ-H2AX and anti-HA signals were quantified, and the ratio ±SD is reported relative to that obtained for the untreated cells (lane 1). n=3. *, $p<0.05$; **, $p<0.01$.

FIG. 2.2


A
+ etoposide
vehicle
vehicle
caffeine
KU-55933
KU-60019
anti-γ-H2AX
anti-HA
(H2AX)
anti-Tubulin
1
2
3
4
5
B
Normalized γ-H2AX/H2AX
0
1
2
3
4
5
6
7
8
*
*
**
**
vehicle
vehicle
caffeine
KU-55933
KU-60019
+ etoposide

SF21 cells, etoposide increased γ-H2AX levels when compared to vehicle-treated cells (Fig. 2.2A, lanes 1 and 2). In contrast, caffeine, KU-55933, and KU-60019 reduced the level of etoposide-induced γ-H2AX (Fig. 2.2A, lanes 3, 4, and 5). The immunoblot signal intensities were quantified and the ratio between γ-H2AX and H2AX compared (Fig. 2.2B). We concluded that caffeine and the ATM specific inhibitors KU-55933 and KU-60019 blocked DNA damage-induced ATM-mediated phosphorylation of H2AX at concentrations that maintained SF21 cell viability. Thus, ATM is sensitive to each of these inhibitors in SF21 cells.

2.5.2 AcMNPV induces DDR kinase substrate phosphorylation.

During infection, AcMNPV DNA replication triggers DDR activation [107, 181], as judged by host H2AX phosphorylation. Levels of γ-H2AX are highest in absence of AcMNPV replication factor LEF-7 [180], which functions to reduce or limit γ-H2AX levels early in infection. Because of ATM's initiator role in the DDR, we hypothesized that pharmacological inhibition of ATM would block these DDR-mediated phosphorylations during infection. To test this hypothesis, we infected control and ATM inhibitor-treated SF21 cells with AcMNPV, and assessed DDR kinase-mediated phosphorylations using an antibody specific for the S/TQ phosphorylation motif of ATM/ATR kinases. ATM and ATR preferentially phosphorylate serines and threonines which are immediately followed by a glutamine (pS/TQ), including that of H2AX [122]. S/TQ phosphorylation was monitored by immuno-chemical staining of fixed cells early in infection. Etoposide increased S/TQ phosphorylations relative to control cells (Fig. 2.3 and 2.4), confirming DNA damage-induced activation of the DDR. AcMNPV also increased phosphorylations at S/TQ motifs when compared to mock-infected cells. S/TQ phosphorylations were exclusively localized to the nucleus of AcMNPV-infected cells (Fig. 2.3), consistent with DDR activation. Moreover, these phosphorylations were distributed throughout the

nucleus and were not confined to viral DNA replication centers. Viral replication centers were marked by the accumulation of immediate early transactivator IE1 (Fig. 2.3), which is a origin of DNA replication-binding protein [120]. The presence of IE1 in S/TQ phosphorylated cells also confirmed that all cells were infected.

Caffeine treatment prior to infection reduced levels of S/TQ phosphorylation in infected cells as predicted (Fig. 2.3 and 2.4). Furthermore, caffeine eliminated the appearance of IE1-marked viral replications centers in the nucleus (Fig. 2.3). Thus, a caffeine-sensitive kinase is responsible for S/TQ phosphorylation during infection and contributes directly or indirectly to replication center formation. In contrast, neither of the ATM-specific inhibitors, KU-55933 or KU-60019, prevented AcMNPV-induced phosphorylation at S/TQ sites (Fig. 2.3 and 2.4). The KU-55933 and KU-60019 concentrations used here were sufficient to block etoposide-induced phosphorylation of H2AX (Fig. 2.2). These findings suggested that ATM isn't solely responsible for phosphorylation at S/TQ sites during AcMNPV infection, and that a caffeine-sensitive kinase other than or in addition to ATM phosphorylates S/TQ sites following AcMNPV infection.

2.5.3 Specific pharmacologic inhibition of ATM does not affect AcMNPV multiplication.

To determine the role of ATM in viral replication, we next treated SF21 cells with caffeine or the two ATM specific inhibitors (KU-55933, KU-60019) for 24 h, and measured yields of extracellular budded virus. Caffeine reduced the budded virus yields by more than 1,000-fold relative to the DMSO vehicle-treated control (Fig. 2.5), consistent with previous reports [107, 181]. In contrast, neither ATM-specific inhibitor reduced budded virus yields. We concluded that caffeine, which broadly inhibits PIKKs,

FIG. 2.3. Comparison of S/TQ motif phosphorylation in the presence of DDR kinase inhibitors. SF21 cells were untreated, or treated with DMSO (Vehicle), 10 mM caffeine, 32 µM KU-55933, or 16 µM KU-60019 for 24 h, and then infected with AcMNPV recombinant vAcIE1^{HA} (MOI: 3). Drugs were replaced following removal of the virus inoculum. Cells were fixed 6 h post infection, and stained with anti-pST/Q (green), anti-HA to detect IE1^{HA} (red), and Hoechst (blue). Cells were visualized via confocal microscopy; representative fields are shown from triplicate experiments. Yellow bars, 10 µm.

FIG. 2.3

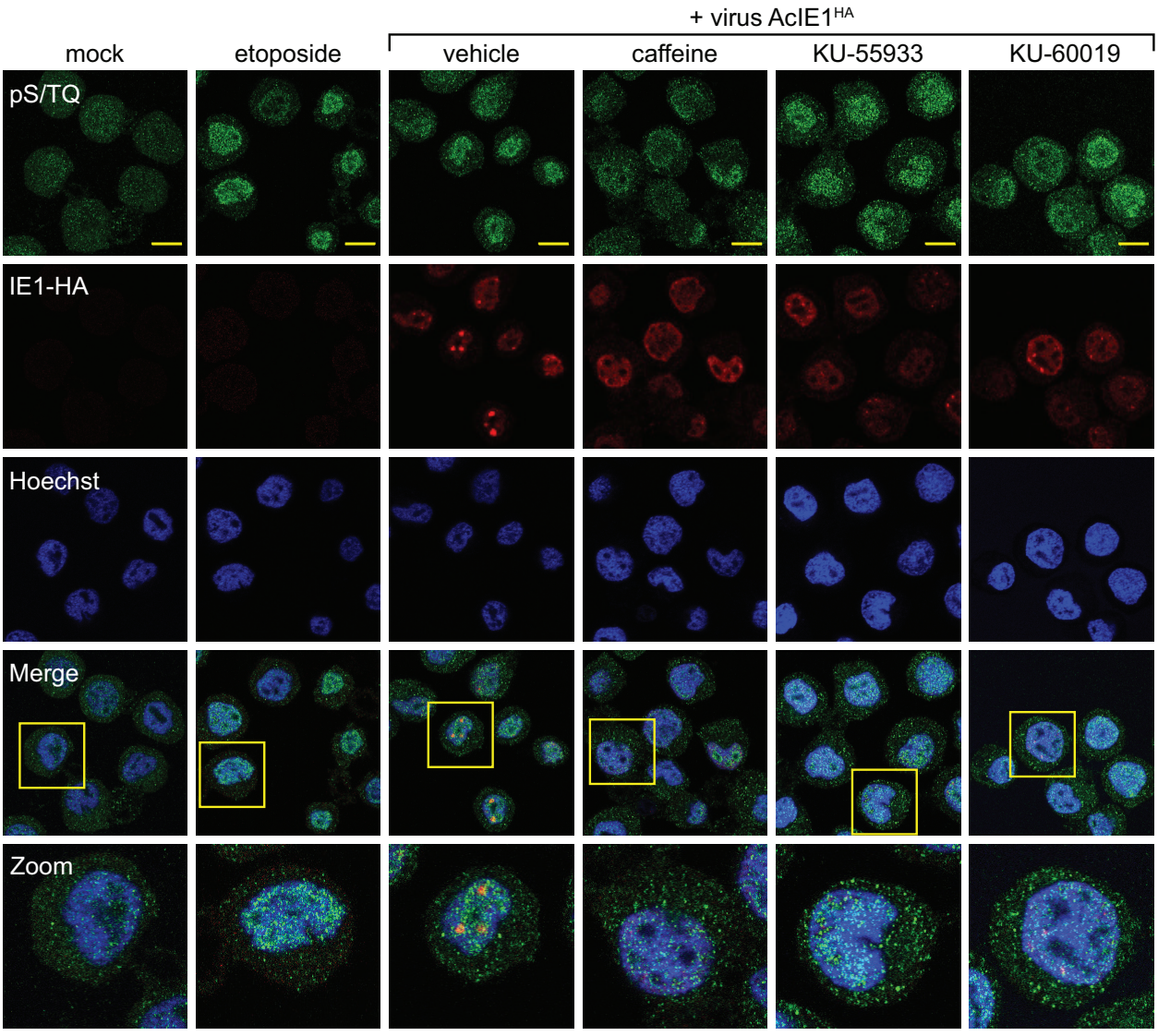
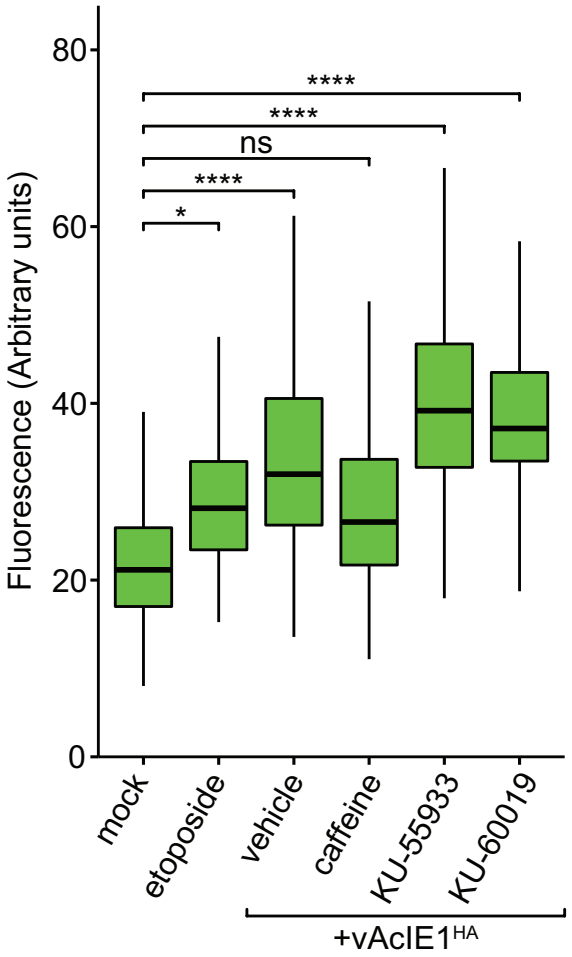

+ virus AcIE1HA
mock
etoposide
vehicle
caffeine
KU-55933
KU-60019
pS/TQ
IE1-HA
Hoechst
Merge
Zoom

FIG. 2.4. Quantitation of AcMNPV-induced S/TQ motif phosphorylation. Fields of immunostained cells, imaged as described in Fig. 2.3, were quantified using FIJI. Immunofluorescence values are reported as the mean nuclear intensity of the anti-pST/Q signal. Lower and upper hinges represent the first and third quartile, and the whiskers are 1.5 times the interquartile range. The number of cells examined for each condition were: Mock, 247; etoposide, 201; DMSO vehicle, 250; caffeine, 216; KU-55933, 250; KU-60019, 193. Statistical analysis was carried out using one-way ANOVA followed by Tukey's honest significant difference test. ns, $p>0.05$; *, $p<0.05$; ****, $p<0.0001$.

FIG. 2.4


80
60
40
20
0
Fluorescence (Arbitrary units)
****
****
ns
****
*
mock
etoposide
vehicle
caffeine
KU-55933
KU-60019
+vAcIE1HA

inhibits a target in AcMNPV-infected SF21 cells that is critical for AcMNPV multiplication. Moreover, caffeine's target(s) of inhibition is likely independent of the target(s) of either ATM inhibitor. These findings suggest that ATM function is not required for AcMNPV multiplication.

2.5.4 *Spodoptera* ATM (SfATM) mediates etoposide-induced H2AX phosphorylation.

We next sought an independent strategy to test the impact of ATM kinase activity on AcMNPV multiplication. To this end, we identified the *Spodoptera* ATM gene (*sfatm*) and generated SfATM knockout SF21 cells. Using sequence similarity with human ATM, we identified a candidate ATM sequence (GenBank: GFAB01000496.1) via a BLAST search of a sequenced SF21 cell transcriptome [116]. Conserved domain analysis [165] of the candidate ATM identified a conserved Telomere-length maintenance and DNA damage repair (TAN) motif, a FRAP, ATM and TRRAP (FAT) domain, an ATM kinase catalytic domain, and a FAT C-terminal (FATC) domain. These ATM domains are evolutionarily conserved (Fig. 2.6A). Importantly, the N-terminal TAN domain within ATM is exclusive to all ATM orthologs and is notably absent in other PIKKs, including the closely related PIKK ATR [236]. Alignment of several ATM orthologs, including that of *Homo sapiens, Saccharomyces cerevisiae, Drosophila melanogaster, Bombyx mori*, and the identified *S. frugiperda* ATM sequences using Clustal Ω [241] showed high conservation within the TAN domain (Fig. 2.6B). Taken together, we concluded that the *S. frugiperda* ATM ortholog SfATM was identified.

To initiate Cas9-mediated genome editing, SfATM was used to query *Spodoptera* whole genome shotgun sequences. We identified the *sfatm* genomic region (Genbank: JQCY02002655.1), which included 35 exons. We next generated two sgRNAs for

FIG. 2.5. AcMNPV multiplication in the presence of kinase inhibitor. SF21 cells were treated with DMSO (vehicle), 10 mM caffeine, 32 μM KU-55933, or 16 μM KU-60019 for 24 h and infected with AcMNPV (MOI 0.5). Extracellular budded virus was collected 72 h later, and quantified by using $TCID_{50}$. Yields are reported as average plaque forming units per ml ±SD (n = 3).

FIG. 2.5

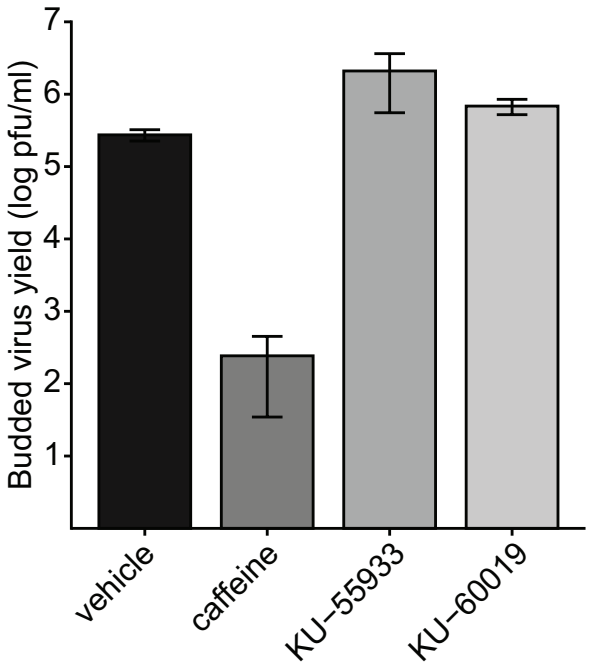
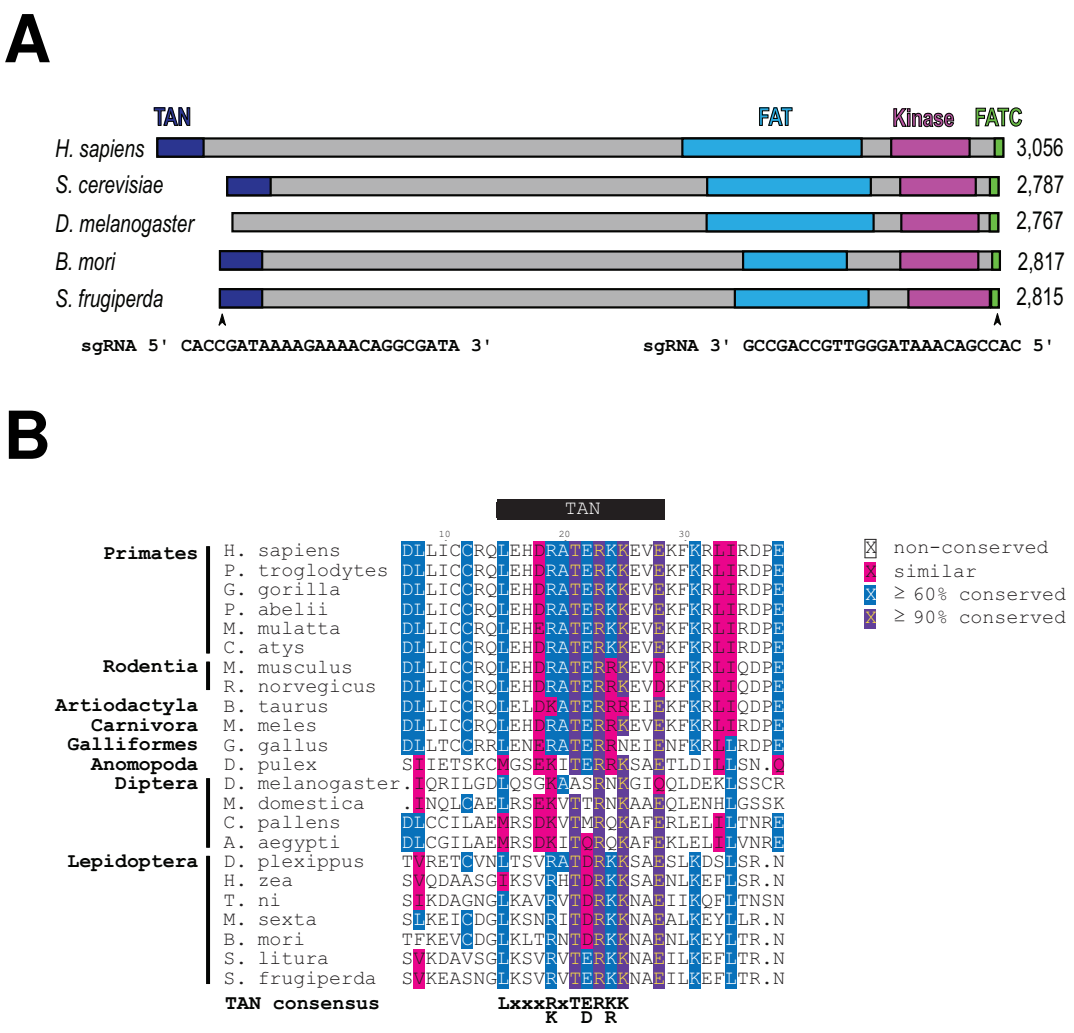

Budded virus yield (log pfu/ml)
7
6
5
4
3
2
1
vehicle
caffeine
KU−55933
KU−60019

FIG. 2.6. ATM orthologs. (A) Protein schematics. *H. sapiens* ATM (3,056 residues), *D. melanogaster* ATM (2,767 residues), *S. cerevisiae* ATM (2,787 residues), *Bombyx mori* ATM (2,817 residues), and *S. frugiperda* ATM (2,815 residues) are shown. Domains identified from NCBI's conserved domain search are indicated: Telomere-length maintenance and DNA damage repair (TAN, purple), FRAP, ATM, and TRRAP domain (FAT, cyan), catalytic domain of ATM kinase (Kinase, magenta), and FRAP, ATM, TRRAP C-terminal domain (FATC, green). The sgRNAs used to target Cas9 to the genomic location of SfATM are shown; arrows indicate approximate locations. **(B) TAN motif conservation.** The amino acid sequences of selected ATM orthologs were aligned using Clustal Ω [241] and the N-terminal region containing the TAN motif is shown. Numbering at top corresponds to the amino acid sequence of human ATM. Shading indicates conservation, with pink indicating similar residues, blue $>$60% conserved, and purple $>$90% conserved. Consensus for the TAN motif is shown at the bottom.

FIG. 2.6


A
TAN
FAT
Kinase
FATC
H. sapiens
S. cerevisiae
D. melanogaster
B. mori
S. frugiperda
3,056
2,787
2,767
2,817
2,815
sgRNA 5' CACCGATAAAAGAAAACAGGCGATA 3'
sgRNA 3' GCCGACCGTTGGGATAAACAGCCAC 5'
B
TAN
Primates
Rodentia
Artiodactyla
Carnivora
Galliformes
Anomopoda
Diptera
Lepidoptera
H. sapiens
P. troglodytes
G. gorilla
P. abelii
M. mulatta
C. atys
M. musculus
R. norvegicus
B. taurus
M. meles
G. gallus
D. pulex
D. melanogaster
M. domestica
C. pallens
A. aegypti
D. plexippus
H. zea
T. ni
M. sexta
B. mori
S. litura
S. frugiperda
TAN consensus
LxxxRxTERKK
K D R
non-conserved
similar
≥ 60% conserved
≥ 90% conserved

targeting Cas9 to *sfatm* using CRISPOR.org [56]; sgRNA #1 targeted the exon 1 region of *sfatm*, and sgRNA #2 targeted the exon 34 region, which contains the critical FATC domain (Fig. 2.6A). Following transfection of SF21 cells with the CRIPSR/Cas9 plasmids pIE1-Cas9-2A-GFP-U6 and pSfU6-neo, which expressed sgRNA #1 and sgRNA #2 respectively, DNA from single cell clones was isolated, screened by PCR, and appropriate portions of *sfatm* were sequenced. We generated multiple single cell clones that contained lesions at the target sites within *sfatm*.

