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DISSERTATION TITLE

Functional Implications of the BAF-B1 Axis During Vaccinia Virus Infection

BY

Nouhou Ibrahim, MSc.

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University of Nebraska Lincoln

FUNCTIONAL IMPLICATIONS OF THE BAF-B1 AXIS DURING THE VACCINIA VIRUS LIFE CYCLE

by

Nouhou Ibrahim

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

(Microbiology and Molecular Biology)

Under the Supervision of Professor Matthew S. Wiebe

Lincoln, Nebraska

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FUNCTIONAL IMPLICATIONS OF THE BAF-B1 AXIS DURING THE VACCINIA VIRUS LIFE CYCLE

Nouhou Ibrahim, MSc., Ph.D.

University of Nebraska, 2014

Advisor: Matthew Wiebe

Vaccinia virus is the prototypic member of the *Poxviridae* family, which includes variola virus, the agent of smallpox. Poxviruses encode their own transcriptional machinery and a set of proteins to evade the host defense system, and thus are able to replicate entirely in the cytoplasm of their host. The poxvirus life cycle occurs in sequential stages: early gene expression, DNA replication, intermediate gene expression and then late gene expression and morphogenesis. The temporally staged poxvirus life cycle makes viral DNA replication a required event for post-replicative events to occur. However, viral DNA replication itself depends on an early viral Ser/Thr kinase B1, which inactivates the cellular DNA-binding protein, the barrier-to-autointegration factor or BAF. BAF is a well-conserved protein with essential roles in mitotic nuclear reassembly. In the absence of B1, BAF colocalizes with viral replication sites and inhibits viral DNA replication.

Results from these studies demonstrate that BAF relocalizes to any cytosolic dsDNA to form BAF-DNA nucleoprotein complexes. Further, DNA-binding and homodimerization properties but not LEM-domain interaction are essential for BAF's activity. Several cellular proteins are present at BAF-DNA complexes, but their contribution to BAF's activity remains to be elucidated. Also,

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a previous report showed that B1 regulates viral intermediate gene expression through an unknown mechanism. Our studies showed that in the absence of B1, BAF inhibits viral intermediate gene expression at the transcriptional level. Surprisingly, BAF modestly inhibits reporter gene activity under non-vaccinia nuclear promoters and has no apparent effect on T7 promoter. Further, the absence of active B1 can lead to defects in morphogenesis and/or viral release /spread independently of B1's role in DNA replication. Collectively, studies presented in this dissertation highlight the significance of the BAF-B1 axis during vaccinia life cycle.

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This work is dedicated to my wife and sons!

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ABBREVIATIONS LIST

aa	amino acid
AraC	cytosine β-D-arabinoside
ATP	Adenosine-tri-phosphate
BAF	Barrier-to-autointegration
BAF-L	BAFF-like
bp	Base pair
BCA	Bicinchoninic acid
BHK	Baby hamster kidney
BrdU	5-bromo-2'-deoxyuridine
CAT	chloramphenicol acetyltransferase
c-Jun	transcription factor
CMV	Cytomegalovirus
CPE	Cytopathic effect
Crx	cone-rod homoeobox
DAPI	4'6'-Dianidino-2-phenlindole
DDT	dithiothreitol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Dideoxyribonucleic acid
ds	Double-stranded
EDTA	Ethylenediaminetetraacetic acid
EFC	Entry-Fusion Complex
eIF-2α	eukaryotic initiation factor-2
ERK	Extracellular signal-regulated kinase
ESCs	embryonic stem cells
EV	enveloped virions
FACS	Fluorescence-activated cell sorting

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FBS	Phosphate buffered saline
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent protein
HIV-1	human immunodeficiency virus, type 1
HMGA	high mobility group protein
IFN	Interferon
IL	interleukin
IMV	intracellular mature virions
ISG	IFN-stimulated genes
IV	immature virions
JIP1	JNK interacting protein-1
kD	Kilo Dalton
LEM	Lap2α-Emerin-MAN1
LMNA	gene encoding lamins A and C
Luc	Luciferase
ml	mililiter
mM	miliMolar
MMLV	Moloney murine leukemia virus
mRNA	messenger RNA
MV	mature virions
MVA	modified vaccinia virus Ankara
NFkB	nuclear factor kB
NHK-1	nucleosomal histone kinase-1
OAS	oligoadenylate synthetase
ORF	Open Reading Frame
PAMP	pathogen-associated molecular pattern
PCR	Polymerase Chain Reaction

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pfu	Plaque-forming unit
PIC	pre-integration complexes
POCs	poxvirus orthologous clusters
PRR	pattern recognition receptor
Rb	Retinoblastoma protein
RFP	Red Fluorescent protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
RPA	Replication Protein A
RPO	Vaccinia RNA polymerase subunit
RT-qPCR	Reverse Transcriptase-quantitative PCR
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	serine
shRNA	Short interfering RNA
siRNA	short interfering RNA
TBP	TATA-binding protein
Thr	threonine
TLR	Toll-like receptors
ts	Temperature-sensitive
VACV	Vaccinia virus
VETF	Vaccinia virus early transcription factor
VITF	Vaccinia virus intermediate transcription factor
VLTF	Vaccinia virus late transcription factor
VPEF	vaccinia virus penetration factor
VRK	virus-related kinases

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VSV-G	Vesicular stomatitis virus-glycoprotein G
WHO	World Health Organization
WR	Western Reserve
WT	Wild type
YY1	Transcription factor Yin Yang 1

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CHAPTER I

INTRODUCTION

A. BARRIER-TO-AUTOINTEGRATION FACTOR

The cytoplasm of mammalian cells is the first line of defense against pathogen such as viruses. The cytoplasmic innate effectors, such pattern recognition receptors (PRR), recognize pathogen associated molecular patterns (PAMP) and activate innate immune responses against viral infection (1, 2). Although PRRs are established as critical activators of the innate immune pathways in the presence of viruses with a cytoplasmic life cycle, such as poxviruses, other cytosolic DNA sensing effectors have been proposed to exist (3, 4). The role of these proteins in innate responses as well as how they recognize cytoplasmic DNA are poorly understood. One such protein is the barrier-to-autointegration factor, BAF.