Using two of our deletion clones, we tested for SfATM kinase function by screening for H2AX phosphorylation in response to etoposide-mediated DNA damage. In parental cells, etoposide induced accumulation of γ-H2AX relative to untreated cells (Fig. 2.7, lanes 1 and 2), as expected. In contrast, neither of two chosen CRISPR-Cas9-edited clones showed increased levels of γ-H2AX in response to etoposide (Fig. 2.7A, lanes 3 to 6). When quantified, both of the two knockout clones showed significantly reduced accumulations of γ-H2AX compared to parental SF21 cells (Fig. 2.7B). We concluded that the CRISPR-Cas9-edited cells are SfATM deficient and that SfATM is responsible for DDR-activated H2AX phosphorylation.

2.5.5 AcMNPV yields are unaffected in SfATM knockout cells.

To further investigate the potential role of ATM in AcMNPV multiplication, we compared the yields of AcMNPV budded virus from parental and SfATM knockout (KO) cells at intervals after infection. Wild-type AcMNPV multiplied in both SfATM KO cell clones to levels indistinguishable from those in parental SF21 cells (Fig. 2.8). Thus, SfATM is not required for AcMNPV multiplication. This finding indicated that SfATM does not account for the caffeine-induced inhibition of AcMNPV replication (Fig. 2.5). Consequently, we hypothesized that caffeine, but not ATM inhibitor KU-55933 or

FIG. 2.7. Comparison of H2AX phosphorylation in SfATM KO cell lines. SF21 cells, including the parental cell line, and two SfATM KO clones (KO #1 and #2) were transfected with a plasmid expressing GFP-HA-SfH2AX. After 48 h, cells were either treated with DMSO (-), or 100 μM etoposide (+) for 1 h. (A) Immunoblots. Cell lysates were prepared and subjected to immunoblot analysis using phospho-specific anti-γ-H2AX (top), anti-HA (middle), or anti-Tubulin (bottom). The results are representative of three independent experiments. (B) Quantitation. The intensities of the anti-γ-H2AX and anti-HA signal were quantified, and the normalized ratio is reported relative to that obtained for the untreated cells (lane 1). n=3. *, $p<0.05$; **, $p<0.01$.

FIG. 2.7

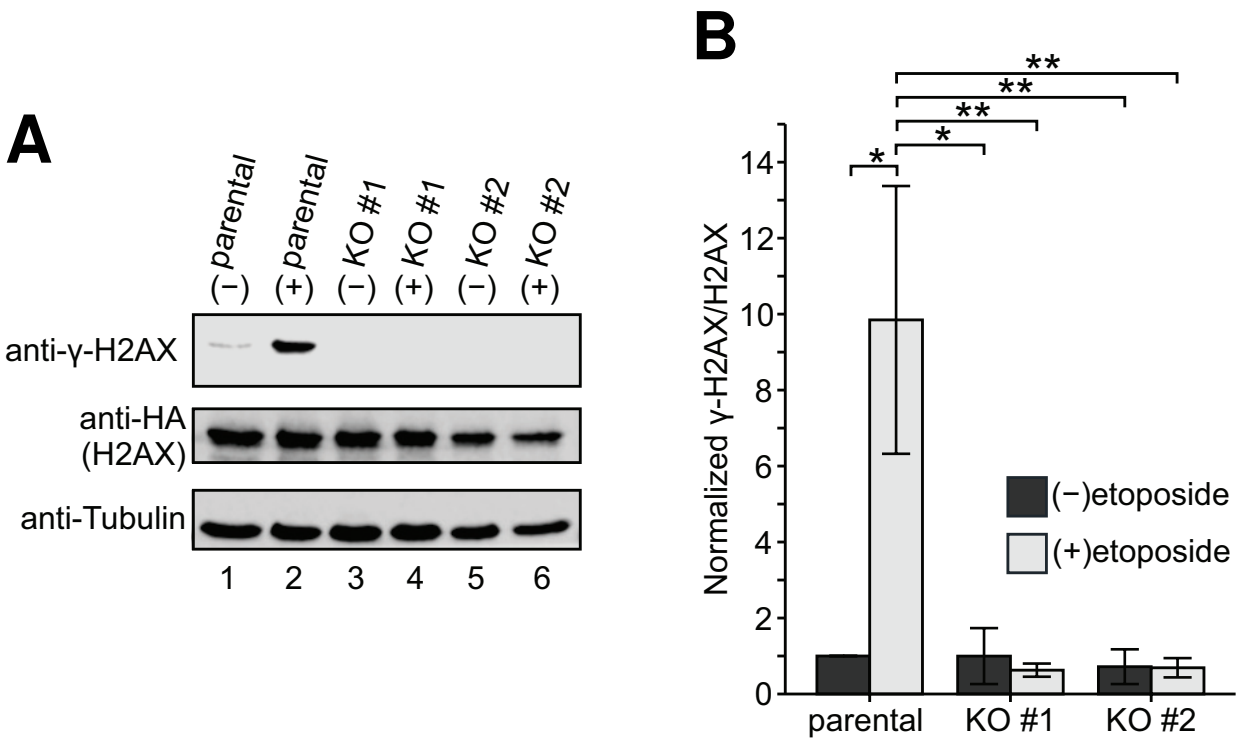
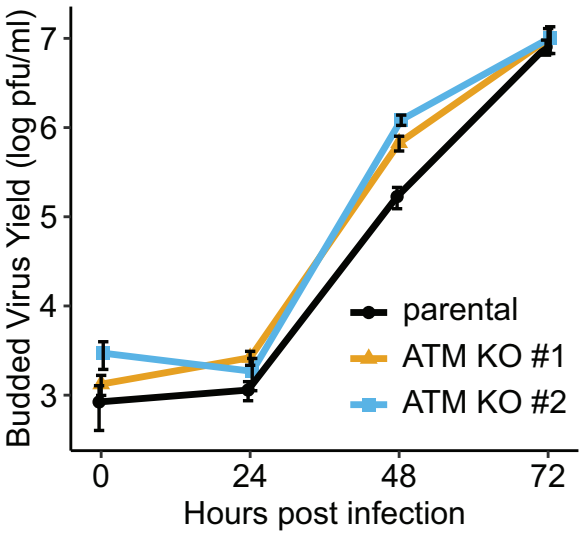

A
(−) parental
(+) parental
(−) KO #1
(+) KO #1
(−) KO #2
(+) KO #2
anti-γ-H2AX
anti-HA (H2AX)
anti-Tubulin
1 2 3 4 5 6
B
Normalized γ-H2AX/H2AX
0 2 4 6 8 10 12 14
*
*
**
**
**
(−)etoposide
(+)etoposide
parental
KO #1
KO #2

FIG. 2.8. Comparison of AcMNPV multiplication in SfATM KO cells. SF21 parental cells and two SfATM KO clones (ATM KO #1 and # 2) were infected with AcMNPV (MOI: 0.5). Extracellular budded virus was collected at the indicated times and quantified by using $TCID_{50}$. BV yields are reported as average pfu ±SD per ml. (n = 3).

FIG. 2.8


Budded Virus Yield (log pfu/ml)
7
6
5
4
3
0
24
48
72
Hours post infection
parental
ATM KO #1
ATM KO #2

KU-60019, would restrict productive AcMNPV multiplication in SfATM KO cells. To test this hypothesis, we first determined the relative toxicity of the ATM-specific inhibitors in SfATM KO cells since the absence of ATM may have affected cell sensitivity to each drug. Caffeine, KU-55933, and KU-60019 affected the viability of the SfATM KO cells (Fig. 2.9) at concentrations comparable to those for parental SF21 cells (Fig. 2.1). We next infected cells pretreated with caffeine, KU-55933, or KU-60019 with AcMNPV and measured budded virus yields. As expected, caffeine reduced yields of budded virus relative to vehicle control cells (Fig. 2.10). In contrast, neither KU-55933 nor KU-60019 affected virus yields from SfATM KO cells. (Fig. 2.10). We concluded that a caffeine-sensitive kinase other than SfATM is required for efficient AcMNPV multiplication.

2.6 Discussion

The host DDR plays an important role during infection by diverse viruses. The invertebrate DDR is activated upon initiation of viral DNA synthesis during baculovirus infection, including that by AcMNPV [107, 180, 181]. Whether the DDR positively or negatively impacts baculovirus multiplication, including virus DNA replication, is of interest because of the important role these viruses play as efficient and popular vectors for foreign gene expression. Our findings here that SfATM contributes to the baculovirus-induced host DDR but is not solely responsible for baculovirus replicative productivity provides insight into invertebrate virus-host interactions by suggesting that additional host PIKKs are involved.

Pharmacological inhibition of SfATM. In this study, we observed that early in infection AcMNPV dramatically increases the accumulation of phosphorylated PIKK substrates possessing S/TQ motifs, including *Spodoptera* histone H2AX (Fig. 2.2 and

FIG. 2.9. SfATM KO cell viability in the presence of DDR inhibitors. SfATM KO cells #1 and #2 were treated with caffeine, KU-55933, or KU-60019 at the indicated concentrations. Cell viability was determined via MTT assay 72 h later. Data is reported relative to values ±SD obtained from cells treated with vehicle control alone. (n = 3). Arrows indicate highest drug concentration that exerted limited effects on cell viability.

FIG. 2.9

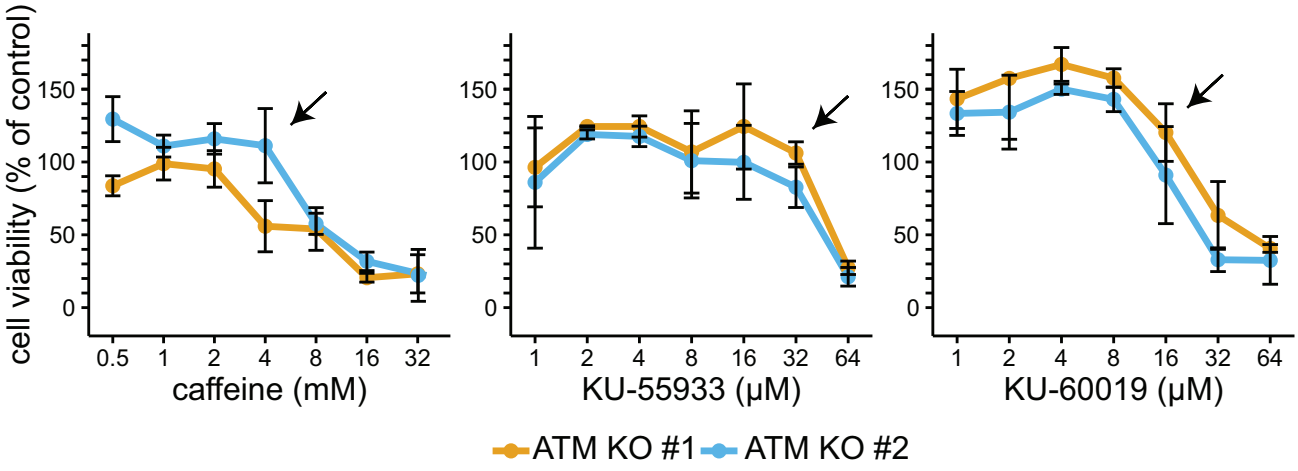
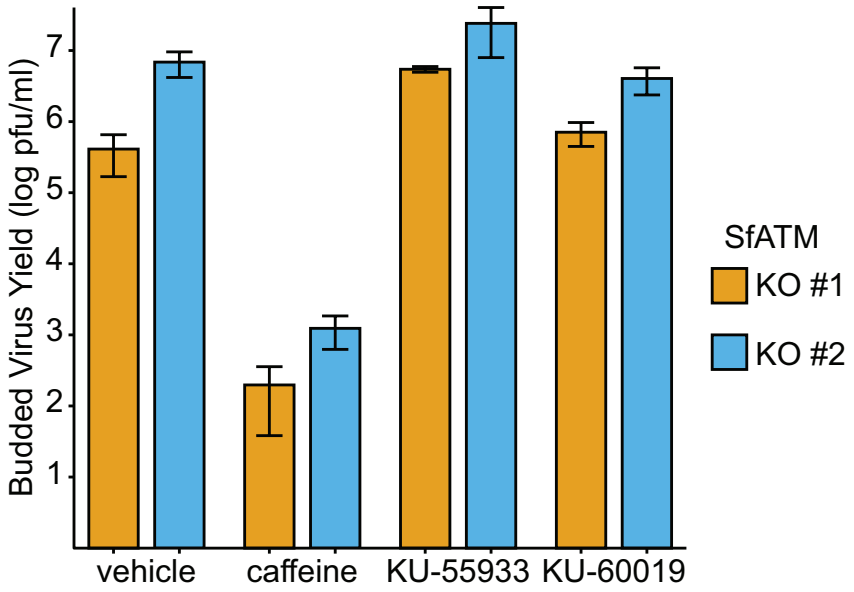

cell viability (% of control)
150
100
50
0
0.5
1
2
4
8
16
32
caffeine (mM)
1
2
4
8
16
32
64
KU-55933 (μM)
KU-60019 (μM)
ATM KO #1
ATM KO #2

FIG. 2.10. AcMNPV multiplication in SfATM KO cells in the presence of DDR inhibitors. Two SfATM KO clones (ATM KO #1 and KO # 2) were treated with DMSO (vehicle), 10 mM caffeine, 32 µM KU-55933, or 16 µM KU-60019 for 24 h, then infected with AcMNPV (MOI: 0.5). Extracellular budded virus was collected 72 h later and quantified using $TCID_{50}$. Budded virus yields are reported as average pfu ±SD per ml. (n = 3).

FIG. 2.10


Budded Virus Yield (log pfu/ml)
7
6
5
4
3
2
1
vehicle
caffeine
KU-55933
KU-60019
SfATM
KO #1
KO #2

2.3). Due to ATM's role as an apical DDR kinase and its contribution to the replication of multiple viruses [72, 113, 242], we focused on ATM functions during AcMNPV multiplication. Caffeine, a known PIKK inhibitor, reduced PIKK-mediated S/TQ motif phosphorylation and dramatically reduced AcMNPV BV yields, which confirmed previous findings [107, 181]. To our surprise, ATM-specific KU-55933 failed to reduce DDR phosphorylation or AcMNPV multiplication when used at nontoxic levels. Our viability assays (Fig. 2.1) suggested that the previously reported KU-55933 (50 μM) inhibition of AcMNPV [181] was possibly due to unknown off-target effects that compromised cell viability. Similarly, when used at nontoxic levels, ATM-specific KU-60019 reduced DDR phosphorylations but failed to affect AcMNPV yields (Fig. 2.3 and 2.5). Thus, the suggestion that ATM plays a central role in AcMNPV multiplication required further investigation.

***Spodoptera* SfATM is distinct from ATR.** To directly assess ATM's functions, we used CRISPR/Cas9-mediated genome editing of cultured SF21 cells, a model system for baculovirus studies. The availability of ATM knock-out cells obviated the dependence upon PIKK inhibitory drugs with their potential off-target effects. Using available data bases, we identified a *Spodoptera* ATM candidate (2,815 amino acid residues) with striking sequence similarity to ATM from humans, yeast, and other insects (Fig. 2.6). In particular, SfATM possesses multiple highly conserved ATM domains, including the kinase catalytic domain near the C terminus that functions in ATP binding and phosphate transfer to ST/Q motif-containing substrates. Importantly, SfATM possesses a TAN (Tel1/ATM N-terminal) motif (Fig. 2.6B), which distinguishes it from other PIKKs, including ATR, DNA-PK, or mTOR that lack a TAN motif [236]. Because the principal PIKKs share significant sequence similarity, it was necessary to differentiate SfATM from other *Spodoptera* PIKKs. Indeed, using a *Spodoptera* transcriptome data base, we identified a putative ATR with high sequence similarity to the human and *Drosophila* ATRs (data not shown). The *Spodoptera* ATR lacks a distinct TAN motif located near the

N-terminus of SfATM. Unlike ATR, ATM and DNA-PK are not essential genes (reviewed by [230]). Our confirmed SfATM knockout cell lines were viable. Finally, we demonstrated that ATM-specific DDR functions, including DNA damage-induced H2AX phosphorylation (Fig. 2.7), were absent in the SfATM knockout cells. Taken together, we concluded that SfATM is the *Spodoptera frugiperda* ATM ortholog. To our knowledge, this is the first report of a lepidopteran ATM.

AcMNPV multiplication in the absence of ATM function. Our SfATM deletions and pharmacological inhibition studies indicated that although SfATM is responsible for DNA damage-induced phosphorylation of SfH2AX, SfATM is not required for AcMNPV multiplication. Thus, in SF21 cells, ATM-generated γ-H2AX is not needed for AcMNPV replication, despite its contribution to replication of other DNA viruses [72, 113, 242]. Indeed, AcMNPV early protein LEF-7 reduces DDR-mediated phosphorylation of SfH2AX during viral DNA replication [180]. AcMNPV may therefore modulate the insect DDR to avoid ATM-mediated anti-viral effects or to subvert an arm of the host DDR to promote viral multiplication. In mammals, ATM is the central kinase contributing to DNA repair by homologous recombination; it contributes to multiple stages of the homologous recombination process [241]. During infection, ATM substrates involved in homologous recombination relocate to sites of viral replication, including Rad51, which traffics to replication centers of human papillomavirus (HPV), HSV-1, Epstein-Barr virus (EBV), and Simian virus 40 (SV40) [22, 85, 130, 277]. Like many large DNA viruses, the mechanisms of baculovirus DNA replication are poorly understood. However, it is likely that recombination-dependent DNA replication contributes to the generation of baculovirus genomic DNA [225]. Multiple virus-encoded factors that are either required for or stimulate AcMNPV DNA synthesis promote homologous recombination [59], indicating that virus DNA replication stimulates homologous recombination. Thus, our findings here raise the possibility that AcMNPV promotes homologous recombination by a mechanism independent of ATM.

Which PIKK contributes to baculovirus replication? Our findings indicate that AcMNPV replication events, including AcMNPV-induced phosphorylation of S/TQ motifs, is mediated by a caffeine-sensitive kinase other than SfATM. The most likely candidate is one of the other PIKK kinases, ATR or DNA-PK. Both enzymes phosphorylate S/TQ motif-containing substrates [20, 122] and are activated by other DNA viruses. ATR localizes to HSV-1 DNA replication centers and is required for efficient HSV-1 multiplication [72, 183]. HPV also activates the ATR pathway [222]. KSHV lytic reactivation causes DNA-PK activation [103] while Adeno-associated virus (AAV) also recruits DNA-PK to AAV replication centers when co-infected with adenovirus [235]. ATR is an DDR regulatory component that is essential for genome integrity and cell survival. It monitors replication stress, often detected as aberrant replication forks with exposed single-stranded DNA regions [17, 53, 230, 274]. ATR activation leads to phosphorylation of downstream substrates, including CHK1, which are involved in cell cycle arrest and slowing replication fork processing. Considering that baculoviruses use multiple origins of viral DNA replication to expedite an enormous production of viral DNA, often exceeding the host cell's own DNA content [225], it is anticipated that these prolific viruses have evolved mechanisms to handle this potentially catastrophic stress. Activation and manipulation of the host ATR pathway may be one such mechanism.

It is noteworthy that caffeine directly or indirectly inhibits phosphorylation of the AcMNPV immediate early 1 (IE1) transactivator [257], which contributes to virus DNA replication as an origin binding protein and stimulates recombination [59]. Phosphorylation of the N-terminal replication domain containing a consensus cyclin-dependent kinase site is required for IE1-mediated DNA replication events but not transcriptional activation. Thus, caffeine's inhibition of AcMNPV multiplication may also be due in part to its inhibition of a cellular or viral kinase responsible for IE-1 phosphorylation. Thus, an important question that remains to be answered is how the host DDR contributes to AcMNPV multiplication. Given that caffeine reduces the

synthesis of AcMNPV DNA during infection [181], caffeine-sensitive factors play an important but poorly understood role in viral DNA synthesis. Further study on the role of these host DDR factors in AcMNPV multiplication will provide mechanistic insight of insect virus DNA synthesis and likely uncover novel yet evolutionarily conserved host anti-viral responses.

Acknowledgments

We thank Dr. Kinjal Majumder (UW-Madison) for his advice on microscopy and providing access to his microscope. We thank David Taggart (Friesen lab) for the construction of AcMNPV recombinant vIE1^{HA}. We thank the members of the Kalejta laboratory for helpful comments. This work was supported by National Institutes of Health grants AI130089 and AI139180 to RFK. In addition, support was provided by National Institutes of Health grant AI40482 to PDF and the Office of the Vice Chancellor for Research and Graduate Education (UW-Madison).

Author Contributions

All experiments were conceived and designed by JRE, RFK, and PDF. JRE performed all of the experiments. JRE, RFK, and PDF analyzed the data and wrote the manuscript.

Chapter 3

Development of Site-Specific Gene Editing in *Spodoptera frugiperda* Cells

3.1 Abstract

The development of the CRISPR-Cas9 system has revolutionized genome editing. The CRISPR-Cas9 system can produce sequence specific cuts in the targeted genome loci and can disrupt the loci through non-homologous end joining (NHEJ) or potentially cause directed repair through homologous recombination (HR). However, a successful CRISPR-Cas9 genome editing system in a lepidopteran *Spodoptera* cell line had not been reported. In other cell lines, DNA polymerase III promoter-based DNA vectors are used to express small RNAs, including the single guide RNA (sgRNA) necessary for targeting of the Cas9 nuclease. Here I show that a *Spodoptera* U6 small nuclear RNA (snRNA) can successfully express small RNAs from a DNA vector, and this promoter can be used as part of a *Spodoptera* CRISPR-Cas9 gene editing strategy through generation of a *Spodoptera frugiperda* (*S. frugiperda*) SF21 H2AX knockout cell line.

3.2 Introduction

The CRISPR-Cas9 gene editing system is an important advancement in the ability of researchers to perform genome engineering in a targeted site-specific manner and perform genetic studies. CRISPR-Cas9 has been used to delete, replace, edit, or tag a gene in multiple cell lines and species, including insect cell lines (Reviewed in [68, 106, 260]). CRISPR-Cas9 is a genome engineering technology based upon the CRISPR-associated RNA-guided endonuclease Cas9, which in combination with a sgRNA can be targeted to virtually any genomic location [106]. CRISPR-Cas9 technology has enhanced investigations into virus-host interface, as CRISPR/Cas9 is a more accessible platform for cellular genome editing compared to previous systems including zinc-finger nucleases and TALENs.

At the beginning of my efforts, there were no reported methods for site specific targeted genome engineering in common insect cell lines used to study *Autographa californica* nucleopolyhedrovirus (AcMNPV) infection, including *S. frugiperda* Sf21 cells lines and *Trichoplusia ni* (*T. ni*) High Five cells. The limiting factor in developing these tools was the lack of an RNA polymerase III promoter that could express the sgRNAs necessary for directing Cas9 nucleases as part of the CRISPR-Cas9 system. Expression of sgRNAs require nuclear localization and a 5'-3' trimming function, both of which are provided by RNA polymerase III [61]. Efforts in other insect systems, including *Drosophila melanogaster* (*D. melanogaster*) and *Bombyx mori* (*B. mori*) have used endogenous U6 snRNA promoters for sgRNA expression [14, 151, 159]. However, unpublished reports suggested that neither *D. melanogaster* nor *B. mori* RNA polymerase III promoters would function well in *S. frugiperda* cells.

Here I show CRISPR-Cas9 technology can function in *S. frugiperda* SF21 cells to perform gene editing. My studies, together with a concurrent report [159], revealed the

U6 snRNA promoter effectively expresses the sgRNA necessary for guiding Cas9 endonuclease to specific locations within the *S. frugiperda* genome. Using CRISPR-Cas9 I specifically targeted the *sfh2ax* gene to generate a SF21 knockout cell line. More broadly, this technology will enable a wide range of investigations into baculovirus host cell interactions. Specifically, I will be utilizing the CRISPR-Cas9 tools for generating genetic knockouts of *S. frugiperda* DNA damage response (DDR) components to further understanding of the role of the DDR in virus infection.

3.3 Material & Methods

Cells and Infections. *Spodoptera frugiperda* IPLB-SF21 (SF21) cells [266] were maintained at 27°C in TC100 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone) Wild-type AcMNPV (L-1 strain) [134] was previously described. In all infections, virus inoculum was allowed to adsorb for 1 h at room temp with rocking. The inoculum was then replaced with fresh medium at time zero. Extracellular budded virus (BV) was quantified by $TCID_{50}$ assays as previously described [197].

Plasmids and Transfections. All plasmids were constructed using a pBluescript KS+ (Invitrogen) backbone containing an ampicillin resistance cassette. Primers used in plasmid construction are given in 3.1. Sequences were amplified via PCR using Q5 high-fidelity polymerase (New England Biolabs). PCR products were gel purified using a gel extraction and PCR cleanup kit (IBI Scientific). PCR products were cloned into plasmid backbones using NEBuilder Hifi (New England Biolabs). Constructs were cloned into Stellar competent cells (Takara Bio). Plasmid maxi-prep was performed using Maxi Fast-Ion kits (IBI Scientific). Transfections were carried out using *Trans*IT-Insect (Mirus Bio) with 2 µl of *Trans*IT-Insect per µg of plasmid DNA. Transfections were carried out in 6 well plates using 10 µg of plasmid DNA per well containing $1x10^6$ SF21 cells. Drug

selection was performed in TC100 medium containing G418 (500 µg/ml) (Gibco) for 7 days.

Fluorescence assays. Fluorescence assays were performed using a Cytation 5 multi-mode plate reader (BioTek). Reads were made in 6 well plates using a 13x13 matrix with a 485 nm excitation and a 516 nm emission for GFP. Values reported represent the average fluorescence measured in arbitrary units for all points in each well.

qPCR. For RNA analysis total RNA was isolated using an RNA isolation kit (IBI Scientific) and treated with DNAse. 250 ng of RNA was converted to complementary DNA (cDNA) using Maxima H minus cDNA Synthesis Master Mix (ThermoFisher). cDNA was amplified using iTaq Universal SYBR Green Supermix (BioRad). Transcripts were compared to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts and are reported as relative fold change calculated using the $2^{-\Delta\Delta Ct}$ method.

PCR screens. For screening SF21 cells were harvested and the DNA was purified using a genomic DNA mini kit (IBI Scientific). The DNA was diluted to 100 µg/mL and 1 µl was used as a template for a PCR reaction using GoTaq polymerase (Promega) and sfH2AX ontarget FWD and sfH2AX ontarget REV primers (Fig. 3.1). Samples were run on an 0.7% agarose gel at 100V for 15 minutes and then imaged on a LI-COR Odyssey FC (LI-COR). DNA from parental cells and DNA from pooled sfH2AX knockouts were used as controls.

FIG. 3.1. Primers used. List of primers used for cloning the sfU6 sequence, for shRNA knockdowns, and for generating and screening sfH2AX KO cells.

FIG. 3.1

Primer	Sequence
sfU6 (+400) FWD	AGCCCGGGGGATCCACTAGTAGACCTTCCTCCTTTTTTG
sfU6 (-202) REV	TATAGGGCGAATTGGAGCTCTAAATTGCATACAAATAAACAATCG
EGFP shRNA FWD	GTGTGCTGTCCTTGTGGCTGTTGTAGTTGTACTTTTTTGAAGAGTTTCAGTTTGGTATGG
EGFP shRNA REV	AAGGACAGCACACTTGTGACTATTATAGTTATACACTTGCCAAGGCAAAATTATTGTACC
sfATM(701) shRNA FWD	CCTGTCGTGTGACATGGTCAACCGATACTCGCTTTTTTGAAGAGTTTCAGTTTGGTATGG
sfATM(701) shRNA REV	TGTCACACGACAGGACATGGTCAACCGATACTCGCACTTGCCAAGGCAAAATTATTGTAC
sfChk2(882) shRNA FWD	GTGTGCTGTCCAGATCTCTGTGTGTAATACCCTTTTTTGAAGAGTTTCAGTTTGGTATGG
sfChk2(882) shRNA REV	TCTGGACAGCACACAGATCTCTGTGTGTAATACCCACTTGCCAAGGCAAAATTATTGTAC
sfUBR5(4278) shRNA FWD	GTGTGCTGTCCGTATGGAACTCAGATGGCTGCTTTTTTGAAGAGTTTCAGTTTGGTATGG
sfUBR5(4278) shRNA REV	TACGGACAGCACACGTATGGAACTCAGATGGCTGCACTTGCCAAGGCAAAATTATTGTAC
sfU6 sgRNA scaffold FWD	TCGTGCTCGACCACCGAAACACCGGGCTTCGAG
sfU6 sgRNA scaffold FWD	AAACTCTTCAAAAAAGCACCGACTCGGTGC
sfH2AX sgRNA sense A	CACCGTGGAGGTCGGCATATTTAA
sfH2AX sgRNA anti-sense A	AAACTTAAATATGCCGACCTCCAC
sfH2AX sgRNA sense B	CACCGCTTTGCAGAAACTGTGATTA
sfH2AX sgRNA anti-sense B	AAACTAATCACAGTTTCTGCAAAGC
sfH2AX ontarget FWD	TTCGATATCAAGCTTGAACGCCAATTTTCCCGCTA
sfH2AX ontarget REV	CCCCTCGAGGTCGACGTGAAGCAACCGGCTCCACC

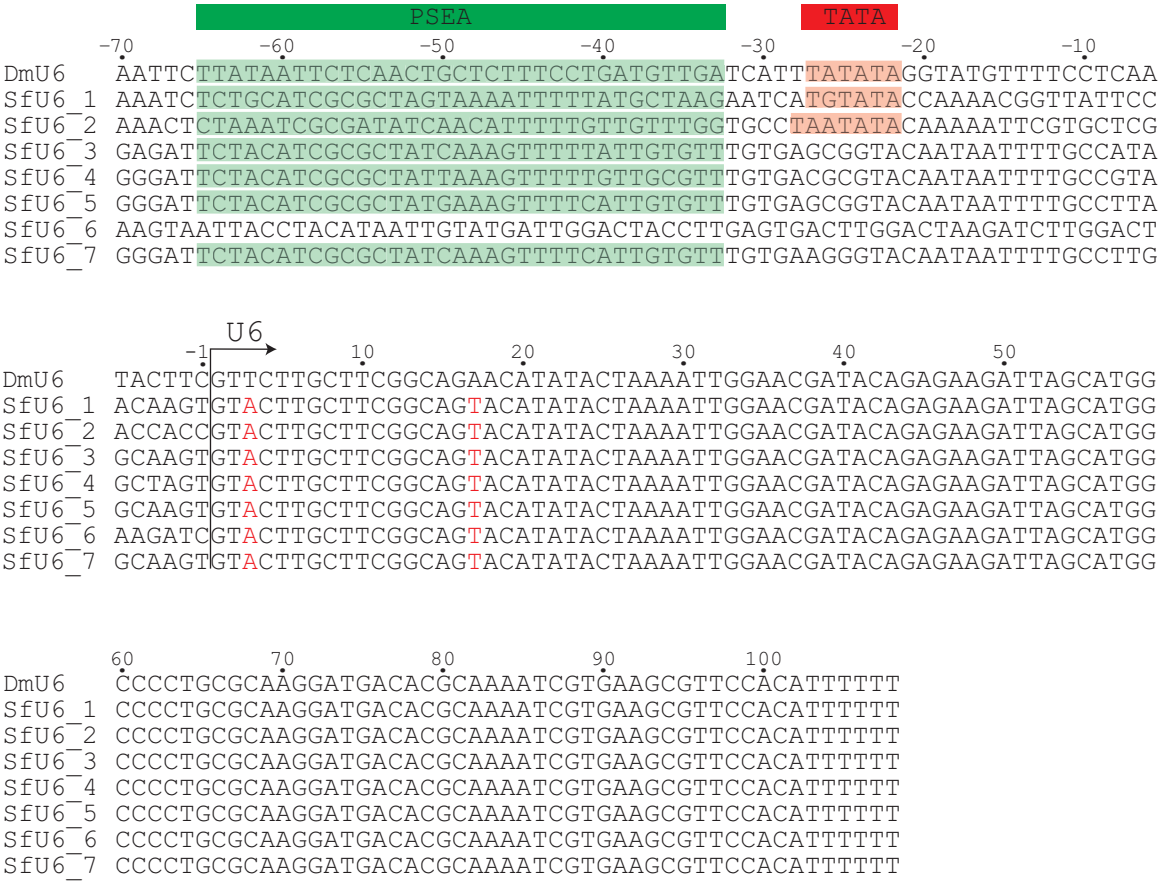
3.4 Results

3.4.1 Identification of *Spodoptera frugiperda* putative U6 promoters and snRNA sequence.

The ability to utilize CRISPR-Cas9 in *S. frugiperda* SF21 cells was limited by the inability to express the sgRNA necessary for specific targeting of the CRISPR associated protein 9 (Cas9) endonuclease. The U6 snRNA gene promoter of *D. melanogaster* and *B. mori* has been used to direct RNA polymerase III expression of small RNAs in the respective species ([200, 268]. The U6 snRNA promoter has also successfully been used to express sgRNA for clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing in *D. melanogaster* [91]. While these appear to be attractive candidates for use in CRISPR/Cas9 gene editing in SF21 cells, unpublished data suggested that these wouldn't function well as heterologous promoters in *S. frugiperda*. Thus, I hypothesized that the *S. frugiperda* U6 promoter would express small RNAs in SF21 cells. Using the DmU6 snRNA sequence (nucleotides 96-202, GENBANK, accession number M24605) (Fig. 3.2 DmU6) I BLAST queried *S. frugiperda* whole genome shotgun contigs. This search identified 7 putative *S. frugiperda* U6 snRNA sequences (Fig. 3.2. While the U1-U5 snRNA genes are expressed by RNA polymerase II, the U6 snRNA gene is transcribed by RNA polymerase III and the U6 promoter contains both a proximal sequence element A (PSEA) and a TATA box. Several of these sequences may be copies of true U6 genes and promoters, however it is also possible that these may be pseudogenes with non-functional promoters. While several of the putative U6 upstream promoters regions contained a PSEA sequence, only SfU6_2 contained both the PSEA and a TATA box region (Fig. 3.2. Thus I selected SfU6_2 promoter as a putative *S. frugiperda* RNA polymerase III promoter.

FIG. 3.2. Identification of the *S. frugiperda* U6 promoter. Multiple sequence alignment of *D. melanogaster* U6 promoter region and snRNA, and 7 putative *S. frugiperda* U6 genes. The PSEA sequences are highlighted in green, and TATA box is highlighted in red. The U6 snRNA transcription start site is indicated by the arrow. Mismatched bases between *D. melanogaster* and *S. frugiperda* U6 snRNA are shown in red. The numbering of the putative *S. frugiperda* U6 genes is arbitrary.

FIG. 3.2


PSEA
TATA
−70 −60 −50 −40 −30 −20 −10
DmU6 AATTCTTATAATTCTCAACTGCTCTTTCCTGATGTTGATCATTTATATAGGTATGTTTTCCTCAA
SfU6_1 AAATCTCTGCATCGCGCTAGTAAAATTTTTATGCTAAGAATCATGTATACCAAAACGGTTATTCC
SfU6_2 AAACTCTAAATCGCGATATCAACATTTTTGTTGTTTGGTGCCTAATATACAAAAATTCGTGCTCG
SfU6_3 GAGATTCTACATCGCGCTATCAAAGTTTTTATTGTGTTTGTGAGCGGTACAATAATTTTGCCATA
SfU6_4 GGGATTCTACATCGCGCTATTAAAGTTTTTGTTGCGTTTGTGACGCGTACAATAATTTTGCCGTA
SfU6_5 GGGATTCTACATCGCGCTATGAAAGTTTTCATTGTGTTTGTGAGCGGTACAATAATTTTGCCTTA
SfU6_6 AAGTAATTACCTACATAATTGTATGATTGGACTACCTTGAGTGACTTGGACTAAGATCTTGGACT
SfU6_7 GGGATTCTACATCGCGCTATCAAAGTTTTCATTGTGTTTGTGAAGGGTACAATAATTTTGCCTTG
U6
−1 10 20 30 40 50
DmU6 TACTTCGTTCTTGCTTCGGCAGAACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGG
SfU6_1 ACAAGTGTACTTGCTTCGGCAGTACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGG
SfU6_2 ACCACCGTACTTGCTTCGGCAGTACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGG
SfU6_3 GCAAGTGTACTTGCTTCGGCAGTACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGG
SfU6_4 GCTAGTGTACTTGCTTCGGCAGTACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGG
SfU6_5 GCAAGTGTACTTGCTTCGGCAGTACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGG
SfU6_6 AAGATCGTACTTGCTTCGGCAGTACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGG
SfU6_7 GCAAGTGTACTTGCTTCGGCAGTACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGG
60 70 80 90 100
DmU6 CCCCTGCGCAAGGATGACACGCAAAATCGTGAAGCGTTCCACATTTTTT
SfU6_1 CCCCTGCGCAAGGATGACACGCAAAATCGTGAAGCGTTCCACATTTTTT
SfU6_2 CCCCTGCGCAAGGATGACACGCAAAATCGTGAAGCGTTCCACATTTTTT
SfU6_3 CCCCTGCGCAAGGATGACACGCAAAATCGTGAAGCGTTCCACATTTTTT
SfU6_4 CCCCTGCGCAAGGATGACACGCAAAATCGTGAAGCGTTCCACATTTTTT
SfU6_5 CCCCTGCGCAAGGATGACACGCAAAATCGTGAAGCGTTCCACATTTTTT
SfU6_6 CCCCTGCGCAAGGATGACACGCAAAATCGTGAAGCGTTCCACATTTTTT
SfU6_7 CCCCTGCGCAAGGATGACACGCAAAATCGTGAAGCGTTCCACATTTTTT

3.4.2 The *sfU6* promoter supports expression of shRNA.

In order to test the ability of the SfU6_2 promoter to express RNA in SF21 cells, I constructed a short hairpin RNAs (shRNAs) expression vector using the SfU6_2 promoter region (Fig/ 3.3A). Since the U6 promoter and terminator sequences are not defined in *S. frugiperda*, this vector contained 400 bp upstream of the *S. frugiperda* U6 snRNA transcription start site, and the 100 bp downstream of the snRNA gene. To test the ability of the SfU6_2 promoter region to express RNA, I inserted a shRNA specific for the EGFP coding sequence that has been previously shown to function to knockdown EGFP in insects [268]. This vector and a vector containing an irrelevant control shRNA were each transfected into SF21 cells expressing EGFP. At 48 h post transfection, the fluorescence of the cells was measured. While the cells transfected with the control shRNA vector showed bright fluorescence, the cells transfected with the SfU6_2 EGFP shRNA vector had significantly reduced fluorescence (Fig. 3.3B). From this observation I concluded that the EGFP shRNA expressed from the sfU6_2 promoter was efficiently knocking down the EGFP transcripts in SF21 cells.

While the sfU6_2 promoter was able to reduce fluorescence in SF21 cells, I also wanted to examine if the sfU6_2 promoter was able to express shRNAs and knockdown cellular transcripts. To identify transcripts of interest, I performed BLAST searches of *S. frugiperda* transcribed RNA sequence libraries using known ataxia-telangiectasia mutated (ATM), checkpoint kinase 2 (Chk2), and ubiquitin protein ligase E3 component n-recognin 5 (UBR5) sequences from other organisms. I identified transcript sequences for *S. frugiperda* ATM (Accession: GFAB01000496), Chk2 (Accession: GCTM01021998), and UBR5 (Accession: GESP01131696). Using these transcript sequences I designed shRNAs with the Thermo Fisher BLOCK-iT^{TM} RNAi designer (https://rnaidesigner.thermofisher.com/rnaiexpress/insert.do). These shRNAs were

cloned into the pU6-shRNA expression vector (Fig. 3.3A). The shRNA vectors, and control shRNA vectors, were transfected into SF21 cells. At 48 hours following transfection RNA was harvested from the cells. The RNA was analyzed via reverse transcription quantitative real-time PCR (RT-qPCR) and reported as the relative fold change between the transcript of interest and transcripts of GAPDH housekeeping transcripts. While the ATM specific shRNA didn't show a significant drop in transcripts relative to control, both the Chk2 specific shRNA and UBR5 specific shRNA significantly reduced the Chk2 and UBR5 transcript levels respectively (Fig. 3.3C). Thus, the sfU6_2 promoter efficiently expressed both the Chk2 and UBR5 specific shRNAs. It is likely that the ATM shRNA was also well expressed, however the specific shRNA didn't cause significant RNAi mediated reduction in ATM transcripts. Additional optimization of shRNA for ATM would likely result in a reduction of ATM transcripts.

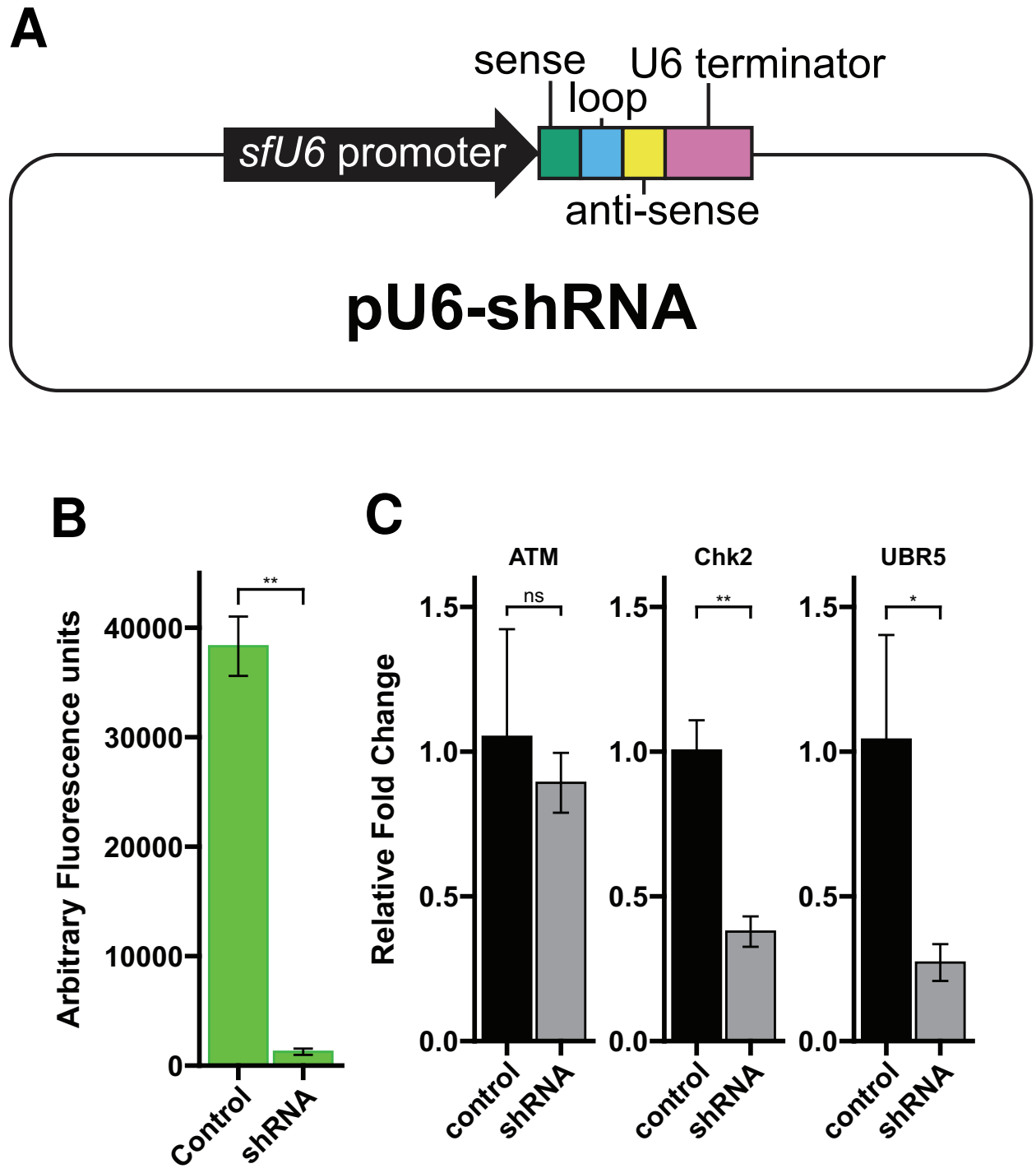
3.4.3 The *sfU6* promoter allows for successful CRISPR-Cas9 gene editing in SF21 cells

I next wanted to test if the sfU6_2 promoter was able to drive sgRNA expression in support of CRISPR-Cas9 editing in SF21 cells. I used the sfU6_2 promoter to construct the generic sgRNA expression vector pU6-sgRNA-neo (Fig. 3.4A). This vector also contained a neomycin resistance gene under control of a *hr5* enhancer and an *ie1* promoter to allow for drug selection of transfected cells. Additionally, I constructed a generic Cas9 and sgRNA expression vector pIE1-Cas9-2A-GFP-U6-sgRNA (Fig. 3.4B). This vector contained Cas9 and GFP, separated by a 2A self-cleaving peptide, along with an sgRNA expression site under control of the sfU6_2 promoter.