BAF, an 89-amino acid and 10kDa, is a cellular dsDNA-binding protein (see figure A1.1) present both in the nucleus and the cytoplasm (5-7). Although there is a significant presence of BAF at the nuclear inner membrane, both nuclear and cytoplasmic pools of BAF do not readily exchange, but are mobile during interphase (8). With the exception of the thymus and peripheral blood leukocytes, BAF is widely expressed in most tissues (9).

Several species, invertebrates (*C. elegans, D. melanogaster*) and vertebrates (mouse, human) express a protein with high similarity to BAF, and BAF's sequence alignment analysis from several organisms showed that BAF is a highly conserved protein (see figure A1.2). For example, the degree of identity

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varies from 100% in *P. troglodytes* to 58% in *C. elegans* compared to Homo sapiens BAF (10).

A.1. BAF is a potent inhibitor of poxvirus DNA replication

BAF has been established as a potent inhibitor of poxvirus DNA replication, but much remains to be determined regarding its function and regulation as a host defense protein (11). During infection with a vaccinia virus (VACV) mutant that expresses a defective B1 (called ts2, see more detail in poxviruses section), BAF relocalizes to the viral replication sites while no relocalization is observed in WT-infection. The relocalization of BAF results in the inhibition of viral DNA replication in ts2 infection (11). Further, BAF is highly phosphorylated during VACV WT but not during ts2 infection, an indication that the phosphorylation status of BAF is a regulatory mechanism employed by VACV (11). The viral B1 kinase phosphorylates Thr2/Thr3/Ser4 residues at N-terminus of BAF in the same fashion as a group of cellular kinases, called vaccinia related kinases (VRKs, see more detail in poxviruses section). This results in the inhibition of BAF's dsDNA-binding abilities as well as its interactions with cellular proteins (12).

A.2. BAF is intrinsically a homodimeric DNA-bridging protein

The non-specific DNA-binding activity of BAF is associated with the existence of a helix-hairpin-helix motif within BAF, a DNA-binding motif found in most proteins that bind to dsDNA in non-sequence specific manner (13, 14). The non-sequence-specific binding to dsDNA by BAF involves hydrogen bonds

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between amide groups of BAF and phosphate groups of the dsDNA backbone (13, 15).

Based on data consistent with a dimeric state as determined by gel filtration, crystallography and NMR, it is reported that BAF exists as a homodimer (5, 13). The crystal structure of BAF-dsDNA complex showed that a dimer of BAF interacts with the phosphate backbone of dsDNA on the minor groove (figure A1.1). This interaction involves two pairs of helix-hairpin-helix motifs located at both opposite sides of the dimeric BAF, and causes the bridging of the dsDNA molecule (16). Based on internal reflection fluorescence microscopy (TIRFM), it is shown that BAF compacts dsDNA by forming intramolecular loops rather than collapsing (17). The intramolecular cross-bridging of dsDNA results in the formation of high-order nucleoprotein BAF-DNA complexes stable even at high ionic strength of 100mM NaCl and 1M NaCl (16-18). Although BAF binds to as little as 7 base pairs (bp) of DNA to form a nucleoprotein complex (16), considering its dimerization's capability, it is suggested that one BAF dimer is sufficient to condense 100bp of DNA (17, 18).

It is believed that BAF-DNA nucleoprotein complexes involve many BAF multimers, although it is not clear how far from each other these multimers of BAF are arranged on dsDNA molecules. Mutational and biochemical studies uncovered several residues whose mutations affect dsDNA-binding (K6A, G25E, G47E, V51E, and L46E), dimerization (G47E) and interaction with other proteins such as emerin (G25E, L46E, K53E) (19, 20).

The dimerization of BAF is essential for binding to dsDNA, and through its

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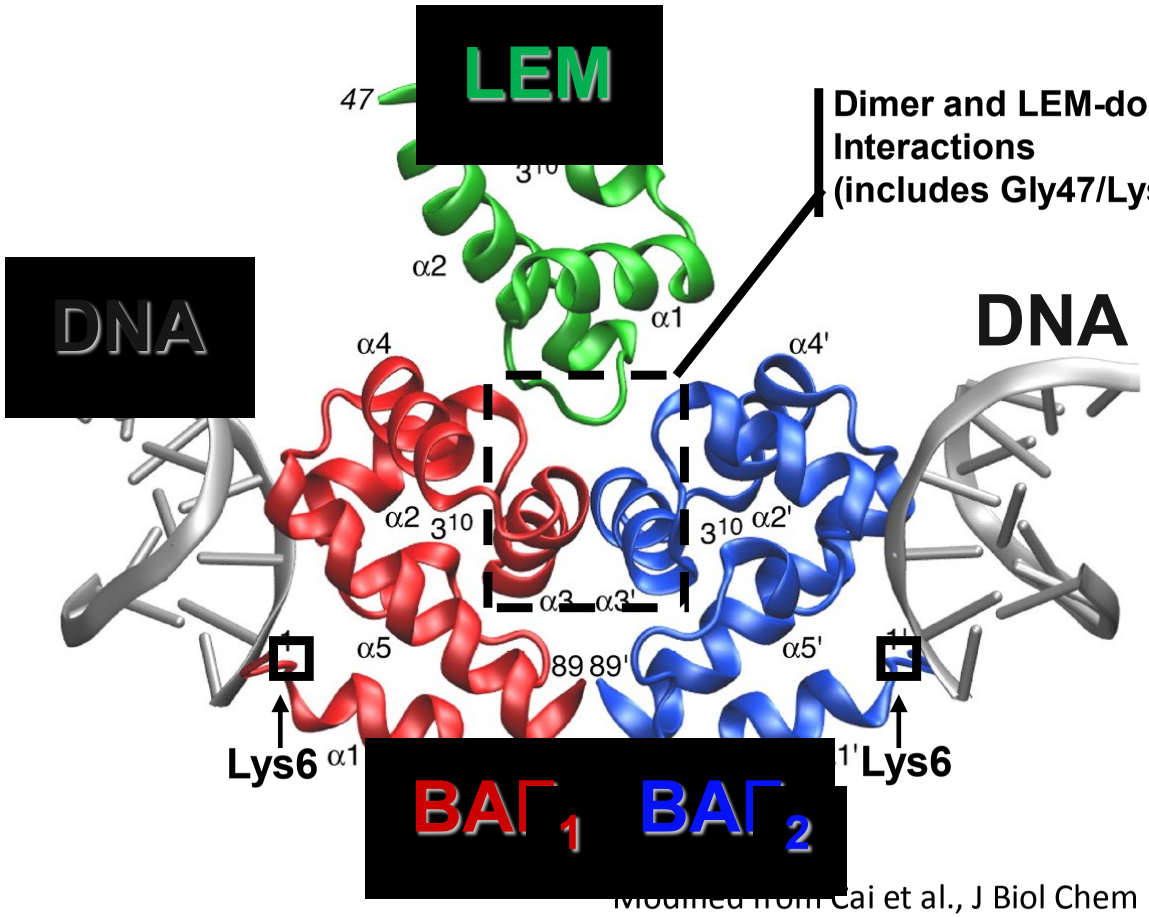

Modified from Cai et al., J Biol Chem (2007) 282:14525-35.

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Figure A1.1: Ribbon diagram of BAF dimer-LEM-dsDNA complex. This is the resolved crystal structure of BAF dimer-dsDNA-LEM-domain complex. The BAF dimer is in the middle of the complex, in interactions with two DNA molecules as well as a LEM-domain at its binding surface. The two subunits of the BAF dimer are shown in blue and red, dsDNA in black and LEM-domain in green. Residues shown as involved in dimerization and interactions with dsDNA and LEM-domain are used in this study.

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interaction with several nuclear proteins, BAF is involved in many essential nuclear functions such as nuclear organization, cell cycle progression. These intrinsic properties of BAF, dsDNA-binding, homodimerization and interactions with other proteins, are central for this work, as our hypothesis to determine whether the relocalization of BAF in the presence of a cytosolic dsDNA depends on any these properties and the molecular mechanisms governing them.

A.3. BAF interacting partners are mostly nuclear proteins

Several studies showed that BAF is enriched at the inner nuclear membrane, and this is due to its interaction with several nuclear proteins (8, 20-22). The nuclear envelope protects the chromatin from the cytosol, and it is composed of the nuclear lamina, a double nuclear membrane and nuclear pores complexes. The nuclear envelope also serves as an anchor for chromatin attachment, thus influences chromatin organization. The attachment of chromosomes to the inner nuclear envelope is mediated in part by A- and B-type lamins. Lamins are considered as a platform for chromatin organization and are involved in other nuclear functions such as replication, transcriptional regulation, and DNA damage repair (23, 24). As a component of the nuclear lamina, BAF interacts with histones, lamins and the LEM (lamina-associated polypeptide)-domain proteins to regulate key nuclear functions (24-26).

One well-characterized set of interacting partners of BAF are the LEM (Lap2α-Emerin-MAN1)-domain proteins (figure B1.1), a group of several inner nuclear envelope proteins sharing a domain containing around 40-residues initially identified in Lap2α-Emerin-MAN1 (27, 28). Other members of the LEM-

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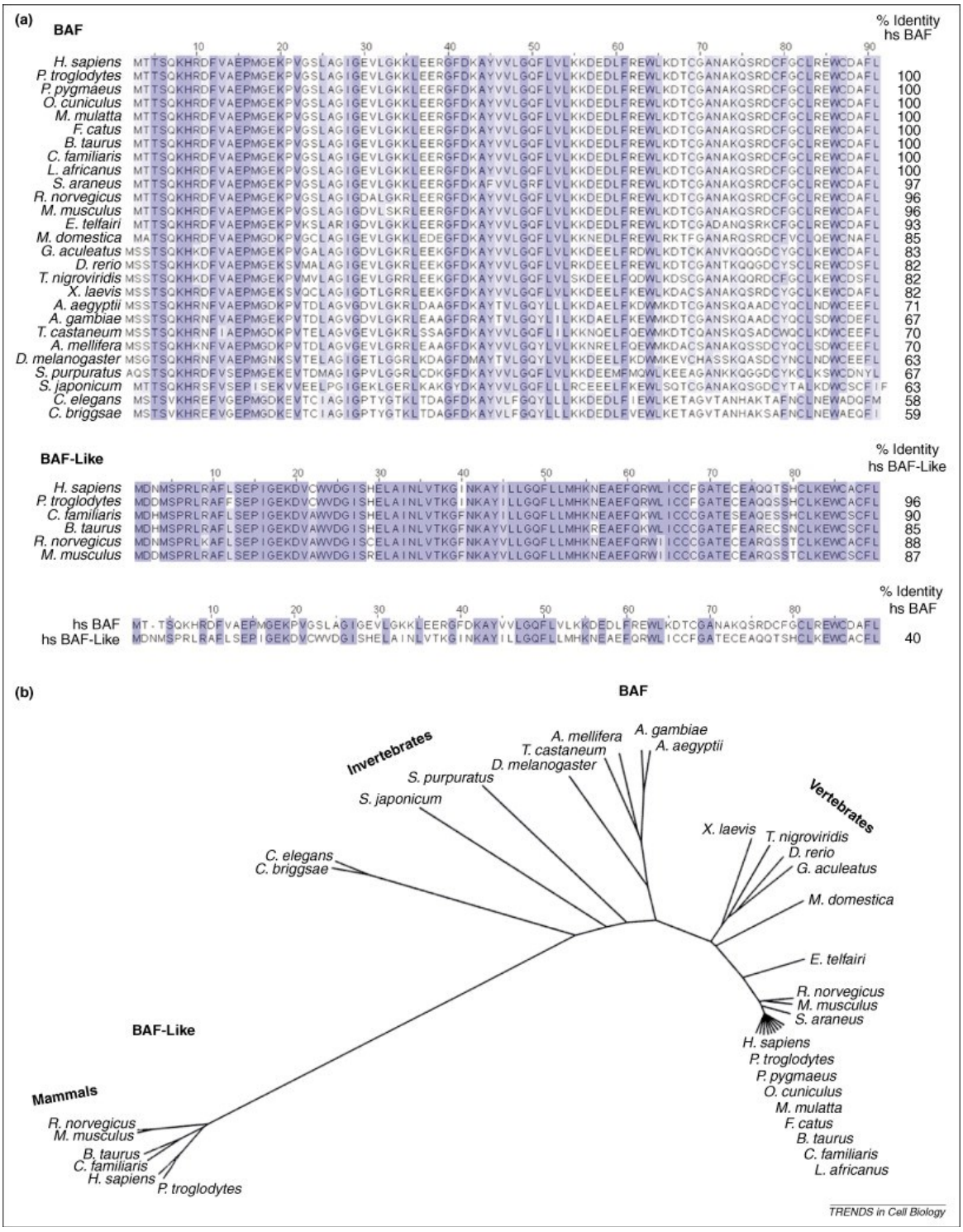

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Figure A1.2. BAF is a conserved protein across species. A. Multiple alignment of BAF (top) and BAF-like (bottom) primary sequences from several species showing percent identity to *Homo sapiens* sequence (number on the right). B. The phylogenetic tree generated based on alignment shown above. Adopted from Margalit et al., Trends in Cell Biology 2007 Volume 17 (4):202-208.