To test the ability of these vectors to carry out gene editing in SF21 cells, I targeted the *S. frugiperda* ortholog of the variant histone variant histone H2AX (H2AX) (sfH2AX)

FIG. 3.3. The *S. frugiperda* U6 promoter expresses shRNA (A) Diagram of a generic pU6-shRNA vector containing a 21 bp sense sequence that corresponds to the target in the transcript, an 11 bp loop, and a 21 bp anti-sense sequence under control of the *S. frugiperda* SfU6_2 promoter (Fig. 3.2) and 100 bp terminator sequence. (B) SF21 cells expressing EGFP were transfected with a pU6-shRNA expression vector expressing shRNA against EGFP or a control shRNA. Fluorescence intensity was measured 48 h following transfection. n=3. Statistical analysis was performed using a t-test. **, $p<0.01$. (C) SF21 cells were transfected with a pU6-shRNA expression vector expressing shRNA against *S. frugiperda* ATM, *S. frugiperda* Chk2, *S. frugiperda* UBR5, or a control shRNA. At 48 h following transfection mRNA was isolated from cells and analyzed via RT-qPCR. n=3. Statistical analysis was performed using two-way ANOVA followed by a t-test with Bonferroni correction. ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$.

FIG. 3.3


A
sense
loop
U6 terminator
sfU6 promoter
anti-sense
pU6-shRNA
B
Arbitrary Fluorescence units
40000
30000
20000
10000
0
**
Control
shRNA
C
Relative Fold Change
ATM
Chk2
UBR5
ns
**
*
1.5
1.0
0.5
0.0
control
shRNA

in SF21 cells. To determine the genomic locus of sfH2AX, I used the coding sequence of sfH2AX [181] to BLAST search *S. frugiperda* whole genome shotgun contigs. I identified a genomic sfH2AX locus (accession: JQCY02002352) containing the sfH2AX coding sequence in 3 exons (Fig. 3.4C). I then used the sfH2AX locus sequence to identify potential sgRNA target sites within the locus using CRISPOR (crispor.tefor.net) [56]. Two sgRNAs were selected, one which targeted near the start of the coding region in exon 1, and a second that targeted near the end of exon 3 (Fig. 3.4C sgRNA #1 & sgRNA #2). The goal was to have the Cas9 nuclease cut at both sgRNA, and have DNA repair occur with a loss of the region between the two sgRNA, eliminating most of the sfH2AX coding region. To perform the gene editing of sfH2AX, sgRNA #1 and sgRNA #2 (Fig. 3.4C) were cloned into pIE1-Cas9-2A-GFP-U6-sgRNA (Fig. 3.4B) and pU6-sgRNA-neo (Fig. 3.4A) respectively. These constructs were used to co-transfect SF21 cells. The transfected cells were placed under G418 selection, followed by single cell dilution and scoring for GFP expression. Single-cell clones (57 in total) were screened using a PCR assay for a deletion of sfH2AX using primers just outside the coding region (Fig. 3.4C). An example of the PCR assay is shown in Fig. 3.4D. Two clones were identified as lacking full length sfH2X. I concluded that the sfU6_2 promoter was able to express sgRNAs in support of CRIPR-Cas9 genome editing in SF21 cells.

3.4.4 AcMNPV yields are indistinguishable in parental and SfH2AX knockout (KO) cells.

H2AX has previously been implicated in AcMNPV infection, as the viral early protein LEF-7 has been shown to block phosphorylation of H2AX during infection [180]. I hypothesized that SfH2AX wasn't required for AcMNPV multiplication, and that wild-type (WT) AcMNPV would replicate well in SfH2AX KO cells. To investigate the potential role of H2AX in AcMNPV multiplication, I compared the yields of AcMNPV budded virus from

parental and two clonal SfH2AX KO cell lines at various times post infection. At all time points WT AcMNPV multiplied in SfH2AX cells to levels indistinguishable from those in parental SF21 cells (Fig. 3.5). From this I concluded that SfH2AX is not required for AcMNPV multiplication, and that CRISPR-Cas9 edited SF21 cells can successfully be infected with AcMNPV.

3.5 Discussion

My results show that it is now possible to efficiently generate modifications of the *S. frugiperda* genome using CRISPR-Cas9 technology. Prior to this effort, no promoter had been shown to effectively express small RNAs in SF21 cells. The identification of a U6 snRNA RNA polymerase III promoter allows for the expression of heterologous small RNAs in SF21 cells, including shRNAs and sgRNAs. I showed how a Cas9 endonuclease in combination with a sgRNA expressed from the *S. frugiperda* U6 snRNA promoter could be used to make site specific genetic deletions in SF21 cells. Importantly, this same system could be used to make more sophisticated genomic edits, including point mutations and the insertions of epitope tags to *S. frugiperda* proteins. The ability to tag *S. frugiperda* proteins creates many possibilities for future investigations of viral infection using the SF21 cell baculovirus system, as a lack of specific antibodies for *S. frugiperda* proteins has limited investigations.

Tool development with *Spodoptera* U6 promoter This study serves as a proof of concept for further investigations using CRISPR-Cas9 editing in SF21 cells. I have shown that a combination of insect and baculovirus promoters can be used to express critical CRISPR-Cas9 components in *S. frugiperda* cells. My identification of the sfU6_2 promoter and its ability to express small RNAs was critical to this effort. The construction of CRISPR-Cas9 vectors using the sfU6_2 promoter will facilitate further investigations

FIG. 3.4. The *S. frugiperda* U6 promoter supports sgRNA expression for CRISPR-Cas9 editing (A) Diagram of a generic pU6-sgRNA expression vector containing an sgRNA and a Cas9 binding scaffold under control of the *S. frugiperda* sfU6_2 promoter (Fig. 3.2) and 100 bp terminator sequence, and a neomycin resistance gene under control of a baculovirus *hr5* enhancer and *ie1* promoter. (B) Diagram of a generic Cas9-sgRNA expression vector containing an sgRNA and a Cas9 binding scaffold under control of the *S. frugiperda* sfU6_2 promoter (Fig. 3.2) and 100 bp terminator sequence, and a Cas9-2A-GFP under control of a baculovirus *hr5* enhancer and *ie1* promoter. (C) Diagram of the *S. frugiperda* H2AX ortholog (sfH2AX) genomic locus, containing 3 exons. Approximate location of the sgRNA and primers used in KO screenings are shown. (D) Representative PCR assay results on parental SF21, pooled H2AX KO cells (KO Pool), and five clonal H2AX KO cells (Clone A-E) using primer 1 and 2 shown in Fig. 3.4C. The band at 1000 bp represents full length, or nearly full length, sfH2AX locus. The band at 250 bp corresponds to the expected size if DNA repair occurred between the sgRNA #1 and sgRNA #2 sites shown in 3.4C, resulting in loss of the majority of the sfH2AX coding region.

FIG. 3.4

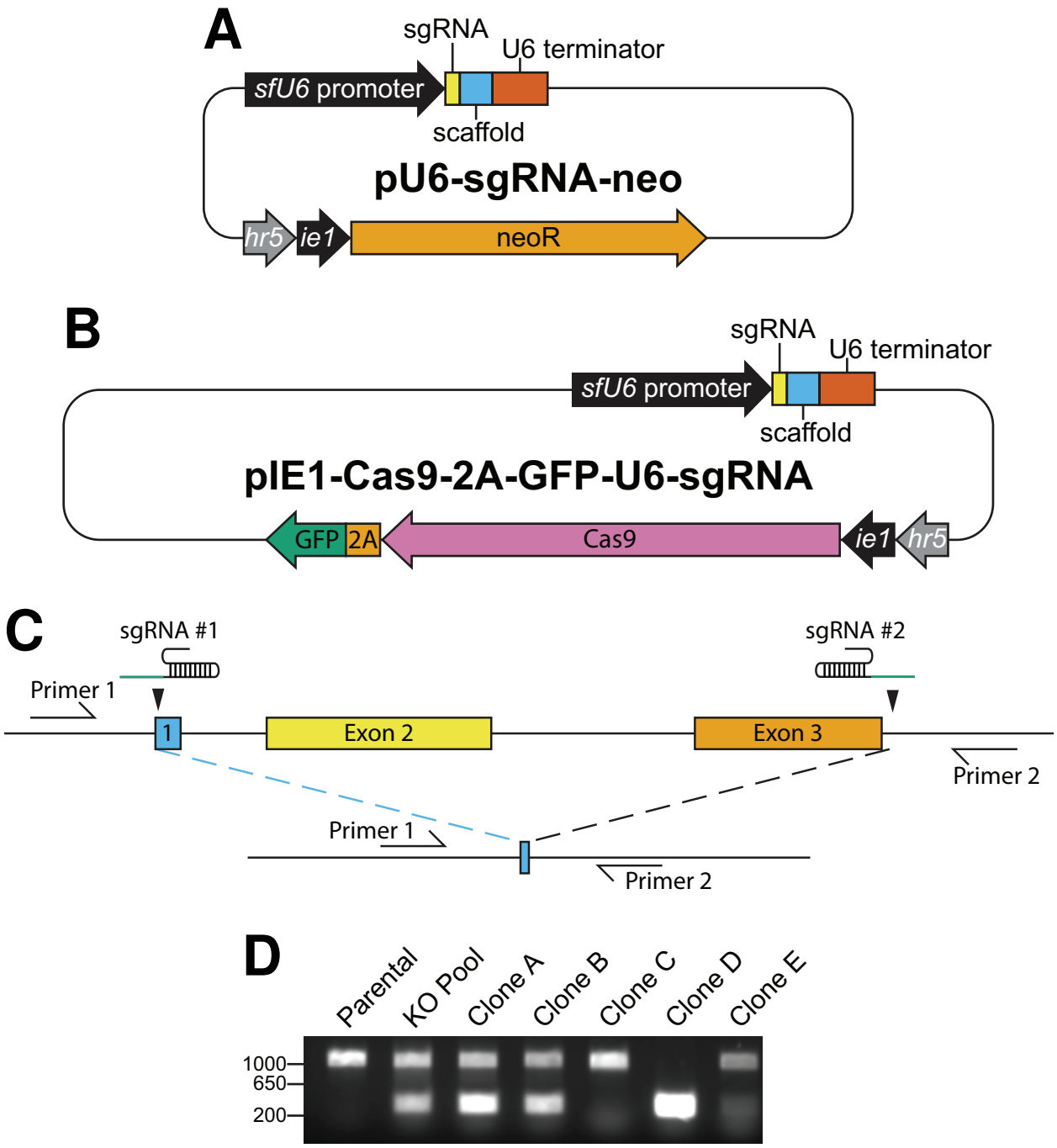
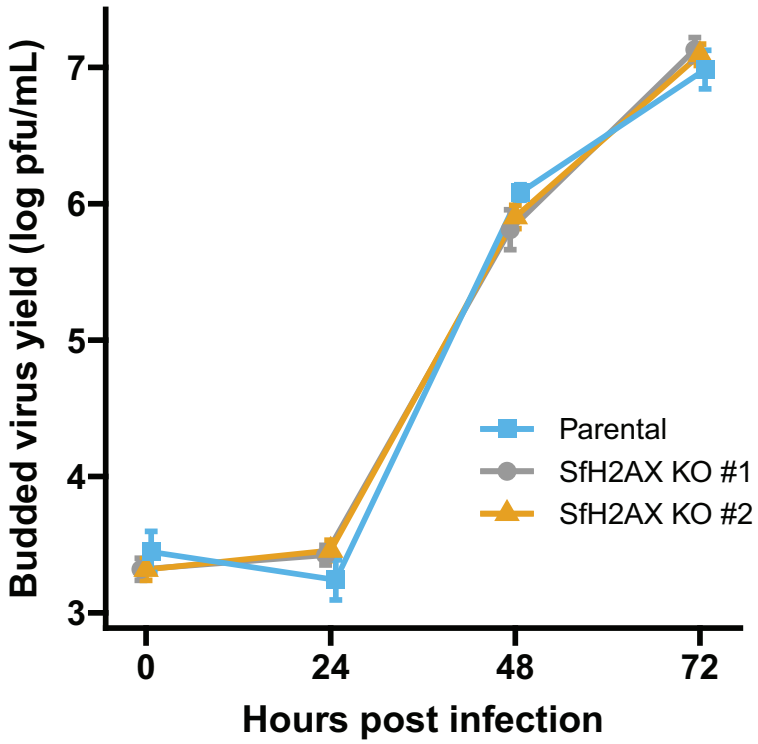

A
sgRNA
U6 terminator
sfU6 promoter
scaffold
pU6-sgRNA-neo
hr5
ie1
neoR
B
sgRNA
U6 terminator
sfU6 promoter
scaffold
pIE1-Cas9-2A-GFP-U6-sgRNA
GFP
2A
Cas9
ie1
hr5
C
sgRNA #1
sgRNA #2
Primer 1
1
Exon 2
Exon 3
Primer 2
Primer 1
Primer 2
D
Parental
KO Pool
Clone A
Clone B
Clone C
Clone D
Clone E
1000
650
200

FIG. 3.5. Comparison of AcMNPV multiplication in SfH2AX KO cells. SF21 parental cells and two SfH2AX KO clones (SfH2AX KO #1 and #2) were infected with AcMNPV (MOI 0.5). Extracellular budded virus was collected at the indicated times and quantified using $TCID_{50}$. Budded virus yields are reported as average pfu ±SD per ml. (n=3).

FIG. 3.5


Budded virus yield (log pfu/mL)
7
6
5
4
3
0
24
48
72
Hours post infection
Parental
SfH2AX KO #1
SfH2AX KO #2

into broad areas of lepidopterans, baculoviruses, and virus host interactions. Additionally, I have shown the sfU6_2 promoter functions to express shRNAs. This will allow for knockdowns of *S. frugiperda* proteins for further study in baculovirus experiments.

Generation of an *h2ax* knockout This study examined the deletion of the *S. frugiperda* ortholog of *h2ax*, a key modulator of the DDR. Since baculovirus infection has been shown to interact with the host DDR, this made *h2ax* a high priority target for gene editing. During AcMNPV infection the activation of H2AX through DNA damage-induced phosphorylation is blocked [181]. The AcMNPV early protein late expression factor 7 (LEF-7) is necessary and sufficient for blocking this DNA-damage induced H2AX phosphorylation [180]. The use of the CRISPR-Cas9 tool to knockout *h2ax* allows for further investigation into the role of H2AX with LEF-7 and AcMNPV multiplication.

Host targets for baculovirus infection. The DDR is a series of conserved pathways that in response to DNA damage regulate the cell cycle, trigger DNA repair, and in the case of irreparable damage trigger pro-apoptotic signaling within the cell (Reviewed in [17]). Additionally, it has become increasing clear the DDR, or a portion of the DDR, function within the innate immune response to viral infection (Reviewed in [42, 256]). The DDR functions to promote baculovirus multiplication [107, 181] However the mechanism through which the host DDR promotes baculovirus multiplication remains elusive. Baculovirus has also been shown to promote homologous recombination, an important outcome of the DDR [59]. Thus, DDR factors that promote homologous recombination, including BRCA1, and Rad51 [40], are high priority targets for knockdown and knockout studies involving AcMNPV.

The ability to utilize CRISPR-Cas9 technology in *S. frugiperda* cells also benefits research beyond virus research. Baculovirus vectors are commonly used to express high levels of protein for a variety of uses, from scientific study to the production of therapeutics and vaccines [77, 178]. The total protein expressed from baculovirus vector

infection of *S. frugiperda* cells is limited by the baculovirus-induced cell lysis that occurs late during infection. Using CRISPR-Cas9 technology for genome engineering of *S. frugiperda* cell lines has the possibility of enhancing protein yields by delaying cell lysis. Additionally, proteins undergo post-translational modifications. Some post-translational pathways differ between *S. frugiperda* and humans and limit the utility of the baculovirus expression system [5]. The CRISPR-Cas9 genome engineering technology could be used to ”humanize” *S. frugiperda* cell lines to express, creating post-translational pathways which are more human like. This could allow for research using the baculovirus expression system that was previously limited. Together, the development of a CRISPR-Cas9 technology for editing *S. frugiperda* cells has exciting implication for baculovirus research.

Chapter 4

Autographa californica Multiple Nucleopolyhedrovirus early protein LEF-7 targets *Spodoptera frugiperda* cGAS homolog

4.1 Abstract

The host DNA damage response (DDR) is frequently activated following infection by a diverse array of viruses, including insect cells by the baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV). Baculoviruses manipulate the host DDR in part through the viral LEF-7, which is an F-box protein which reduces the accumulation of phosphorylated H2AX (γ-H2AX). LEF-7 promotes AcMNPV DNA synthesis and homologous recombination. However, the mechanism by which LEF-7 functions are the proteins it targets for ubiquitination are unknown. To illuminate this mechanism, I utilized a strategy that involved a LEF-7 proximity labeling fusion protein in *Spodoptera frugiperda* (*S. frugiperda*) SF21 cells to identify LEF-7 substrates. The fusion LEF-7 interacted with *S. frugiperda* ortholog of cGAS (SfcGAS) in that was identified, cloned, and expressed in *S. frugiperda* SF21 cells. Interaction was strongest when the LEF-7 F-box domain was disabled by mutation. LEF-7 may target SfcGAS for polyubiquitination and possible proteasomal degradation. Thus, cGAS is an innate immune sensor, activating a Stimulator of Interferon genes (STING) and downstream immunity genes in response to foreign nucleic acids, including baculovirus nucleic acids. An additional

function of cGAS is repression of homologous recombination following DNA damage, potentially linking LEF-7/SfcGAS interaction to the DDR. This study suggests that baculovirus LEF-7 targets the host cGAS for degradation to promote homologous recombination, which contributes to viral DNA generation and virus multiplication.

4.2 Introduction

Baculoviruses have long been used as a model system to study virus-insect host interactions. Recent investigations using the prototypic baculovirus AcMNPV have revealed a significant interaction between the virus and the host DDR during infection [107, 181]. Baculovirus infection and subsequent vDNA synthesis resulted in DDR activation and this activation contributed to virus multiplication [107, 181]. Moreover, the conserved baculovirus protein late expression factor 7 (LEF-7) is intimately linked to successful baculovirus infection and the host DDR [180].

LEF-7 is expressed early during infection and contributes to viral DNA (vDNA) replication and homologous recombination [43, 59, 87]. Deletion of LEF-7 from the virus (vΔLEF-7) causes a 65-fold reduction in budded virus production [180]. Interestingly, LEF-7 was shown to modulate the host DDR and block the AcMNPV induced accumulation of γ-H2AX early during infection [180]. In *S. frugiperda* SF21 cells, expression of LEF-7 alone is sufficient to block γ-H2AX accumulation in response to pharmacologically-induced DNA damage. Thus, baculoviruses show an intriguing modulation of the DDR, being both reliant on the DDR and inhibiting its functions. Taken together, baculovirus LEF-7 targets γ-H2AX, a key regulator of the DDR, promotes homologous recombination, and enhances viral multiplication. Additionally, homologs of LEF-7 are present in all of Group I alphabaculoviruses, three Group II, and two betabaculoviruses, indicating LEF-7 shares a conserved function to promote baculovirus

multiplication [225]. LEF-7 also limits γ-H2AX accumulation in response to DNA damage when expressed in human cells [34]. Thus LEF-7, a conserved baculovirus protein, targets an evolutionarily conserved DDR protein and has an positive affect on viral multiplication.