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-domain family include several splicing forms of Lap2, Lem-3 and otefin (29-31).

The LEM-domain proteins are involved in a wide range of cellular functions, such as nuclear envelope morphology, cell cycle, chromatin organization, gene expression and signaling pathways (32-34). LEM-domain proteins are essentially vital in *C. elegans* (35), while mutations within their genes (LMNA and LMNB) cause muscular dystrophy and laminopathic diseases, which are rare ageing syndromes in human (36).

Structural studies of Lap2α splicing forms showed that the LEM-domain is composed of two large and parallel α-helices, and a short α-helix at the N'terminus (37-39). Most LEM-domain proteins, including Lap2β, Emerin and MAN1, have a transmembrane domain anchored in the inner nuclear membrane. But Lap2α, an isoform of Lap2, is soluble in the nucleus. In addition to the LEM-domain, a DNA-binding domain is found in some LEM-domain proteins such as Lap2β; but MAN1, Emerin and Lap1 do require BAF to interact with chromatin (40, 41). Whitin the nucleus BAF interacts with histone H3 and the linker histone H1.1 (20). Histones (H2A, H2B, H3 and H4) compose the nucleosome, a fundamental unit of the chromatin maintained as a structural entity by the linker histone H1 (H1 has many subtypes) (42). Therefore BAF provides an attachment link between the chromatin and LEM-domain proteins with no DNA-binding ability.

In efforts to understand the functional implications of BAF in a wide range of nuclear processes, affinity purification coupled to mass spectrometry methods was employed to identify proteins interacting with BAF (43). In addition to the

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known partners such as LEM-domains proteins, novel BAF-interacting partners were discovered including transcription factors, DNA repair proteins, histone chaperones and chromatin remodeling proteins (43). The association of BAF with each of these proteins may associate BAF to specific cellular processes. It is conceivable to say that in function of its interacting partners, BAF may be involved in a wide range of cellular processes, although the extent and significance of these interactions are not well understood.

A.4. Phosphorylation and interacting partners of BAF regulates its activity

Early studies using *D. melanogaster* and *C. elegans* provided evidence of BAF's role in key cellular functions (8, 21, 25, 26). These studies, including mutational and depletion approaches, showed that BAF is involved in several mitotic processes, and this is regulated by BAF's phosphorylation status and its interaction with several inner nuclear proteins involved in chromatin and nuclear envelope organization (25, 26). The *C. elegans* kinase VRK-1 and the *D. melanogaster* kinase nucleosomal histone kinase-1 (NHK-1) phosphorylate residues Thr2/Thr3/Ser4 at the N-terminal domain of BAF (12, 25, 26).

A.4.1. The phosphorylation of the N-terminus of BAF regulates its location

In *C.elegans* and *D. melanogaster,* BAF's phosphorylation regulates its activity (25, 26). In addition of NHK-1 and the *C. elegans* VRK1, the cellular VRKs and the viral B1 phosphorylate BAF. The phosphorylation of BAF by these kinases abrogates the DNA-binding properties of BAF and reduces its interactions with LEM-domain proteins (12, 44). Further, using a series of BAF

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mutants carrying a single or combined amino acid substitutions of Thr3/Thr2/Ser4 (MTTQ (WT) --> MTTAQ, MAASQ, MTTDQ and MAAAQ), showed that the phosphorylation of BAF also regulates its location (11, 12). During VACV infection, the phosphorylation status of residues at the N-terminus of BAF is a critical determinant of BAF's ability to localize and exert its anti-poxvirus activity (11).

A.4.2. BAF-L regulates BAF function via heterodimerization.

BAF is also regulated by its interaction with BAF-L (barrier-to autointegration factor-Like), and possibly its interacting partners such as LEM-domain proteins. Encoded a paralog of BANF1 gene, BANF2, BAF-L is expressed in several organisms (figure A1.2, bottom). BAF-L is highly expressed in pancreas and testis, but absent in heart, kidney and muscle (45). BAF-L is a 10kDa protein with 40% identity and 53% similarity to BAF at amino acid level (figure A1.2, bottom), but does not bind to dsDNA (45). There is no indication that BAF-L is phospho-regulated. BAF-L can homodimerize and interacts with BAF to form a heterodimer.

The interaction of BAF-L to BAF disrupts BAF's ability to bidn to dsDNA and form high order nucleoprotein complexes (45). When interacting with BAF-L, BAF binds to dsDNA using only one site instead of both sites when BAF is a homodimer (16, 45). This consequently affects the stability of BAF-DNA complexes.

A.5. BAF is essential in key cellular processes

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BAF is an essential protein involved in several cellular processes including mitosis. Depletion of BAF in the gonads of *C. elegans* and *D. melanogaster* resulted in an early developmental arrest, and death in early stage of embryonic development (24, 25, 46). In human and mouse, depletion of BANF1 gene with a BAF-specific shRNA resulted in a decrease in the survival and self-renewal ability of ESCs (47). BANF1 gene knockdown altered cell cycle by increasing cells in G2-M phase (47).

A mutation within BANF1 gene, Ala12Thr, is associated with a atypical form of progeria in humans (48). Progeria, a premature aging in humans is associated with alterations and mutations in genes governing DNA repair and nuclear envelope formation (49). The mutation, Ala12Thr, affects the stability of protein, and causes the formation of blebs (48, 50, 51).

A.6. Nuclear organization, a critical step in cell cycle, is regulated by BAF

During mitosis, the nuclear envelope is disassembled and reassembled to establish the nuclear architecture. Several nuclear membrane proteins are recruited to chromosomes to mediate nuclear disassembly/reassembly, a critical step for cell cycle progression (52). The expression of GFP-BAF in *C. elegans* showed that BAF is ubiquitously present in the cell, and both cytosolic and nuclear pools of BAF are dynamic during mitosis (8, 10).

The localization pattern of BAF is cell type dependent. In primary cell lines, such as TIG-1, BAF is predominantly nuclear, but in cancer-derived cell lines such as HeLa, BAF is present in both cytoplasm and nucleus (53). During mitosis, the accumulation of BAF in the nucleus of HeLa cells is essential for S-

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phase progression, and nuclear envelope recruitment of lamin A (53). Specifically, during telophase, several nuclear proteins, including Lap2α, A-type lamins and emerin, are recruited to a core region near spindle attachment sites on chromosomes to form complexes. Once formed, these complexes spread on the chromosomes during nuclear assembly and contribute to DNA condensation (21, 46). The core region on telophase chromosomes depends on BAF for chromatin structure, as well as the reformation of nuclear envelope (54-56).

The role of BAF in the reformation of nuclear envelope during nuclear reassembly is mediated by the recruitment of Lap2α, A-type lamins and emerin to core chromosomes, stabilizing the interaction between nuclear envelope and chromatin (54). In *C. elegans*, depletion of BAF resulted in abnormal condensed chromatin in interphase and anaphase-bridged chromatin, and defective chromosome segregation (18, 57). Further, depletion of *C. elegans* VRK-1 led to constitutive associations of chromatin with LEM-domain proteins, and an abnormal formation of nuclear envelope, similar to those observed in RNAi-depletion of BAF (26).

Using nuclear assembly extracts from *Xenopus* eggs, it was shown that large concentrations of BAF promote chromatin hypercondensation and nuclear assembly arrest, but small concentrations of BAF enhance chromatin decondensation and nuclear growth (7). These studies showed that BAF level of expression is tighly regulated during mitosis. However, in *C. elegans* and *D. melanogaster*, overexpression of BAF did not interfere with nuclear assembly

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(24, 26). The studies showed that BAF plays essential roles during mitosis, cell cycle progression, chromosome segregation and nuclear organization.

A.7. BAF is involved in gene expression via its interacting partners

BAF interacts with Crx (cone-rod homoeobox), a transcription factor for photoreceptor and pineal genes (58). The Crx protein is a member of the OTX Homeobox gene family, a group of transcription factors regulating sensory organs and anterior head structure development (59). *In vivo* studies showed that BAF and Crx colocalize within the nucleus, and BAF represses the transactivation activity of Crx. The mechanism of this repression is not yet understood, but BAF does not interfere with the DNA-binding activity of the Crx (58). Recently, it was shown that the interaction between BAF and Crx requires the presence of DNA, establishing that no direct interaction exists between these two proteins (60). In this context, it is possible that when Crx-mediated transcription is activated, BAF is recruited to the same region of DNA, and therefore interferes the recruitment of other transcription factors.

A.8. BAF is involved in the biogenesis of pre-integration complexes

Studies on the preintegration complexes (PIC) of Moloney murine leukemia virus (MMLV) showed that the pre-treatment of PIC with high salt increased its autointegration activity and interferes with the integration of retroviral cDNA into the host genome (6, 61). The addition of BAF to the salt-treated PICs restored the intermolecular integration activity of PICs (6, 61, 62). In *in vitro* studies showed that the DNA-binding function of BAF contributes to the

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reconstitution of PICs of HIV-1 (human immunodeficiency virus, type 1), while BAF compacts the MMLV cDNA to stimulate its association with the PICs (63, 64). The mechanism by which BAF contributes to the activity of PICs is not well understood, but it is proposed that BAF compacts the retroviral cDNA by blocking autointegration catalyzed by the viral integrase.

The activity of MMLV's PIC can be disrupted by VRK1, showing the importance of BAF within the PIC (65). However, using cell lysates from BAF knockdown cells, it was recently reported that BAF had no effect on HIV-1 infection. This report suggest also that blocking autointegration may not be the mechanism by which BAF contributes to HIV-1 infection (66)

BAF-interacting partners also contribute to PIC activities. Lap2α stabilizes the association of BAF with the MMLV's PICs resulting in the stimulation of intermolecular integration (67). However, there is a conflicting data on the role of emerin during HIV-1 infection. BAF and the viral integrase are required for emerin association with HIV-1 cDNA in vivo (68). For example, another report showed that depletion of emerin in HeLa cells did not affect the infectivity of HIV-1 and MMLV (69). Depletion of BAF and Lap2α in HeLa cells did not affect HIV-1 and MMLV infections (69). Based on these studies, the role of BAF during retroviral infections is controversial and requires further study before any conclusion can be drawn.

B. POXVIRUSES

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B.1. Poxviruses have vertebrate and invertebrate hosts

Poxviridae is a family of large and linear double-stranded (ds) DNA viruses. They are characterized by their unique ability to complete their life cycle in distinctive cytoplasmic "mini-nuclei" sites independently of cellular machinery (70). Based on host tropism, the *Poxviridae* family is divided in two subfamilies: *Chordopoxvirinae* and *Entomopoxvirinae*, infecting respectively vertebrate mammals and birds and invertebrate (insects) hosts (71). Within the *Chordopoxvirinae*, there eight genera: *Avipoxvirus, Capripoxvirus, Cervidpoxvirus, Leporipoxvirus, Molluscipoxvirus Orthopoxvirus, Parapoxvirus, Suipoxvirus and Yatapoxvirus* (70). Vaccinia virus (or VACV) is considered as the prototypic member of the orthopoxvirus (72), because it is used routinely in genetic and biochemical studies on poxviruses. The orthopoxvirus members are morphologically identical, and are genetically and antigenically related (70), and include members with important health implications for their hosts.

B.2. Smallpox is caused by a member of the *Poxviridae* family

Poxviridae includes members that are pathogenic to human and other animals such as variola virus or smallpox (human), ectromelia virus or mousepox (mice), camelpox virus (camel). Variola virus is a highly lethal and contagious virus causing smallpox, a disease characterized by pocks and blisters on the skin. After several years of global vacciniation program, the World Health Organization (WHO) certified that smallpox has been eradicated in 1980, although some laboratories-associated cases of smallpox have been reported. To date, the *orthopoxviridae* remains a class of important human and animal

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pathogens due to the re-emergence of pathogenic orthopoxviruses such as monkeypox virus (73-75). In addition, the cross-species transfer of some orthopoxviruses and the risk of using smallpox virus as a biological weapon are of much concern.