AcMNPV LEF-7 is a F-box protein that interacts with the *S. frugiperda* Skp, Cullin, F-box containing (SCF) complex, likely as a substrate recognition component to target substrates for polyubiquitination [180]. F-box proteins are found throughout eukaryotes, with 11 F-box proteins found in budding yeast, 22 in *Drosophila*, and at least 38 in humans [123]. By binding substrates and the SCF complex, cellular F-box proteins control the levels of diverse regulatory proteins [243]. Some viruses also encode F-box proteins including P0 from *Turnip yellows virus* [175] and C9L from Vaccinia virus [109].

LEF-7 modulates the host insect DDR during baculovirus infection. However, the mechanism through which LEF-7 exerts its effects remain unclear. The proposed model of LEF-7 function is one which LEF-7 binds the host SCF and its target substrate(s), triggering polyubiquitination and subsequent proteasomal degradation. Thus, LEF-7 may promote the degradation of a DDR component and limit γ-H2AX accumulation. The identification of LEF-7 substrates has remained elusive [34]. As is the case with other E3 ligase substrates, the identification of LEF-7 substrates is challenging due to low affinity ligase:substrate interactions and the rapid degradation that substrates undergo.

To overcome these challenges, I attempted to identify LEF-7 substrates through the use of a proximity-based biotinylation (BioID) strategy [121]. This strategy has been successfully used to identify other F-box protein substrates [58]. BioID functions by creating a fusion protein between a biotin conjugating enzyme (BirA) and a protein of interest, in this case, LEF-7. The BirA domain functions to covalently label with biotin those proteins in close proximity. The biotin-labeled proximal proteins could then be isolated by standard procedures that would normally disrupt protein-protein interactions.

Here, I demonstrate that expression of BioID-LEF-7 resulted in biotin labeling of a FLAG tagged-SfcGAS. This preliminary result suggests that SfcGAS is a LEF-7 substrate, which may be targeted for polyubiquitination and proteasomal degradation. Loss of SfcGAS would avoid activation of innate immune signaling or possibly promote homologous recombination of viral DNA.

4.3 Materials & Methods

Cells and infections. *Spodoptera frugiperda* IPLB-SF21 (SF21) cells [266] were maintained at 27°C in TC100 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone). For cells expressing BioID fusion proteins the TC100 medium was supplemented with 50 µM of biotin (Sigma-Aldrich) to stimulate biotinylation

Sequence identification. The coding sequences for *S. frugiperda* ortholog of PARP-1 (SfPARP-1) and SfcGAS were identified by using a tblastn search of a whole cell transcriptome of SF21 cells (NCBI Accession: PRJNA271593). The human PARP-1 (NCBI Reference Sequence: NM_001618.4) and cGAS (NCBI Reference Sequence: NM_138441.3) amino acid sequences were used as the query. The tblastn search identified a transcript containing the SfPARP-1 (GenBank: GCTM01001987.1) and SfcGAS (Genbank: GCTM01003329.1) coding sequences. Subsequently, the SfPARP-1 and SfcGAS sequences were used as queries using NCBI's Conserved Domain Database showing domains matching those within human PARP-1 and cGAS [165].

Plasmids and Transfections. BioID fusion proteins were constructed using a myc-BioID2 fusion protein [121]. The plasmid mycBioID2-13X Linker-MCS was a gift from Kyle Roux (Addgene plasmid # 92308; http://n2t.net/addgene:92308 ; RRID: Addgene_92308). The mycBioID2-13X Linker-LEF-7 fusion proteins were cloned into an expression plasmid using NEBuilder® Hifi (New England Biolabs). Fusion proteins were

expressed under control of the AcMNPV IE1 promoter and *hr5* enhancer sequence. SfSKP2, SfPARP-1 and SfcGAS were identified by using a BLAST search of a whole cell transcriptome of SF21 cells (NCBI Accession: PRJNA271593). The human SKP2 (Accession: AY029177.1), PARP-1 (Accession: NM_001618.4) and cGAS (Accession: NM_138441.3) sequences were used as the query. The BLAST search identified SfSKP2 (GenBank: GCTM01011926.1), SfPARP-1 (GenBank: GCTM01001987.1) and SfcGAS (GenBank: GCTM01003329.1) sequences. SfSKP2 was cloned using SF21 cDNA into a BioID expression plasmid in a manner identical to LEF-7 above. SfPARP-1 and SfcGAS were cloned using SF21 cDNA into an expression plasmid under control of an IE1 promoter. Transfections were carried out using *Trans*IT-Insect (Mirus Bio) with 2 µl of *Trans*IT-Insect per µg of plasmid DNA. Transfections were carried out in 6 well plates using 10 µg of plasmid DNA per well containing 10^6 SF21 cells.

Pulldowns. Cell lysates were prepared by washing cells 2x with insect PBS pH 6.2, then harvesting cells in 1.5 mL of insect PBS using a cell scraper. Cells were pelleted at 1,800 x g, 5 min, 4 °C. The supernatant was removed, and the cells were lysed in 50 mM Tris pH 7.4, 500 mM NaCl, 0.4% SDS, 5 mM EDTA, 1 mM DTT, 1x PhosSTOP™(Roche) phosphatase inhibitor, and 1x cOmplete™Mini (Roche) protease inhibitor for 15 min on ice. Lysates were then sonicated for 30s on 50% duty cycle using a Fisher Scientific Sonic Dismembrator Model 100 and passsed through a 25 G needle. Lysates were adjusted to 2% Triton X-100 and 150 mM NaCl using 50 mM Tris pH 7.4. Samples were incubated with Dynabeads MyOne Streptavain T1 (Invitrogen). Beads were collected on a magnetic stand and washed 4x as follows; Wash 1: 2% SDS in water; Wash 2: 50 mM HEPES pH7.4, 1 mM EDTA, 500 mM NaCL, 1% Triton X-100, 0.1% Na-deoxycholate; Wash 3: 10 mM Tris pH 8, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na-deoxycholate; Wash 4: 50 mM Tris pH 7.4, 50 mM NaCl, 0.1% NP-40. Samples were eluted from the beads by incubating at 98 °C for 15 min in 10 mM Tris pH 7.4, 2% SDS, 5% B-mercaptoethanol, and 2 mM Biotin. Beads were removed using magnetic rack and

samples stored at -20 °C.

Immunoblots and antisera. Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Osmonics, Inc.). Membranes were blocked in 0.2% (w/v) I-Block (Applied Biosystems) dissolved in PBS containing 0.1% Tween-20. The indicated primary antibodies were used followed by IRDye 680-secondary antibodies (LI-COR) and IRDye 800CW Streptavidin (LI-COR 926-32239). Membranes were imaged on a LI-COR Odyssey FC. The following commercial antiserum were used: mouse monoclonal anti-Myc Tag (Cell Signaling 9B11) and mouse monoclonal anti-FLAG (M2 clone Sigma F3165).

4.4 Results

4.4.1 BioID-LEF-7 proximity labels proteins in SF21 cells.

I hypothesized that a proximity-based protein labeling approach in SF21 cells would capture LEF-7 protein-protein interactions that had been difficult to identify through other means. I therefore made use of proximity-dependent biotinylation (BioID) [121, 227] to identify potential targets of LEF-7. Previous reports indicated an N-terminally tagged LEF-7 retains function, including inhibition of DNA-damage induced γ-H2AX and promoting AcMNPV multiplication [180]. A BioID LEF-7 fusion protein was generated containing an N-terminal Myc-tagged BirA biotin ligase (FIG. 4.1B) and expressed in SF21 cells from a plasmid containing an AcMNPV IE1 promoter and *hr5* enhancer. This produced a LEF-7 bait protein which biotinylates proximal proteins. A key feature of the BioID system is the BirA domain self-labels the fusion protein with biotin [121]. Whole cell lysates were prepared, and proteins were purified from lysates using streptavidin magnetic beads. Immunoblot analysis of the pulldown showed BioID-LEF-7 was able to

FIG. 4.1. LEF-7 constructs used in this studyA. LEF-7 mutants. LEF-7 contains a nuclear localization signal (NLS), an F-box domain (F-box), and a C-terminal leucine rich domain (Leu-rich). From top to bottom: LEF-7 is the complete wild-type sequence, ΔF-box is missing the F-box domain (amino acid residues 15 through 52), LP15AA has two conserved amino acids in the F-box domain mutated to alanines, L15A & P16A. **B. Model of BioID-LEF-7.** The BioID fusion protein consists of a BirA biotin conjugating enzyme connected via a flexible linker to LEF-7, LEF-7(ΔF-box), or LEF-7(LP15AA). BioID-LEF-7 binds to LEF-7 substrates. The flexible linker allows BirA to come into close proximity and covalently link biotin to the LEF-7 substrate. Following cell lysis the biotinylated LEF-7 substrate can be pulled down with streptavidin beads.

FIG. 4.1

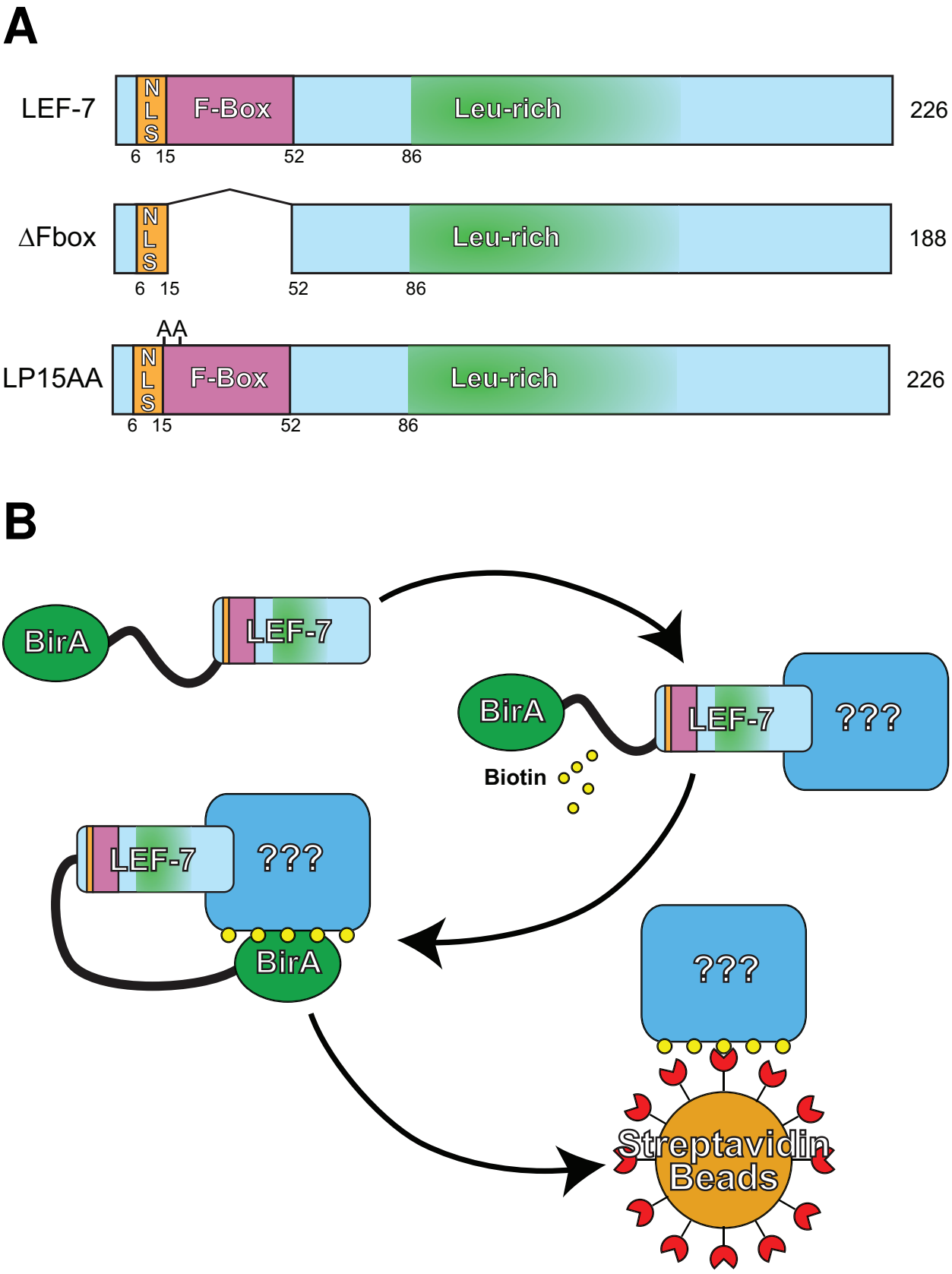
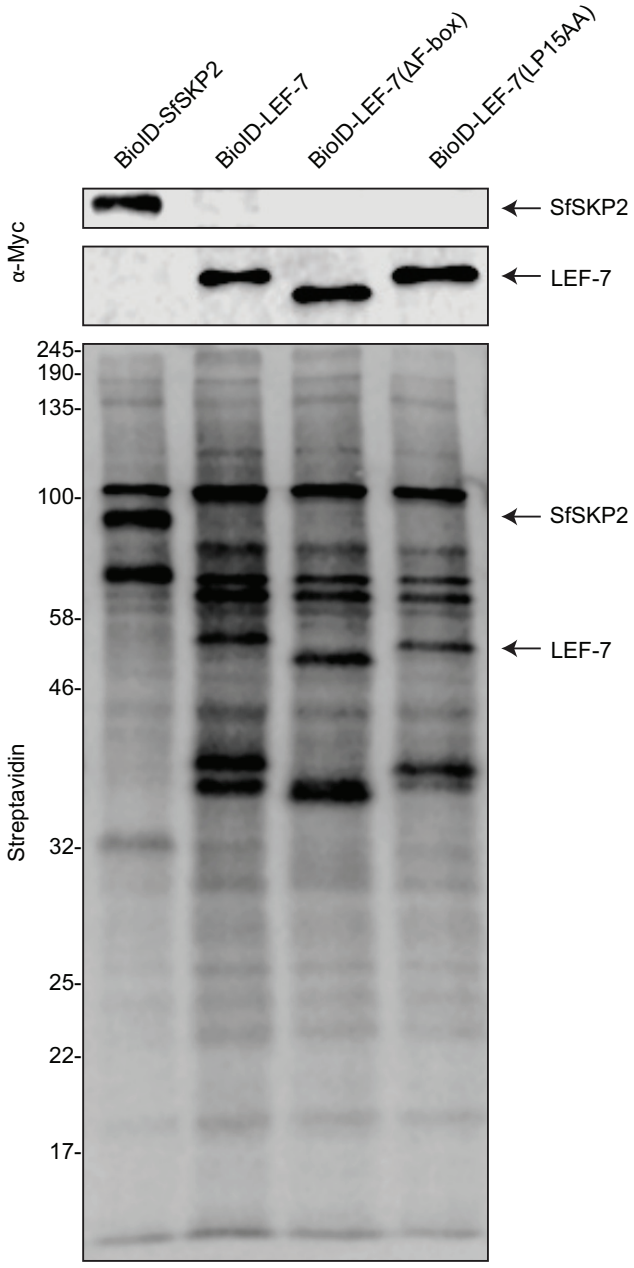

A
LEF-7
NLS
F-Box
Leu-rich
226
6
15
52
86
ΔFbox
188
AA
LP15AA
B
BirA
LEF-7
???
Biotin
Streptavidin
Beads

FIG. 4.2. BioID Expression in SF21 cells. SF21 cells transfected 24 hours earlier with plasmids encoding BioID-SfSKP2, BioID-LEF-7, BioID-LEF-7(F-box domain deletion (ΔF-box)), or BioID-LEF-7(LP15AA) respectively, were lysed and subjected to immunoblot analysis by using anti-Myc (top 2 panels) and a fluorescent dye-labeled streptavidin (bottom panel). The presence of the myc epitope confirmed expression of all BioID fusion proteins. Distinct bands corresponding to biotin labeled BioID fusion proteins are also indicated in the streptavidin blot. Numbers on the left of the streptavidin blot indicate approximate protein sizes in kDa.

FIG. 4.2


BioID-SfSKP2
BioID-LEF-7
BioID-LEF-7(ΔF-box)
BioID-LEF-7(LP15AA)
α-Myc
SfSKP2
LEF-7
245-
190-
135-
100-
58-
46-
32-
25-
22-
17-
Streptavidin
SfSKP2
LEF-7

label various proteins when expressed in the cells (FIG. 4.2).

As an F-Box protein LEF-7 polyubiquitinates its target proteins, signaling them for rapid proteasomal degradation [123]. This activity means it may be difficult to identify LEF-7 targets. Thus even when BioID-LEF-7 is in close enough proximity for substrate proteins to be labeled, they are rapidly degraded. Consequently, I also constructed BioID-LEF-7 fusion proteins with a mutation in LEF-7's F-box region (BioID-LEF-7-ΔF-box and BioID-LEF-7-LP15AA) which abrogates binding to S-phase kinase-associated protein 1 (SKP1) of the SCF complex (FIG. 4.1). These F-box mutations prevent proteasome mediated degradation [180]. In the absence of proteasome degradation, LEF-7 substrate binding was expected to be stabilized, thereby increasing the abundance of LEF-7 interacting proteins in the streptavidin pulldown. As a control, I also constructed a fusion protein containing BirA biotin ligase fused to SfSKP2 (BioID-SfSKP2), the *S. frugiperda* ortholog of the cellular F-box protein S-phase kinase-associated protein 2 (SKP2), as a control for interactions with the SCF. Both LEF-7 F-box mutations, ΔF-box and LP15AA, showed biotin labeling of proteins in SF21 cells (FIG. 4.2). From this observation I concluded the BioID-LEF-7 F-box-mutations were competent to label proteins with biotin. Additionally, BioID-SfSKP2 also showed biotin labeling of proteins, with a pattern distinctive to those obtained from LEF-7 and the LEF-7 F-box-mutations (FIG. 4.2). Together, I concluded that the BioID-LEF-7 system generated a series of biotinylated proteins that were distinct from another F-Box protein.

4.4.2 PARP does not interact with LEF-7.

Due to the known ability of LEF-7 to modify the host DDR [180], Poly ADP-ribose polymerase 1 (PARP-1) was an attractive candidate for a potential LEF-7 target. PARP-1 functions in response to DNA damage to help coordinate the DDR [273]. PARP-1

activation in insect cells is dependent upon its interaction with insect orthologs of variant histone H2AX (H2AX) [128]. Since LEF-7 is known to influence γ-H2AX, I hypothesized that LEF-7 may interact with SfPARP-1 to modify the DDR during infection. To test this possibility, I identified SfPARP-1, and cloned a FLAG-tagged SfPARP-1 into an expression vector under control of an IE1 promoter. This vector was used to overexpress FLAG-sfPARP-1 in SF21 cells in the presence of BioID-LEF-7, BioID-LEF-7-ΔF-box, or BioID-LEF-7-LP15AA. Cell lysates were subjected to streptavidin pulldown. Lysates were then analyzed via immunoblot to determine if FLAG-SfPARP-1 was pulled down by streptavidin. Biotin labeled fusion proteins corresponding to BioID-LEF-7, BioID-LEF-7(ΔF-box), and BioID-LEF-7(LP15AA) were detected by streptavidin, indicating self-labeling by BirA domains (FIG 4.3A, 4.3B, 4.3C). While there was streptavidin recognition binding to a protein of the appropriate size for SfPARP-1 in the input, there is no FLAG-SfPARP-1 detected by the FLAG antibody in the elution in BioID-LEF-7 or either of the LEF-7 mutants (Top panels FIG 4.3A, 4.3B, 4.3C). From this observation, I concluded that LEF-7 does not interact with SfPARP-1.

4.4.3 cGAS may interact with LEF-7

Previous efforts to identify LEF-7 interacting proteins produced a candidate list of proteins through a pulldown mass spectrometry approach [34]. In addition to SCF complex proteins, the *S. frugiperda* ortholog of cGAMP synthase (cGAS) SfcGAS was detected. Canonically, cGAS functions in an innate immune response by detecting cytosolic double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or RNA:DNA species and triggers an interferon response through STING [46]. However, cGAS activation is also involved in crosstalk with the DDR [256]. I hypothesized that LEF-7 may be interacting with SfcGAS and targeting it for degradation to modulate the DDR or temper an innate immune response. To test this possibility, I overexpressed

FIG. 4.3. Streptavidin pulldown of SfPARP-1. SF21 cells transfected 24 h earlier with plasmids encoding FLAG-SfPARP-1 and either **A.** wild-type BioID-LEF-7, **B.** BioID-LEF-7(ΔF-box), or **C.** BioID-LEF-7(LP15AA) were harvested and whole-cell lysates were prepared for streptavidin pulldown. Biotinylated proteins were pulled down using streptavidin beads, and input, flowthrough, wash, and elution fractions were reserved for immunoblot analysis. Immunoblot analysis was carried out using anti-FLAG (top) and streptavidin (bottom). Numbers on the left of the streptavidin blot indicate approximate protein size in kDa.

FIG. 4.3

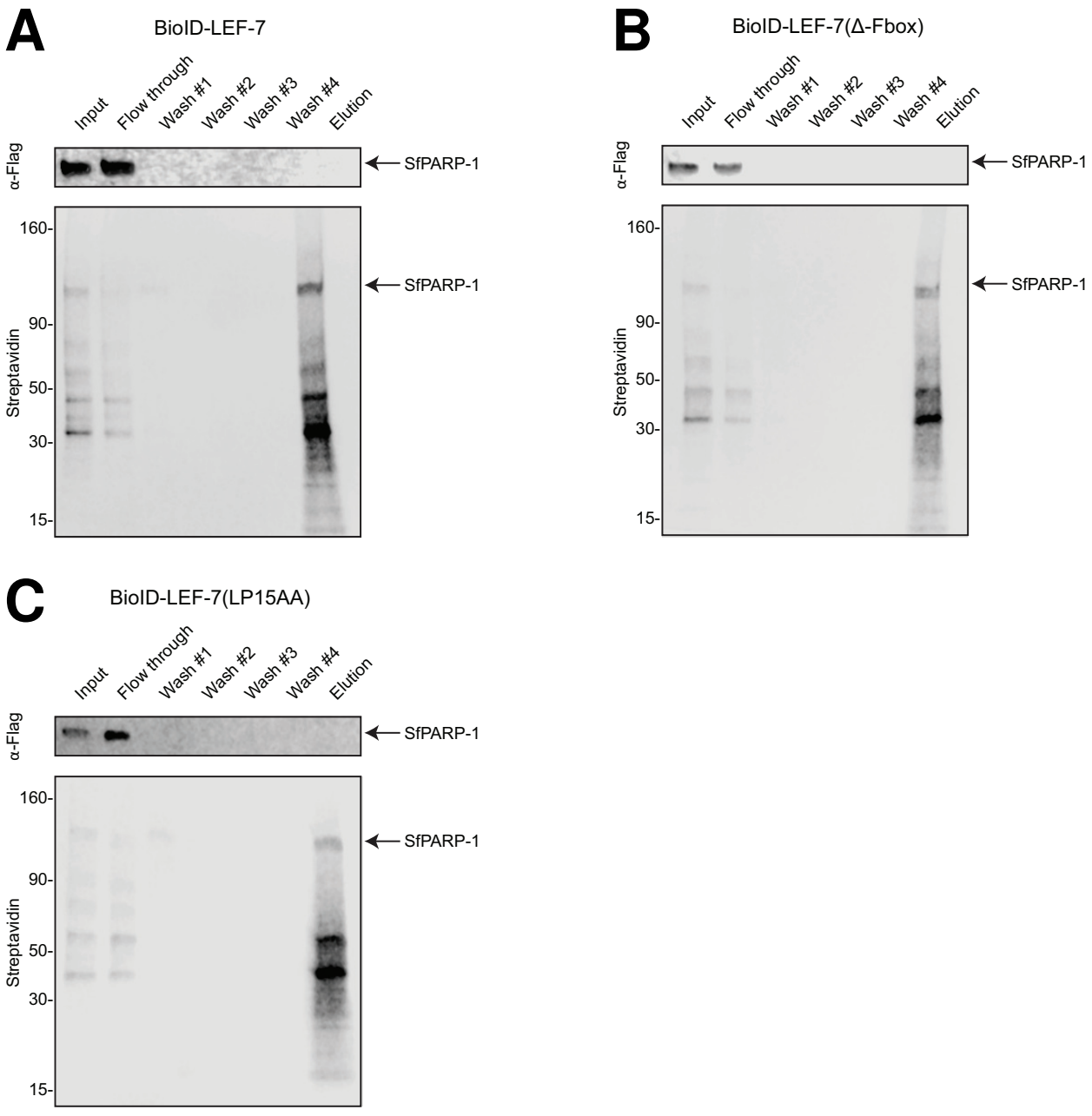
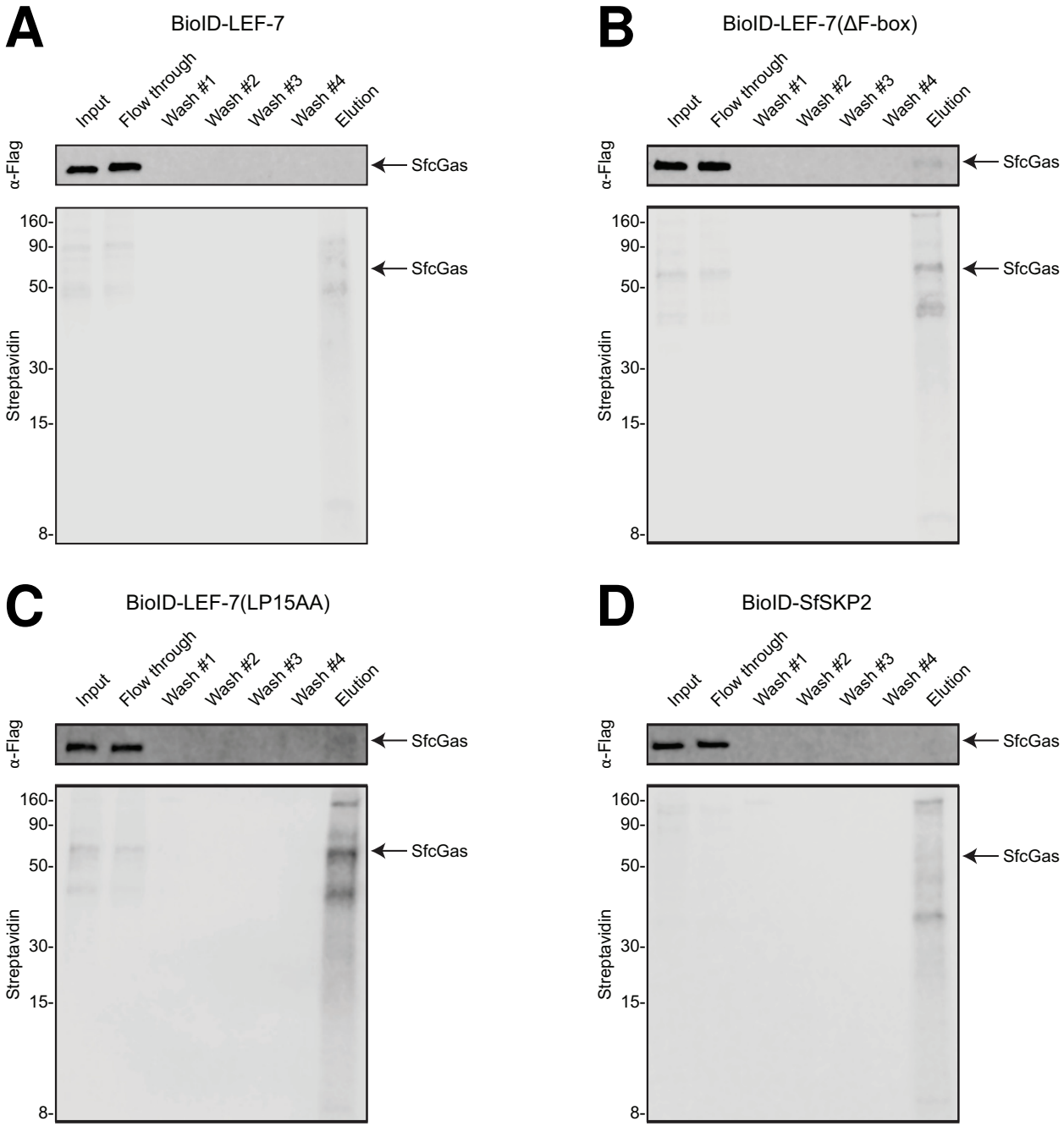

A
BioID-LEF-7
Input
Flow through
Wash #1
Wash #2
Wash #3
Wash #4
Elution
α-Flag
SfPARP-1
160-
90-
50-
30-
15-
Streptavidin
SfPARP-1
B
BioID-LEF-7(Δ-Fbox)
Input
Flow through
Wash #1
Wash #2
Wash #3
Wash #4
Elution
α-Flag
SfPARP-1
160-
90-
50-
30-
15-
Streptavidin
SfPARP-1
C
BioID-LEF-7(LP15AA)
Input
Flow through
Wash #1
Wash #2
Wash #3
Wash #4
Elution
α-Flag
SfPARP-1
160-
90-
50-
30-
15-
Streptavidin
SfPARP-1

FIG. 4.4. Streptavidin pulldown of SfcGAS SF21 cells transfected 24 h earlier with plasmids encoding FLAG-SfcGAS and either **A.** wild-type BioID-LEF-7, **B.** BioID-LEF-7(ΔF-box), **C.** BioID-LEF-7(LP15AA), or **D.** BioID-SfSKP2 were harvested and whole-cell lysates were prepared for streptavidin pulldown. Biotinylated proteins were pulled down using streptavidin beads, and input, flowthrough, wash, and elution fractions were reserved for immunoblot analysis. Immunoblot analysis was carried out using anti-FLAG (top) and streptavidin (bottom). Numbers on the left of the streptavidin blot indicate approximate protein size in kDa.

FIG. 4.4


A
BioID-LEF-7
B
BioID-LEF-7(ΔF-box)
C
BioID-LEF-7(LP15AA)
D
BioID-SfSKP2
Input
Flow through
Wash #1
Wash #2
Wash #3
Wash #4
Elution
α-Flag
SfcGas
Streptavidin
160-
90-
50-
30-
15-
8-

FLAG-tagged SfcGAS in SF21 cells in the presence of BioID-LEF-7, BioID-LEF-7-ΔF-box, or BioID-LEF-7-LP15AA. Cell lysates were subjected to streptavidin pulldown. Lysates were then analyzed via immunoblot to determine if FLAG-SfcGAS interacted with LEF-7. Lysates from BioID-LEF-7 expressing cells failed to show FLAG-SfcGAS in the elution (FIG. 4.4A). However, there is detectable FLAG-SfcGAS in both the lysates from BioID-LEF-7(ΔF-box) (FIG. 4.4B) and the BioID-LEF-7(LP15AA) (FIG. 4.4C). This finding is consistent with SfcGAS being degraded by wild-type LEF-7, but SfcGAS having an increased abundance with the LEF-7 F-box mutations due to the ΔF-box and LP15AA mutations failing to bind the SCF complex. Additionally, BioID-SfSKP2 failed to biotin label SfcGAS (FIG. 4.4D), indicating that cGAS doesn't have a non-specific interaction with the SCF complex or other proteins involved in F-box protein mediated degradation. From this observation, I concluded that LEF-7 is interacting with SfcGAS. Thus, host SfcGAS maybe a target of LEF-7 during baculovirus infection.

4.5 Discussion

The viral modulation of the DNA damage response during infection is important to understand, as the DDR plays an important role in viral oncogenesis, and the medical use of viral vectors may modulate the DDR during therapy. Baculoviruses have been utilized as gene delivery platforms in mammalian cells as they are replication defective, but capable of delivering viral genomes to the nucleus and expressing genes-of-interest from heterologous promoters in mammalian cells [203]. A key viral regulator of the host DDR during baculovirus infection is LEF-7, which has been shown to impair DDR substrate phosphorylation [180]. My findings here indicate that LEF-7 interacts with the lepidopteran ortholog of the DNA-sensor cGAS (SfcGAS).

LEF-7 is a viral F-box protein, which interacts with the SCF complex to polyubiquitinate LEF-7 substrates [180]. The identification of LEF-7 substrates will assist in better understand the role of LEF-7 during baculovirus infection. The identification of F-box protein ligase substrates can be difficult, F-box protein interactions with their substrates can be low-affinity or transient, and short-lived as substrates are rapidly degraded by the proteasome following interaction with the F-box protein. To overcome these limitations, I employed a proximity labeling technique allowing the LEF-7 bait protein interacting proteins to be covalently labeled with biotin by the BirA biotin ligase. To overcome the rapid degradation, I utilized LEF-7 F-box mutants that are unable to bind SfSKP1 of the SCF complex.

I report here that baculovirus AcMNPV LEF-7 interacts directly with the *S. frugiperda* ortholog of cGAS (SfcGAS). While wild-type BioID-LEF-7 didn't biotin label SfcGAS (FIG. 4.4A), likely due to rapid proteasomal degradation of SfcGAS. However, the two BioID-F-box mutations ΔF-box and LP15AA biotin labeled SfcGAS (FIG. 4.4B and 4.4C). The F-box mutations allowed for detectable biotin labeled SfcGAS due to their ability to bind SfcGAS through C-terminal interactions of the leucine rich repeats, but inability to interact with the SCF and subsequently degrade SfcGAS. I would expect that treatment of cells with the 26S proteasome inhibitor MG132 would stabilize SfcGAS levels in the presence of WT LEF-7, and I would observe biotin-labeled polyubiquitinated SfcGAS. It is important to note that this is a preliminary result, additional investigation should be undertaken to confirm this interaction, and determine if SfcGAS can be used to pulldown LEF-7, LEF-7(ΔF-box), and LEF-7(LP15AA).

Further investigations are needed to understand the role of cGAS in baculovirus infection. Deletion of *lef-7* from the baculovirus genome (vΔLEF-7) results in reduced vDNA replication and a defect in budded virus production [180]. If the degradation of SfcGAS by LEF-7 is important for viral multiplication, knockdown or knockout of SfcGAS

should rescue vΔLEF-7 budded virus production. Additionally, how SfcGAS functions during baculovirus infection needs investigation, as SfcGAS function as a DNA sensor, be involved in cross-talk with the DDR, have an additionally as-of-yet unknown function, or some combination of these possibilities. The ability of LEF-7 to interact with SfcGAS also needs to be investigated in the context of DDR activation and viral infection. DDR activation results in cGAS phosphorylation [147], and baculovirus infection changes the phosphorylation status of DDR components [107, 180, 181]. Phosphorylation of SfcGAS may enhance the interaction with LEF-7.

Role of cGAS. Innate immunity detects and responds to microbial pathogens. Innate immunity is especially important in invertebrates, which lack an adaptive immune system. Studying innate immunity in invertebrates such as *Drosophila melanogaster* has led to discoveries such as the discovery of Toll-like receptors [137]. Canonically, cGAS functions as foreign DNA sensor, which catalyzes cyclic guanosine-monophosphate adenosine-monophosphate (cGAMP) production. cGAMP functions as a second messenger to activate STING. In mammals, activated STING stimulates TANK-binding kinase 1 (TBK1) resulting in phosphorylation of the transcription factor interferon regulatory factor 3 (IRF3) and increased transcription of interferon-β (IFNβ). In insects, cGAS-STING activation is associated with an anti-viral response, however insects lack interferon [217]. A diverse group of viruses activate cGAS including Herpes simplex virus-1 (HSV-1) [253], Vaccinia virus [253], and HIV [82]. Given the conserved function of cGAS-STING pathways in response to viral infection [129], and the presence of anti-cGAMP poxins in baculoviruses [71] baculovirus infection of lepidopteran cells likely activates an anti-viral cGAS mediated response.

Viral regulation of cGAS-STING signaling. Due to the conserved function of the cGAS-STING function in sensing viral nucleic acid, it is not surprising that viruses have developed strategies to antagonize the cGAS-STING response. The gammaherpesvirus

Kaposi sarcoma herpesvirus (KSHV) tegument protein open reading frame 52 (ORF52), and ORF52 homologs in other gammaherpesviruses, interact with cGAS and disrupts the ability of cGAS to bind DNA, preventing activation of the cGAS-STING pathway [283] HSV-1 protein ICP27 interacts with STING and TBK1, preventing downstream signaling [51]. The human cytomegalovirus (HCMV) latency associated-protein UL138 inhibits the cGAS-STING pathway, preventing IRF3 phosphorylation and subsequent IFNB transcription [7]. The DNA tumor viruses Human papillomavirus (HPV) and adenovirus both inhibit the cGAS-STING pathway through their E7 and E1A oncoproteins, respectively [191]. The inhibition of the cGAS-STING pathway through LEF-7 mediated degradation of SfcGAS would be a reasonable mechanism for baculoviruses to antagonize the host innate immune response.

Nuclear cGAS linked to DNA repair. In addition to the cGAS canonical innate immunity functions, cGAS is also involved in the DDR and DNA repair functions. Following activation of the DDR cGAS has been shown to localize to the nucleus [147]. This nuclear translocation is independent of the function of cGAS in DNA sensing [147]. Nuclear cGAS is recruited to double-strand breaks (DSBs) in a γ-H2AX dependent manner, and cGAS inhibits homologous recombination independent of IFNB induction [147]. Since LEF-7 is known to modulate γ-H2AX, it is possible that LEF-7 interaction with SfcGAS may be linked. Alternatively, both LEF-7 and SfcGAS may localize to γ-H2AX and their interaction may be incidental to their independent functions.

LEF-7 polyubiquitination of cGAS may be regulatory. While F-box proteins commonly direct their substrates for proteasomal-mediated degradation, the polyubiquitination of cGAS by LEF-7 may be non-degradative. Ubiquitination is a common post-translational modification used in regulation of the innate immune system [16]. During HSV-1 infection, the E3 ligase RNF185 catalyzes polyubiquitination of cGAS that enhances cGAMP production [272]. cGAS levels in cells are regulated by

polyubiquitination-dependent autophagic degradation, but cGAS is stabilized during viral infection by TRIM14/USP14-mediated cleavage of cGAS polyubiquitin chains [45]. While it may be unlikely that LEF-7 polyubiquitination of is degradative due to the stabilization of SfcGAS due to F-box mutation (FIG. 4.4), it is still possible that LEF-7 has a non-degradative regulatory effect on SfcGAS.

Additionally, the proximity-based labeling technique can be expanded to identify other proteins that interact with LEF-7. The F-box regions of LEF-7 is critical for interactions with the degradative SCF complex, and mutations in this region caused an increase in SfcGAS stability in the presence of LEF-7 (FIG. 4.4A, 4.4B, 4.4C).

Chapter 5

Discussion and Future Directions

5.1 Overview

The DNA damage response (DDR) functions to faithfully maintain the host's genome. However, the DDR also functions in response to viral infection as an antiviral response to restrict the ability of viruses to multiply within the cell. Viruses have overcome this restriction and modify the DDR to escape its antiviral effects. Viruses also usurp the DDR and exploit the host DDR responses to promote viral multiplication. The ability of viruses and viral factors to modify DDR function, block DDR signaling, and ablate DDR directed DNA repair contribute to viral pathogenesis and virus promoted oncogenesis (Reviewed in [193, 194, 276]). Therefore, furthering the understanding of the mechanisms through which viruses interact with the DDR are of importance to human health.

The prototypic baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) is a large DNA virus that, similar to other large DNA viruses, engages the host DDR during infection. The goal of my thesis work was to better understand the role of the DDR in virus infection of an invertebrate host, utilizing AcMNPV infection of *Spodoptera frugiperda* (*S. frugiperda*) SF21 cells as a model system. Previously it was thought the ataxia-telangiectasia mutated (ATM) mediated DDR stimulates AcMNPV multiplication

and viral DNA (vDNA) synthesis [107, 181], yet AcMNPV infection blocks the phosphorylation of variant histone H2AX (H2AX), a key step in the ATM-mediated DDR response [180]. This created a contradiction, as it wasn't understood why AcMNPV inhibits a DDR pathway that promotes its own multiplication. To investigate this contradiction, I expanded the study of ATM and AcMNPV through the use of multiple small molecule inhibitors of ATM (Chapter 2). Contrary to previous reports, I showed that small molecule inhibition of ATM kinase function at concentrations that maintained cell viability had no effect on AcMNPV multiplication (Chapter 2).

To investigate the DDR interaction with baculoviruses more robustly, I expanded the study to include genetic DDR knockout cell lines. Prior to my efforts no other report had shown an effective method to produce site-specific genetic knockouts in *S. frugiperda* cells. In my thesis work, I developed a CRISPR/Cas9 system to perform site specific genomic editing of *S. frugiperda* cells, a host cell type used to study baculovirus infections (Chapter 3). This required the identification of an RNA polymerase III promoter for expression of single guide RNAs (sgRNAs). I identified and verified the *S. frugiperda* U6 small nuclear RNA (snRNA) gene promoter as an effective sgRNA promoter. This CRISPR/Cas9 system was used to generate *S. frugiperda* SF21 H2AX knockout cells (Chapter 3), and SF21 ATM knockout cells to further investigate the role of ATM during AcMNPV infection (Chapter 2). In concert with the ATM knockout cells, I utilized pharmacological DDR inhibitors to further understand the DDR and AcMNPV (Chapter 2). AcMNPV multiplied to wild-type levels in ATM knockout cells, and in ATM-specific inhibitor treated cells, but not in pan-DDR inhibited cells (Chapter 2). This suggested a DDR kinase other than ATM must be responsible for baculovirus multiplication. Notably, this is the first report that a DDR kinase other than ATM is contributing to baculovirus multiplication.

Importantly, I showed that AcMNPV infection in SF21 cells causes an accumulation

of phosphorylated DDR substrates at S/TQ phosphorylation motifs (Chapter 2). Blocking of ATM function through treatment with specific inhibitors failed to reduce these phosphorylations. Since S/TQ phosphorylations are targets of DDR kinases, we have attributed these phosphorylations to the activation of the host DDR. Caffeine ablates these phosphorylations, however it is not clear if caffeine's effects are a result of direct inhibition of the kinase responsible for these phosphorylations, or if caffeine inhibition of DNA replication prevents the activation of the DDR. It is unclear if the AcMNPV induced accumulation of pS/TQ is the result of an antiviral response, an incidental result of infection, an AcMNPV modulation to promote viral multiplication, or some combination of these possibilities. This further supported the conclusion that a different, non-ATM DDR kinase promotes baculovirus multiplication.

The conserved baculovirus replication factor late expression factor 7 (LEF-7) is a potent modulator of the host *S. frugiperda* DDR [180]. LEF-7 is a F-box protein, and interacts with the *S. frugiperda* Skp, Cullin, F-box containing (SCF) complex [180]. LEF-7 contributes to vDNA synthesis, promotes homologous recombination, and represses phosphorylated H2AX (γ-H2AX) accumulation in response to DNA damage [59, 180, 225]. While F-box proteins are known to interact with substrates and promote their polyubiquitination by the SCF complex, the LEF-7 substrate(s) remained unknown. I identified that LEF-7 interacts with *S. frugiperda* ortholog of cGAS (SfcGAS), which functions as an innate immune sensor and is known to suppress homologous recombination at double-strand breaks (DSBs). Thus, my thesis work has uncovered a possible mechanism by which baculoviruses evade the innate immune system and promote homologous recombination.