Monkeypox virus causes a rare viral zoonosis endemic in central and western Africa (76), however, it is now an reemerging zoonosis as it has emerged and caused an outbreak in non-endemic areas such as the United States of America (73-75). While the clinical signs of monkeypox virus infection resemble those of smallpox (symptoms, severity and mortality) (74, 77), the mode of transmission of monkeypox virus is not yet known as well as the mechanism of transmission between monkey and humans (75, 78). The primary reservoir of monkeypox virus for human infection is also unknown (79). In addition to its zoonotic hosts (human and monkeys), monkeyvirus has a wide range of reservoir hosts (squirrels, rodents and non-human primates) (76, 80). Thus, monkeypox virus is likely to persist in these hosts, with the potential to cross-species transfer and become a more frequent disease in human.

B.3. *Poxviridae* members have many conserved genes in common

The size of the linear dsDNA poxvirus genome ranges from 130 kb in parapoxviruses to 360 Kb in avipoxviruses, a variation mostly associated with gene coding capacity (70). The genome is flanked at both ends by terminal inverted repeats forming covalently closed hairpin loops (70, 81).

Genome sequencing showed that the genome of poxviruses is AT-rich (82) and that genes common to VACV, Variola virus and cowpox virus are more

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than 90% identical (70). Furthermore, bioinformatics analysis using the poxvirus orthologous clusters (POCs, found at www.poxvirus.org) identified 49 conserved gene families in both *Chordopoxvirinae* and *Entomopoxvirinae*, and 41 gene families conserved in the *Chordopoxvirinae* subfamily alone (83). The same analysis identified 90 conserved genes shared by all of the *Chordopoxvirinae* subfamily including essential genes for their role in replication, transcription and morphogenesis (83). The viral kinase B1 is a conserved protein as only few poxviruses do not express it and those include molluscum contagiosum, crocodilepox virus.

B.4. Vaccinia virus biology

B.4.1. The virion of poxvirus is larger than other animal viruses

The virion of poxviruses is a complex and large structure (70). Membrane-enveloped, the virion has a brick-shaped structure with a central biconcave core, flanked by two lateral bodies (84). The dimensions of the mature virion are 360X270X250nm. The core contains the viral genome, which is packaged and likely wrapped by viral proteins to form a nucleoprotein complex, and a complete set of transcriptional machinery. There are three different forms of poxvirus virions in an infected cell: immature virions (IV), intracellular mature virions (MV) and the extracellular enveloped virions (EV) (85).

The mature virion, based on dry weight, is composed of proteins (90%), lipids (5%), and DNA (3.2%) (85, 86), and there is no evidence of RNA within the virion (85). Based on the solubility of virions in Nonidet P-40 (a neutral detergent) and reducing agent (2-mercaptoethanol or dithiothreitol, DDT), more

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than 100 proteins of the virions were classified as enzymatic (E10R, G4L), structural (A26L, A27L, A9L) (85, 87). In addition, 22 other membrane proteins were classified as structural proteins, and 2 of them show an enzymatic activity with potential implication in the maintenance of disulfide bonds of the membrane proteins (85, 88).

Another 47 core proteins were identified with enzymatic and structure functions, and a set of transcription factors are also reported as a virion-encapsidated, and those include the multisubunit RNA polymerase, a capping enzyme (RNA phosphatase, RNA guanylyltransferase, and RNA (guanine-7)-methyltransferase), a poly (A) polymerase, and the vaccinia early transcription factor (VETF), a late protein, two subunits with DNA-dependent ATPase and helicase activities (89), whose association with the RNA polymerase is necessary for early transcription (90). Further a viral DNA topoisomerase, the nucleoside-triphosphate phosphohydrolases I and II, and the kinase B1 are also found in the core (91, 92). So far, three proteins, VH1 (dual-specificity phosphatase), F17 (phosphoprotein) and G4 (oxidoreductase) were reported as component of the lateral bodies (93). While many of these virion-encapsidated proteins, such as B1, play role in viral metabolism, a subset of these proteins have immunomodulatory functions against host defenses (93).

B.4.2. Poxvirus genome is a closed linear duplex DNA

The genome of VACV is about 192Kb and its hairpins contain 12 extrahelical bases (82, 94). Adjacent to each hairpin is a motif of 87-bp region playing a role in DNA replication and concatemer formation; this motif is also

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adjacent to large inverted terminal (ITR) repeats (95, 96). The remainder of the genome contains 200 closely packed genes, with very little intergenic spaces and some genes frequently overlap by several nucleotides (97). There is no indication of clustering of genes within the genome but genes are organized to maximize transcription (70). There are no introns and no splicing, and viral mRNAs are transcribed in the cytoplasm (70).

The central region of the genome, or conserved core, constitutes 66% of the whole genome. Genes belonging to the conserved core are essential, because of their role in viral replication (83). Genes at both ends of the genome are not conserved from virus to virus, and are often not essential for viral replication. However, these genes are considered to carry specific functions such as host tropism, virulence and disease patterns and possibly a role in the modulation of host defenses (70). In the central region, the direction of genes in the middle the genome is random, but similar in all chordopoxviruses, and genes located in each terminal third of the genome are oriented such that transcription occurs outward toward the termini of the genome (70).

B.4.3. Poxvirus nomenclature is based on HindIII restriction fragments

The Copenhagen strain of vaccinia virus is the first to be sequenced and was used for the genetic nomenclature of orthopoxviruses ORFs based on HindIII restriction fragments (82). Using HindIII restriction endonuclease, the Copenhagen genome digestion gave 16 fragments (A-P), which were labelled from left to right (C, N, M, K, F, E, P, O, I, G, L, J, H, D, A, B). The naming of each ORF within a given fragment consists of a number (left to right,) and its

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transcriptional direction (L or R) (85). For example, The Ser/Thr protein kinase (B1R) is the first ORF within the HindIII sixteenth DNA fragment, and is transcribed in a rightward direction.