5.2 AcMNPV and activation of the host DDR

The specific mechanisms through which virus infection results in the activation of the host DDR aren't well understood. As shown in Chapter 2 of this thesis, AcMNPV infection of SF21 cells resulted in a activation of the host DDR. Previous reports have shown that blocking AcMNPV DNA replication prevents the AcMNPV induced phosphorylation of the *S. frugiperda* ortholog of p53 [107]. However, AcMNPV infection of *Drosophila* cells also results in DDR activation despite *Drosophila* cells not supporting high levels of viral DNA synthesis [181]. This suggests that if viral DNA synthesis is involved in activating the host DDR, it may be pre-replicative structures or early replication events, and not high levels of vDNA synthesis, that trigger DDR activation. Indeed, the formation of viral pre-replicative structures during Herpes simplex virus-1 (HSV-1) infection are associated with DDR activation [144]. Thus, the accumulation of phosphorylated S/TQ sites in response to AcMNPV infection may DDR proteins localizing to pre-replicative structures (Chapter 2).

The formation of reactive oxygen species (ROS) during viral infection can also activate the DDR. During Epstein-Barr virus (EBV) infection, the latency associated protein EBV nuclear antigen 1 (EBNA-1) leads to DNA damage and activates the DDR through the production of ROS by up-regulating Nox2 [92]. It is possible that AcMNPV may be producing ROS and subsequently inducing DNA damage during viral multiplication through some as-of-yet unknown mechanism which modulates the infected cell metabolism. This effect may be dependent upon or a precursor to the progression through vDNA synthesis. Thus, AcMNPV induced activation of the DDR and accumulation of phosphorylated S/TQ motifs in proteins may be due to DNA damage caused by viral induced ROS.

Additionally, AcMNPV infection may be leading to DDR activation through direct

manipulation of the DDR without the induction of DDR sensors. Direct interaction of viral proteins with host DDR factors may result in a viral induced modulation or activation of the DDR. During Human papillomavirus (HPV) infections, the viral E7 protein directly interacts with ATM, and promotes the phosphorylation of the ATM substrate checkpoint kinase 2 (Chk2) [184]. It has been previously shown that the AcMNPV protein LEF-7 modulates the DDR, preventing DNA damage induced phosphorylation of H2AX [180]. LEF-7 may be interfering with the DDR in a manner which results in a modulation of DDR kinase activity, blocking phosphorylation of substrates associated with damaged chromatin and promoting activation of DDR signaling effectors such as checkpoint kinase 1 (Chk1) or Chk2. Thus, AcMNPV may be redirecting DDR factors from damaged chromatin to replication centers to aid in viral multiplication.

5.3 AcMNPV DNA synthesis and the DDR

The specific mechanisms through which baculoviruses replicate their genome are not clear. DNA replication is thought to occur through a rolling circle mechanism, producing long concatemers of viral DNA [80, 225]. During infection, baculoviruses promote a high level of homologous recombination [167]. It is possible concatemers of baculovirus DNA are resolved into single viral genomes through homologous recombination. However, baculoviruses likely require host factors to undergo homologous recombination, as baculoviruses lack all the proteins necessary for homologous recombination [201, 202, 225]. Baculovirus modulation of the host DDR may redirect host cellular homologous recombination factors to viral replication centers. These homologous recombination factors, in concert with viral replicative proteins, may aid in vDNA synthesis and resolution of these multi-genome fragments into single genomes. Thus, baculoviruses may activate and modulate the DDR in part to promote homologous recombination in viral replication centers for the production of unit length

viral genomes for packaging into virions.

5.4 AcMNPV, the DDR, and the cell cycle

The tumor suppressor p53 is involved in cell cycle regulation. p53 activation promotes cell cycle arrest at the g1/2 and g2/m checkpoints. In response to DNA damage or other genotoxic stresses, p53 is activated through phosphorylated by Chk2 or other kinases [279]. During AcMNPV infection, p53 is phosphorylated in a vDNA synthesis dependent manner [107]. Additionally, AcMNPV infection causes a G2/M cell cycle arrest. However, the mechanisms through which AcMNPV causes cell cycle arrest at the G2/M checkpoint are not clear [29]. The modulation of the DDR by AcMNPV may contribute to this cell cycle arrest. Progression though the G2/M checkpoint is associated with the dephosphorylation and activation of cyclin B/cell division cycle protein 2 (cdc2). It is possible that cellular tumor antigen p53 (p53), which accumulates and is activated during AcMNPV infection [107], is contributing to the G2/M checkpoint restriction. However, a RNAi mediated knockdown of p53 in *S. frugiperda* cells had no effect on viral multiplication [107]. Additionally, Chk1 and/or Chk2 activated through AcMNPV induction of the DDR (Chapter 3) may be inactivating CyclinB:cdc2 complexes directly. Cyclin B levels also vary during AcMNPV infection, with levels dropping off at 10 hours post infection, and then returning at 14 hours post infection [29]. Since ATM isn't required for AcMNPV multiplication, the phosphorylation of Chk1 by ATR may contribute to the viral induced G2/M cell cycle arrest. Interestingly, phosphorylation of the N-terminus of AcMNPV immediate early gene 1 (IE1) at a potential cyclin B/cdc2 site is necessary for vDNA synthesis and subsequent budded virus production [257]. This presents an apparent contradiction in which AcMNPV may promote G2/M arrest through cyclin B/cdc2 inactivation, yet baculoviruses depend upon cyclin B/cdc2 activity for proper IE1 function. This potential cell cycle regulation, along with the varying cyclin B levels during

infection, points to a complex regulation of the DDR by AcMNPV during infection.

5.5 IE1 phosphorylation

The function of AcMNPV IE1 may involve bridging together viral genome replication and the DDR. AcMNPV IE1 has several critical functions, including activation of AcMNPV transcription, binding of viral homologous regions (*hr*s), promotion of homologous recombination, and is essential for AcMNPV DNA replication [59, 126, 209, 219]. Additionally, AcMNPV IE1 contains an N-terminal replication domain that is essential for vDNA synthesis, but not required for transcriptional activation [257]. Phosphorylation of this N-terminal domain at a potential cyclinB/cdc2 phosphorylation site is critical for AcMNPV multiplication, and treatment with the phosphatidylinositol 3-kinase-related protein kinase (PIKK) inhibitor caffeine ablates this phosphorylation [257]. As caffeine is a potent inhibitor of the DDR PIKKs, this suggests a PIKK kinase or downstream kinase contributes to IE1 function in promoting AcMNPV multiplication. Additionally, IE1 together with IE2 are sufficient to promotes high frequency homologous recombination [59] This leads to a model where IE1, along with IE2, are activated by the DDR and modulate cellular homologous recombination factors to promote high frequency recombination. The transcriptional activation and DNA replication activity of IE1 are separable [257]. However, it is not clear if both transcriptional activation an DNA replication activities are required for IE1 and IE2 enhancement of homologous recombination. Further investigations may link DDR activation with core IE1 functions including transcriptional enhancement and initiation of vDNA replication.

5.6 Additional PIKKs and AcMNPV

Further investigation into the relationship between PIKKs and AcMNPV multiplication are required. The ability of caffeine to limit AcMNPV multiplication demonstrates the involvement of a caffeine sensitive kinase, such as member of the PIKKs. However, due to the broad range of inhibition of PIKKs by caffeine, the exact target of caffeine that is critical to AcMNPV multiplication is not clear. The use of specific inhibitors in SF21 cells is also limited, as most inhibitors have not been well validated in *S. frugiperda* cells. Although many DDR components are conserved even between mammals and lepidopterans, my evidence of inhibitor effects in SF21 cells (Chapter 2) contrasted with other reports [107, 181] highlights the limitations of inhibitor studies in lepidopteran cell lines. My progress in developing shRNA and CRISPR-Cas9 to study the role of the DDR kinase ATM in AcMNPV infection can easily be expanded to investigate the other DDR kinases ataxia-telangiectasia and Rad3 related (ATR) and DNA-dependent protein kinase (DNA-PK), along with additional PIKKs. While ATR has been shown to be critical in mammalian cell lines and a homozygous knockout is embryonic lethal in mice [30, 57, 62], ATR knockout *Drosophila* are viable [133]. Further investigation involving knockdowns, and knockouts of ATR and DNA-PK in SF21 cells would further define the role of DDR in baculovirus multiplication.

The non-DDR PIKKs are mammalian target of rapamycin (mTOR), suppressor of morphogenesis in genitalia (SMG-1), and transformation/transcription domain-associated protein (TRRAP). mTOR regulates a variety of cellular functions, including cell growth, cell proliferation, motility, cell survival, transcription, and protein synthesis [231]. Adenovirus E4-ORF3 activity activates mTOR during infection [198]. SMG-1 is involved in regulation of nonsense-mediated RNA decay [287]. TRRAP is involved transcription and recruits histone acetyltransferases, however TRRAP is unlikely

to be involved in baculovirus multiplication as a kinase as it lacks a catalytic site and hasn't been shown to have kinase activity [172]. While these may be involved in baculovirus multiplication, it isn't immediately clear how these non-DDR PIKKs would be involved in baculovirus DDR manipulation.

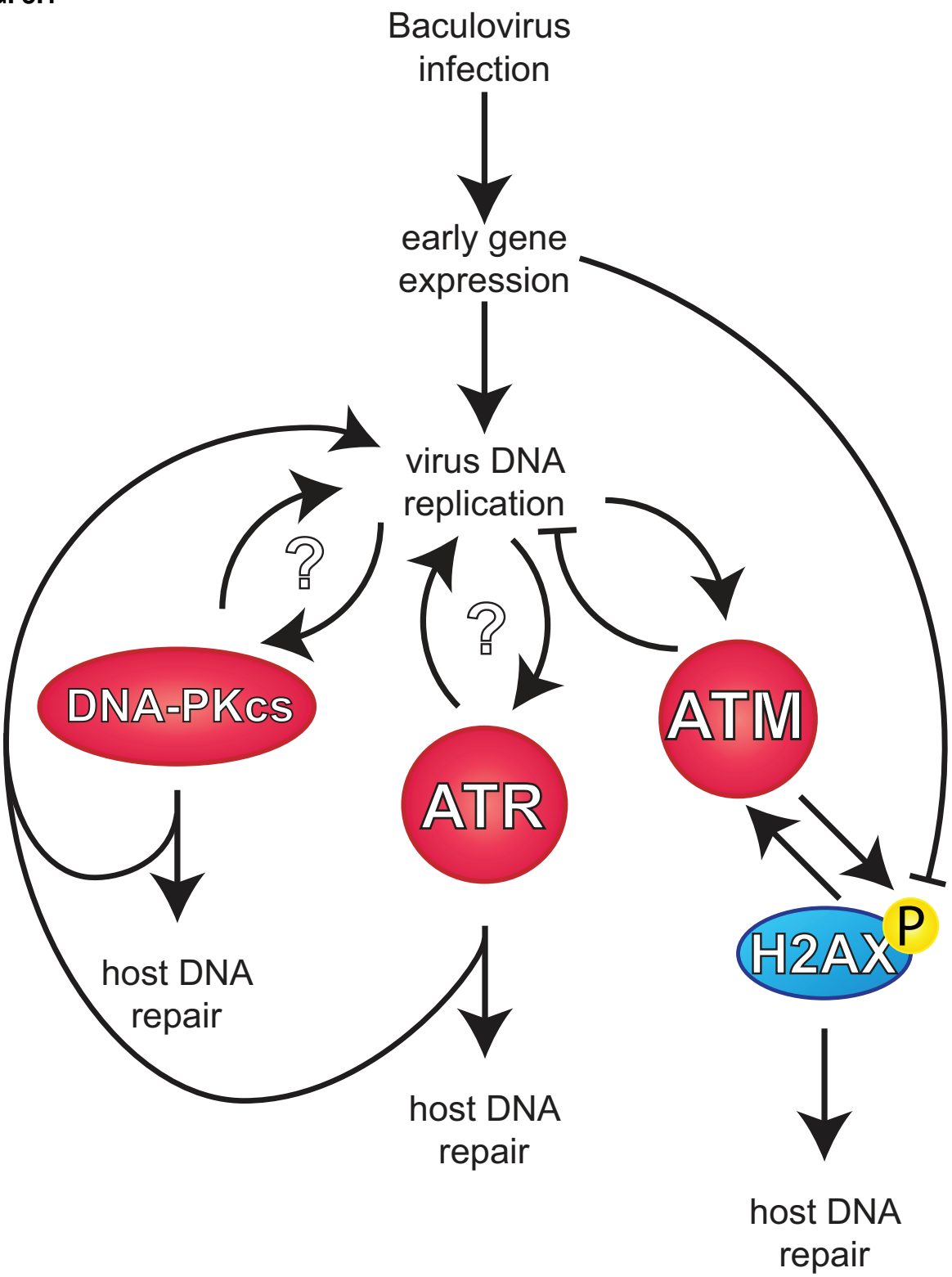
5.7 DDR may contribute host factors for baculoviruses

Homologous recombination is directly linked to baculovirus genome replication, and several baculovirus proteins essential for the synthesis of new genomes also promote homologous recombination [59, 225]. However, baculoviruses do not encode all the factors necessary for homologous recombination, suggesting that they depend on the exploitation of host homologous recombination factors [201, 202, 225]. Therefor baculoviruses may indeed be modulating the host DDR to promote the localization of homologous recombination factors to viral replication centers. I have shown that ATM, the master coordinate of homologous recombination repair [185] is not necessary for AcMNPV multiplication (Chapter 2). However, ATM itself is not essential for homologous recombination repair to proceed [119]. Thus, we should determine if *Spodoptera* orthologs of homologous recombination factors such as BRCA1, RPA, and Rad51 contribute to baculovirus promotion of homologous recombination. Using methods developed in Chapter 3, it is now possible to produce knockouts or shRNA-mediated knockdowns of homologous recombination factors in SF21 cells. The knockout/knockdown of host homologous recombination factors in concert with a previously developed baculovirus homologous recombination assay [59] would define the basis of baculovirus induced homologous recombination.

However, it is interesting to note that loss of ATM and pharmacological inhibition of ATM have distinct effects on homologous recombination. In mouse cells ATM inhibition,

FIG. 5.1. Proposed model of AcMNPV and the DDR. Following baculovirus entry early genes expression commences. Early genes encode viral factors that are necessary for baculovirus DNA replication, including LEF-7. Baculovirus DNA replication activates the DDR, and may involve numerous host kinases, including DNA-PKcs, ATR, and ATM. Baculovirus DNA replication requires the kinase activity of DNA-PKcs and/or ATR, or DNA-PKcs and/or ATR provide the activation of downstream cellular DDR or DNA repair factors that promote baculovirus DNA replication. However, the mechanism through which DNA-PKcs and/or ATR contribute to baculovirus multiplication still remains to be elucidated. It is also possible that the activation of DDR and DNA repair factors results in interference with viral DNA replication and genome packaging. To counter the antiviral effects of the DDR, baculovirus LEF-7 represses the DDR, reducing accumulation of γ-H2AX in response to virus DNA replication.

FIG. 5.1


Baculovirus infection
early gene expression
virus DNA replication
?
?
DNA-PKcs
ATR
ATM
H2AX
P
host DNA repair
host DNA repair
host DNA repair

but not ATM ablation, reduced homologous recombination [119]. Thus, in *S. frugiperda* cells, as in mammalian cells, the loss of ATM kinase activity may not be equivalent to the loss of ATM protein for homologous recombination. Other reports detailing the treatment of baculovirus infected cells with high concentrations of the ATM inhibitor KU-55933 may have inhibited ATM-independent homologous recombination, and thus reduced AcMNPV budded virus yields [107, 181]. However, the loss of SfATM protein (Chapter 2), may still allow for homologous recombination despite a loss of ATM kinase activity. Further investigation into whether or not ATM knockout cells show a reduction in homologous recombination is needed.

ATM and ATR have been shown to be involved in repair of viral genomes during viral DNA synthesis [246, 247]. human cytomegalovirus (HCMV) has been shown to abrogate repair of host DNA while maintaining repair of viral DNA [196]. Thus, baculoviruses may be usurping repair factors to efficiently repair viral genomes and caffeine-mediated inhibition of baculovirus multiplication may be due to reduced repair of nicks and breaks in viral genomes. Thus, DNA repair factors themselves may be responsible for the DDR contribution to baculovirus vDNA synthesis. If this is true, then during infection viral genomes should be repaired with a higher efficiency than host genomes, and caffeine treatment should show a reduction in repair. This could be observed using qPCR or a dual color southern blot [291] to observe repair in viral and host genomes.

5.8 AcMNPV DNA polymerase and helicase

Since AcMNPV infection induces phosphorylation of cellular DDR proteins, it is also possible that the AcMNPV induction of the DDR results in the phosphorylation of viral proteins by a cellular PIKK. Other viruses trigger the phosphorylation of viral proteins by a PIKK during infection, During Hepatitis B virus (HBV) infection ATM phosphorylates the

HBV core protein (HBc) on two SQ motifs in its C-terminal domain [156]. The phosphorylation of HBc by ATM is thought to regulate HBc function, with a process of phosphorylation and subsequent de-phosphorylation by cellular phosphatases to aid in HBV DNA synthesis and nucleocapsid formation. Additionally, The EBV protein EBNA-LP is phosphorylated by DNA-dependent protein kinase catalytic subunit (DNA-PKcs) during infection [97].

If an AcMNPV protein is being phosphorylated by a PIKK, a viral protein involved in viral DNA replication is an attractive candidate based on AcMNPV activation of the DDR coinciding with the onset of vDNA replication (Chapter 2, [107, 181]. There are 6 AcMNPV genes that are absolutly required for viral DNA replication: *dnapol*, *lef-1*, *lef-2*, *lef-3*, *helicase (p143)*, and *ie1* (reviewed in [225]). Several of these genes encode for proteins which contain SQ/TQ motifs, indicating possible targets of DDR kinases. However, both the AcMNPV DNA polymerase (FIG. 5.2) and the helicase (FIG. 5.3) contain S/TQ motifs which are well conserved within the alphabaculoviruses. These sites may be conserved phosphorylation sites of a PIKK such as ATR, and phosphorylation of these S/TQ motifs may be required for their function. Thus, caffeine's ability to reduce baculovirus vDNA synthesis and budded virus production may be due in part to the reduction of phosphorylation at these sites.

Interestingly, the conserved S/TQ site (residues 962-963) in AcMNPV helicase is near a helix-turn-helix motif (residues 967-981). Helix-turn-helix motifs are important for protein to DNA binding. If this site is indeed a target of ATM/ATR, or another kinase, then phosphorylation may enhance the ability of the helicase to bind DNA and function properly. Thus, activation of the DDR kinases may contribute to baculovirus multiplication by enhancing the function of the viral helicase.

While baculoviral proteins may be directly phosphorylated by a host DDR kinase, it is also possible that a downstream kinase is responsible for phosphorylating viral

FIG. 5.2. AcMNPV DNA polymerase. (Top) The 984-residue AcMNPV DNA polymerase is shown in orange with relative positions of SQ/TQ sites indicated. (Bottom) Alignment of AcMNPV DNA polymerase residues 397-428 with the DNA polymerase of 29 indicated baculoviruses. Baculovirus abbreviations are taken from the International Committee of Taxonomy of Viruses (https://talk.ictvonline.org). The red box indicates the conserved ATM/ATR SQ/TQ site found at residue 412 of AcMNPV DNA polymerase. Gray shading indicates conservation between sequences.