B.5. Vaccinia virus Life cycle

B.5.1. Poxvirus entry is a complex process mediated by macropinocytosis

Entry of a virus into a cell is a 2-step process: binding to a cellular receptor, and entry. The complexity of this process is associated withthe type of entry, the number of viral proteins involvedand the steps required to release the viral genome into the cytosol (98). No specific cellular receptor has been identified for poxviruses (98). However, the wide host range of poxviruses suggests the existence of at least one receptor or membrane protein commonly expressed by the host cells (99).

Poxvriuses employ two entry pathways: plasma membrane fusion and endocytic pathway (100, 101). The entry process is finalized when the viral core is released into the cytosol of the infected cell. First, the viral core expand to become morphologically oval (70), and transcription of some 80 early genes occurs within the oval-shaped core (102). Then follows the uncoating process which releases the viral DNA genome, and the lateral bodies, as well as the release of several virion-encapsidated and early proteins within the cytoplasm of the infected cell (see figure B1.1) (93, 103). Many of the early viral proteins are transcription factors, DNA replication proteins and proteins modulators of the host immune responses (70, 93).

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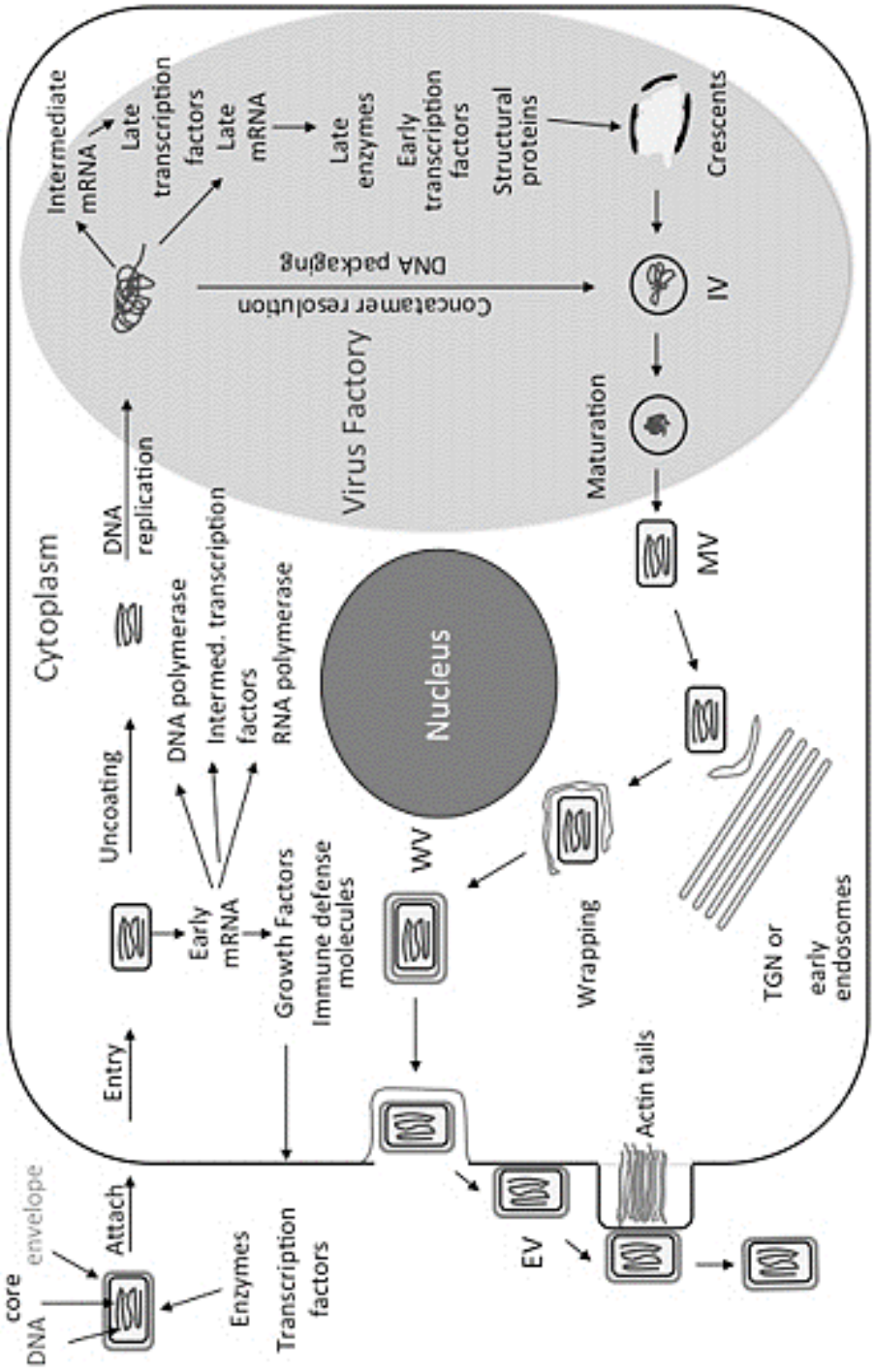

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Figure B1.1. Poxvirus replication cycle: The virion of poxvirus attaches to cell and release the virion core into the cytoplasm. Early transcription occurs within the virion, and several early proteins including viral DNA and RNA polymerases, factors for DNA replication and intermediate transcription and immune defense modulators. Upon uncoating, the viral DNA is released, the replication of DNA results in the synthesis of numerous concatemeric molecules. The replicated DNA serve as a template for intermediate gene expression to produce factors needed for late gene expression. Late gene expression produced structural proteins and enzymes for viral morphogenesis, and early proteins packaged into newly formed virions. The maturation process of the virion is characterized by passages through the Trans-Golgi and early endosomes, and transport to the cell membrane. From Dr. Bernard Moss's website on December 19, 2013 at http://www.niaid.nih.gov/LabsAndResources/labs/aboutlabs/lvd/geneticEngineeringSection/Pages/moss.aspx

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B.5.2. Poxviruses have a conserved temporal regulation of gene expression

The transcription of individual genes in poxviruses is temporally regulated, and both transcription factors and promoter functions are conserved across the family (104). This means that the regulation of gene expression in poxviruses follows a well-coordinated scheme as follows: early gene expression -> DNA replication -> intermediate gene expression -> late gene expression and morphogenesis (105). This cascade in gene expressions is governed by the dependence of each step on the previous step that follows, although both intermediate and late gene expressions are dependent on DNA replication, which serves as a switch to the initiation of intermediate, and then late gene expression.