FIG. 5.2

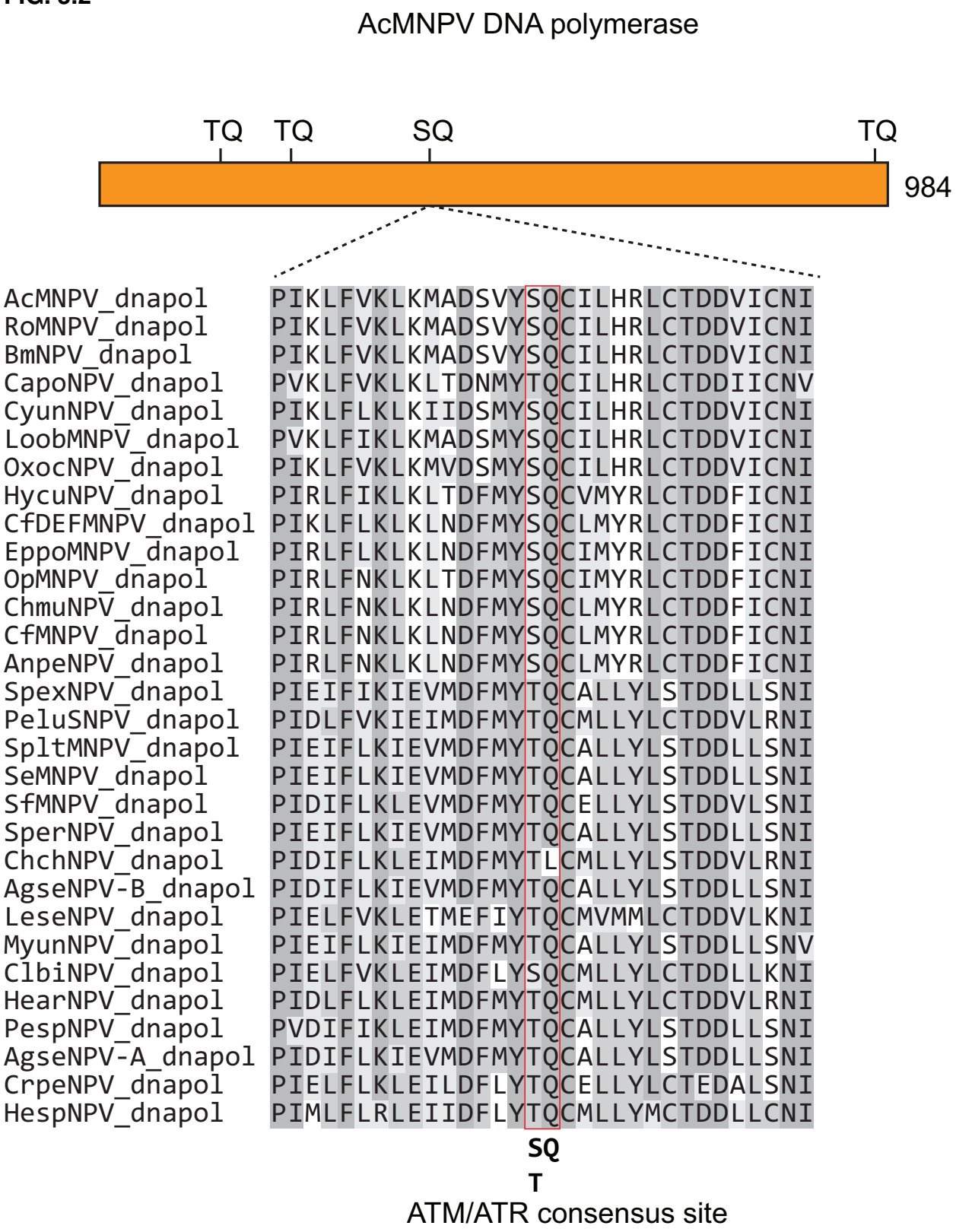
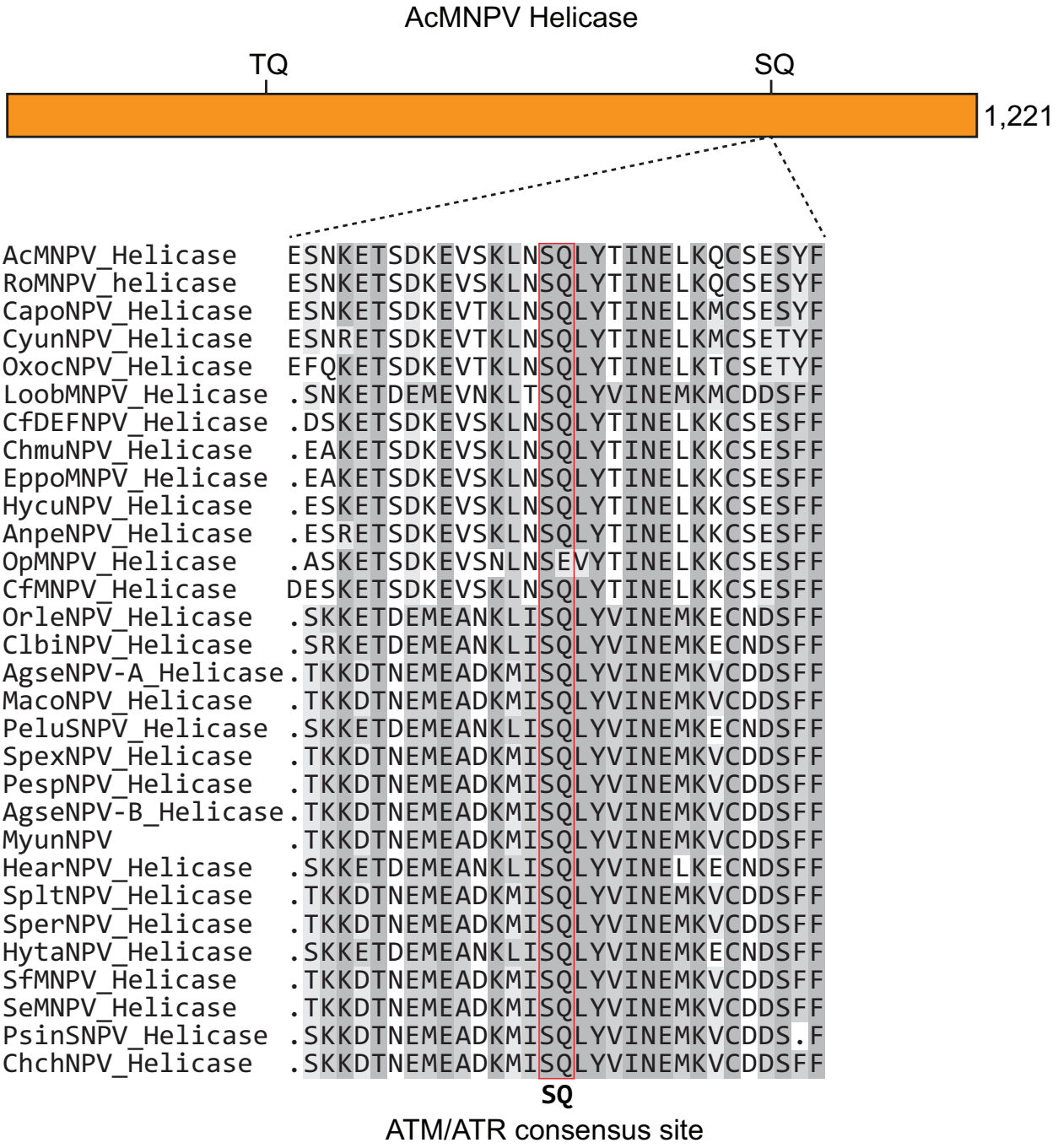

AcMNPV DNA polymerase
TQ
TQ
SQ
TQ
984
AcMNPV_dnapol PIKLFVKLKMADSVYSQCILHRLCTDDVICNI
RoMNPV_dnapol PIKLFVKLKMADSVYSQCILHRLCTDDVICNI
BmNPV_dnapol PIKLFVKLKMADSVYSQCILHRLCTDDVICNI
CapoNPV_dnapol PVKLFVKLKLTDNMYTQCILHRLCTDDIICNV
CyunNPV_dnapol PIKLFLKLKIIDSMYSQCILHRLCTDDVICNI
LoobMNPV_dnapol PVKLFIKLKMADSMYSQCILHRLCTDDVICNI
OxocNPV_dnapol PIKLFVKLKMVDSMYSQCILHRLCTDDVICNI
HycuNPV_dnapol PIRLFIKLKLTDFMYSQCVMYRLCTDDFICNI
CfDEFMNPV_dnapol PIKLFLKLKLNDFMYSQCLMYRLCTDDFICNI
EppoMNPV_dnapol PIRLFLKLKLNDFMYSQCIMYRLCTDDFICNI
OpMNPV_dnapol PIRLFNKLKLTDFMYSQCIMYRLCTDDFICNI
ChmuNPV_dnapol PIRLFNKLKLNDFMYSQCLMYRLCTDDFICNI
CfMNPV_dnapol PIRLFNKLKLNDFMYSQCLMYRLCTDDFICNI
AnpeNPV_dnapol PIRLFNKLKLNDFMYSQCLMYRLCTDDFICNI
SpexNPV_dnapol PIEIFIKIEVMDFMYTQCALLYLSTDDLLSNI
PeluSNPV_dnapol PIDLFVKIEIMDFMYTQCMLLYLCTDDVLRNI
SpltMNPV_dnapol PIEIFLKIEVMDFMYTQCALLYLSTDDLLSNI
SeMNPV_dnapol PIEIFLKIEVMDFMYTQCALLYLSTDDLLSNI
SfMNPV_dnapol PIDIFLKLEVMDFMYTQCELLYLSTDDVLSNI
SperNPV_dnapol PIEIFLKIEVMDFMYTQCALLYLSTDDLLSNI
ChchNPV_dnapol PIDIFLKLEIMDFMYTLCMLLYLSTDDVLRNI
AgseNPV-B_dnapol PIDIFLKIEVMDFMYTQCALLYLSTDDLLSNI
LeseNPV_dnapol PIELFVKLETMEFIYTQCMVMMLCTDDVLKNI
MyunNPV_dnapol PIEIFLKIEIMDFMYTQCALLYLSTDDLLSNV
ClbiNPV_dnapol PIELFVKLEIMDFLYSQCMLLYLCTDDLLKNI
HearNPV_dnapol PIDLFLKLEIMDFMYTQCMLLYLCTDDVLRNI
PespNPV_dnapol PVDIFIKLEIMDFMYTQCALLYLSTDDLLSNI
AgseNPV-A_dnapol PIDIFLKIEVMDFMYTQCALLYLSTDDLLSNI
CrpeNPV_dnapol PIELFLKLEILDFLYTQCELLYLCTEDALSNI
HespNPV_dnapol PIMLFLRLEIIDFLYTQCMLLYMCTDDLLCNI
SQ
T
ATM/ATR consensus site

FIG. 5.3. AcMNPV helicase. (Top) The 1,221-residue AcMNPV helicase is shown in orange with relative positions of SQ/TQ sites indicated. (Bottom) Alignment of AcMNPV DNA helicase residues 947-978 with DNA polymerase of 29 indicated baculoviruses. Baculovirus abbreviations are taken from the International Committee of Taxonomy of Viruses (https://talk.ictvonline.org). The red box indicates the conserved ATM/ATR SQ/TQ site found at residue 962 of AcMNPV helicase. Gray shading indicates conservation between sequences.

FIG. 5.3


AcMNPV Helicase
TQ
SQ
1,221
AcMNPV_Helicase ESNKETSDKEVSKLNSQLYTINELKQCSESYF
RoMNPV_helicase ESNKETSDKEVSKLNSQLYTINELKQCSESYF
CapoNPV_Helicase ESNKETSDKEVTKLNSQLYTINELKMCSESYF
CyunNPV_Helicase ESNRETSDKEVTKLNSQLYTINELKMCSETYF
OxocNPV_Helicase EFQKETSDKEVTKLNSQLYTINELKTCSETYF
LoobMNPV_Helicase .SNKETDEMEVNKLTSQLYVINEMKMCDDSFF
CfDEFNPV_Helicase .DSKETSDKEVSKLNSQLYTINELKKCSESFF
ChmuNPV_Helicase .EAKETSDKEVSKLNSQLYTINELKKCSESFF
EppoMNPV_Helicase .EAKETSDKEVSKLNSQLYTINELKKCSESFF
HycuNPV_Helicase .ESKETSDKEVSKLNSQLYTINELKKCSESFF
AnpeNPV_Helicase .ESRETSDKEVSKLNSQLYTINELKKCSESFF
OpMNPV_Helicase .ASKETSDKEVSNLNSEVYTINELKKCSESFF
CfMNPV_Helicase DESKETSDKEVSKLNSQLYTINELKKCSESFF
OrleNPV_Helicase .SKKETDEMEANKLISQLYVINEMKECNDSFF
ClbiNPV_Helicase .SRKETDEMEANKLISQLYVINEMKECNDSFF
AgseNPV-A_Helicase.TKKDTNEMEADKMISQLYVINEMKVCDDSFF
MacoNPV_Helicase .TKKDTNEMEADKMISQLYVINEMKVCDDSFF
PeluSNPV_Helicase .SKKETDEMEANKLISQLYVINEMKECNDSFF
SpexNPV_Helicase .TKKDTNEMEADKMISQLYVINEMKVCDDSFF
PespNPV_Helicase .TKKDTNEMEADKMISQLYVINEMKVCDDSFF
AgseNPV-B_Helicase.TKKDTNEMEADKMISQLYVINEMKVCDDSFF
MyunNPV .TKKDTNEMEADKMISQLYVINEMKVCDDSFF
HearNPV_Helicase .SKKETDEMEANKLISQLYVINELKECNDSFF
SpltNPV_Helicase .TKKDTNEMEADKMISQLYVINEMKVCDDSFF
SperNPV_Helicase .TKKDTNEMEADKMISQLYVINEMKVCDDSFF
HytaNPV_Helicase .SKKETDEMEANKLISQLYVINEMKECNDSFF
SfMNPV_Helicase .TKKDTNEMEADKMISQLYVINEMKVCDDSFF
SeMNPV_Helicase .TKKDTNEMEADKMISQLYVINEMKVCDDSFF
PsinSNPV_Helicase .SKKDTNEMEADKMISQLYVINEMKVCDDS.F
ChchNPV_Helicase .SKKDTNEMEADKMISQLYVINEMKVCDDSFF
SQ
ATM/ATR consensus site

proteins. The Chk1 and Chk2 both function downstream of ATR and ATM and are responsible for regulating a wide range of factors, including DNA repair factors and cell cycle regulation factors [221]. Baculovirus modulation of the DDR may require functions of these downstream DDR signal transducers, or other factors that are activated by Chk1 or Chk2 in turn. This could explain the baculovirus reduction of γ-H2AX during vDNA synthesis as Chk1 and Chk2 functions don't require γ-H2AX interaction. Thus, a downstream DDR kinase that requires PIKK activity to function may be responsible for promoting baculovirus multiplication.

5.9 LEF-7 and cGAS

As detailed in Chapter 4, preliminary results show LEF-7 interacts with SfcGAS. LEF-7, as an F-box protein, functions as substrate adapter for *S. frugiperda* SCF complexes. Following LEF-7 binding of its substrate and the SCF complex, the substrate is polyubiquitinated by the SCF complex and likely subjected to proteasomal degradation. In Chapter 4 the wild-type LEF-7 doesn't show SfcGAS in the pulldown, consistent with LEF-7 promoting the degradation of SfcGAS. However, the LEF-7 F-box mutants F-box domain deletion (ΔF-box) and LP15AA pulldown SfcGAS as they are unable to interact with the SCF complex, and thus unable to promote polyubiquitination and degradation. If LEF-7 is indeed triggering the proteasomal degradation of SfcGAS then treatment of cells with a proteasome inhibitor such as MG132 should allow for the pulldown of SfcGAS even with wild-type BioID-LEF-7. To further assess if SfcGAS is a LEF-7 substrate, then a proximity labeling BioID-SfcGAS will allow for the labeling and pulldown of LEF-7. Immunofluorescence examination should also show colocalization of SfcGAS and LEF-7(ΔF-box) and LEF-7(LP15AA). SfcGAS should also colocalize with wild-type LEF-7 when cells are treated with a proteasome inhibitor. Additionally, a knockdown or knockout of SfcGAS should rescue the budded virus production defect

observed in AcMNPV∆LEF-7.

The innate immune function of cGAMP synthase (cGAS) is to detect foreign nucleic acids in the cell's cytosol and produce cyclic guanosine-monophosphate adenosine-monophosphate (cGAMP) [105]. In mammals, the production of cGAMP in turn activates Stimulator of Interferon genes (STING), which promotes TANK-binding kinase 1 (TBK1) to phosphorylate interferon regulatory factor 3 (IRF3) [105]. Active IRF3 activates transcription of type I interferon genes [105]. Viruses including HCMV, adenovirus, and HPV, disrupt cGAS or another part of the pathway during infection [7, 191]. Insects, including lepidopterans, lack an interferon response. However, in insects cGAS-STING activation is still associated with an anti-viral response [217]. I hypothesize that LEF-7 is targeting SfcGAS for degradation to avoid the activation of and insect antiviral response. To test this hypothesis a knockdown or knockout of SfSTING and should rescue ∆LEF-7 budded virus production.

Recently it has become clear that cGAS functions in roles outside of the innate immune response. The induction of DNA damage causes cGAS to re-localize to the nucleus, associate with γ-H2AX at DSBs, and suppress homologous recombination [147]. Thus, antagonism of cGAS is an attractive function of LEF-7 as LEF-7 is known to promote homologous recombination and suppress γ-H2AX accumulation. Additionally, the nuclear re-localization of cGAS is associated with phosphorylation of cGAS [147], and F-box proteins are known to bind phosphorylated proteins [123]. I hypothesize that LEF-7 may targeting SfcGAS to shift the balance of repair away from NHEJ and promote homologous recombination. If this hypothesis is correct, then the knockdown or knockout of SfcGAS should enhance baculovirus induced homologous recombination using a homologous recombination reporter system in *S. frugiperda* cells [59].

5.10 Additional targets of LEF-7

While my research has pointed to a possible mechanism through which LEF-7 is promoting viral multiplication and enhancing homologous recombination through SfcGAS antagonism, other LEF-7 functions are still unclear. Specifically, LEF-7 expression in SF21 cells prevents pharmacologically induced γ-H2AX accumulation. While cGAS is known to localize to γ-H2AX [147], cGAS degradation shouldn't impact γ-H2AX levels. Since ATM is known to directly phosphorylate H2AX, the likely remaining mechanisms for LEF-7 are interfering with ATM activation, interfering with ATM directly, promoting the inactivation of ATM, interfering with H2AX directly, and promoting the dephosphorylation of H2AX.

Prior to my work, it was thought that LEF-7 could not be preventing ATM activation or degrading ATM directly, as ATM was critical for baculovirus multiplication. However, I have shown *S. frugiperda* ortholog of ATM (SfATM) is in fact dispensable for baculovirus multiplication. Tip60 histone acetyltransferase (Tip60) is a acetyltransferase responsible for acetylating ATM which allows for ATM activation and autophosphorylation [254]. Tip60 is a known viral target for ATM antagonism, as HPV E6 and adenovirus E1B55k and E4orf6 target Tip60 for proteasome-mediated degradation [96, 112]. LEF-7 may be targeting *S. frugiperda* homolog of Tip60 for degradation in a similar manner to prevent SfATM activation and block the accumulation of γ-H2AX. LEF-7 may also be directly interfering with total ATM levels similar to the EBV protein latent membrane protein 1 (LMP-1) downregulation of ATM [92]. If LEF-7 is degrading ATM, then replication of ΔLEF-7 virus in ATM knockout cells generated in Chapter 2 should rescue the budded virus defect. It is also possible that LEF-7 is promoting the inactivation of the activated phosphorylated ATM. ATM inactivation occurs through a gradual process of dephosphorylation by protein phosphatases and deacetylation by sirtuin 7 [88, 211, 240,

259]. The gradual nature and multiple steps needed makes the promotion of ATM inactivation an unlikely mechanism of action for LEF-7.

Alternatively, LEF-7 may be directly targeting H2AX. Previous work by Dr. Jonathan Mitchell in the Friesen lab demonstrated that *S. frugiperda* ortholog of H2AX (SfH2AX) localized with marginalized host DNA but not viral replication centers by 24 hpi, suggesting that AcMNPV may be excluding H2AX from viral replication centers [181]. LEF-7 may be targeting SfH2AX directly, degrading the variant histone and preventing the accumulation of SfH2AX on vDNA. This may be to prevent association of DDR factors with vDNA, as DDR factors such as mediator of DNA damage checkpoint protein 1 (MDC1) localize to DNA via interactions with γ-H2AX [252]. Indeed, DDR activated cGAS localizes to DNA lesions via γ-H2AX [147]. Additionally, LEF-7 may specifically target γ-H2AX, but not unphosphorylated H2AX, as some F-box proteins are known to be specific for phosphorylated versions of proteins [123]. LEF-7 mediated reduction of γ-H2AX might be a mechanism through which LEF-7 promotes homologous recombination, as accumulation of γ-H2AX reduces C-terminal binding protein 1 (CtBP1) interacting protein (CtIP) mediated end resection necessary for homologous recombination and instead favors non-homologous end joining (NHEJ) [101]. If LEF-7 is degrading SfH2AX during infection, then replication of the ΔLEF-7 in the SfH2AX knockout cells from Chapter 3 should rescue ΔLEF-7 virus yields. It is unlikely that LEF-7 directly dephosphorylates γ-H2AX due to a lack of homology to known phosphatase sequences. It is also unlikely that LEF-7 promotes phosphatase activity as previous work in the Friesen lab showed LEF-7 reduces accumulation of γ-H2AX even in the presence of phosphatase inhibitors (unpublished data).

Another possible target of LEF-7 is the Mre11, Rad50, and Nbs1 (MRN) complex. The MRN complex is involved in ATM activation and localization following DNA damage [52]. Indeed, MRN has anti-viral functions and is targeted by other viral proteins,

including E1b-55k/E4-ORF3 targeting MRN components for proteasomal degradation [237]. If LEF-7 causes disruption of MRN complexes, it would contribute to the ability of LEF-7 to reduce ATM activiation and subsequent γ-H2AX accumulation. However, MRN promotes end resection and homologous recombination [280], which would function in opposition to LEF-7's known function of promoting homologous recombination [59].

To promote homologous recombination and viral DNA replication, LEF-7 may be disrupting the NHEJ pathway mediated by DNA-PK. Interference with Ku70, Ku80, or DNA-PKcs subunits of DNA-PK increase homologous recombination [8, 10]. DNA-PK is a known viral target, as adenovirus E4 34k and E1b 55k interact with and inhibit DNA-PK [12, 28]. LEF-7 may promote homologous recombination through targeting DNA-PK for proteasomal degradation, shifting DNA repair away from NHEJ and towards homologous recombination.

5.11 Summary

Overall, my thesis work has provided new details into how baculoviruses modulate the DDR within their *lepidopteran* hosts. While ATM has been shown to be dispensable for AcMNPV multiplication, evidence points to additional DDR pathways or homologous recombination being targets of baculovirus DDR modulation. My efforts to generate a site-specific genome edit has expanded the tools available for baculovirus research. The *S. frugiperda* CRISPR-Cas9 system can easily be expanded into investigations of other host genes. I have also identified a novel interaction between the viral LEF-7 protein and the host cGAS innate immune sensor. Thus, my thesis work has expanded the understanding of how viruses can modify host responses and opened new areas of research.

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