Each stage uses different sets of cis- and trans-acting factors (70). All the enzymes needed for DNA replication and intermediate gene expression are both the products of early gene expression, and intermediate transcription factors have early promoters (106). The intermediate transcription factors recognize late gene promoters and regulate late gene transcription to produce late proteins. Late proteins include both early transcription factors which are packaged into the progeny virions, and most of the proteins required for morphogenesis (70, 85). A viral RNA polymerase produces poxvirus mRNAs in the cytoplasm. There is no indication of splicing of viral mRNA (85), but they are capped and polyadenylated (107).

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B.5.3. All three classes of viral genes are transcribed by a single virus-encoded RNA polymerase

The virus-encoded and virion-encapsidated RNA polymerase is a 500-kDa complex protein that transcribes all three classes of vaccinia virus genes, and is composed of nine subunits (70). There is a high degree of similarity in amino acid between the subunits of the viral RNA polymerase to those of eukaryotic and prokaryotic cells (108). The capability of the viral complex RNA polymerase to transcribe a specific class of viral genes depends on stage-specific transcription factors encoded by vaccinia virus (70). Thus, the shift from one stage to another is both regulated by the composition of the transcriptional complex, and at the transcriptional level (since all three promoters should be different from each other). For example, the subunit RAP94 and the viral stage-specific early transcription factor (VETF) are required for the transcription of early gene (70).

B.5.3.1. Virion-encapsidated factors mediate early gene expression

The virion core is released into the cytoplasm of the infected cell, and morphological changes of the virion are concomitant with the initiation of the transcription of early genes within the core (108). The intra-core transcription of the genome is mediated by a complete set of transcriptional machinery within the core (89). This is supported by report showing that the presence of DNA and protein inhibitors does not affect the viral early transcription (102). This shows that both DNA and early transcription factors are protected, and de novo translation is not required for the transcription of early genes.

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The virion-encapsidated transcriptional machinery is composed of the viral DNA-dependent RNA polymerase (VETF) and a heterodimeric complex composed of D6R and A7L (70). Other viral proteins such as D1R and D12L (capping enzyme), NPH1 (a DNA-dependent ATPase) and VP55-VP39 (role in poly(A) tail formation) are also shown to associate with the viral RNA polymerase for capping, initiation, elongation and termination of early transcripts (109, 110). Once capped and polyadenylated, the synthesized viral mRNA are released into the cytosol (107). The existence of both cap and poly (A) tail supports a ribosome-mediated translation of the viral mRNA, and suggests that the viral mRNA is stabilized in similar manner as in the eukaryotic system (70).

The transcription of early genes is an essential step during the poxvirus life cycle, because half of the genome is transcribed early to produce not only enzymes and factors required for viral DNA replication and intermediate gene expression, but also several virulence factors to evade the host immune responses (92, 93, 102).

B.5.3.2. Early core replication machinery mediates viral DNA replication

The entry and the disassembly of the virions trigger the creation of dense peri-nuclear sites (111). These sites or viral factories are large aggregates surrounded by membranes, but devoid of cellular organelles, and serve as sites where major viral processes such DNA replication, gene expression and virion morphogenesis take place (112, 113). The second uncoating releases the viral genome, as well as newly synthesized (early) and virion-encapsidated proteins

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from the viral core into the viral factories, which initiates the replication of viral DNA (97).

Poxvirus DNA replication is a complex process and not well understood (97). The complexity of this viral process is due to the large number of proteins associated with viral DNA replication and possibly the complex process of generating progeny genomes (97). Based on the current working model as reviewed by Boyle and Traktman (2009), the replication of poxvirus DNA is divided in two steps: the synthesis of nascent concatemeric intermediate genomes and the resolution of these structures into mature monomeric genomes (97).

The cytoplasmic replication of viral DNA uses virus-encoded DNA polymerase, and it is independent of the cellular DNA replication machinery. For example, poxvirus DNA replication proceeds normally in enucleated cells (114, 115). Using genetic and biochemical approaches, a set of viral proteins have been shown to constitute a "core replication machinery" for their role in DNA replication (97). These "core proteins" are mostly expressed early and include: the catalytic DNA polymerase (E9) (116, 117), the polymerase processivity factor (A20) (118, 119), a single-stranded DNA binding protein (I3) (120), a nucleoside triphosphatase (D5) (121), and B1 kinase (B1 kinase will be discussed later) (11, 122). In this dissertation, I will use individual viruses with mutation in B1 (ts2), E9 (ts42) and D5 (ts24) to assess viral DNA replication and intermediate gene expression in function of BAF level of expression.

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In addition, another group of DNA replication proteins, not essential for viral growth in vitro, are early proteins mediating the nucleotide biosynthesis and precursors metabolism to enhance viral DNA replication (97). Those include the thymidine kinase J2 (123), the thymidylate kinase A48 (124), ribonucleotide reductase (two subunits, F4 and I4) (125, 126) and two dUTPases (D4) (127) and F2 (128). Additional proteins such as the DNA ligase A50 (123) and the topoisomerase H6 (129), are considered to play accessory role during viral DNA replication as their presence or absence does not compromise viral replication (97).

B.5.3.3. Intermediate gene transcription requires DNA replication

The initiation of DNA replication triggers a shift in viral gene expression, to initiate intermediate gene expression, which depends on DNA replication (130). A block in intermediate gene expression as well as the persistence of early gene expression occurs when viral DNA replication is inhibited (130). The transcription factors required for intermediate gene expression are products of early gene expression (131, 132). The requirement of DNA replication is supported by the data showing that extracts prepared from poxvirus-infected cells transcribe a reporter gene under an intermediate promoter in the presence of an inhibitor of DNA replication (130, 132). The presence of intermediate transcription factors prior to DNA replication suggests that either the viral RNA polymerase does not have access to the viral genome as a template for transcription (132), or a threshold of replicated DNA must be reached before intermediate transcription is initiated.

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The virion-encapsidated RNA polymerase mediates the intermediate gene expression, however de novo synthesis of virus-encoded and intermediate-specific factors is required. These factors are early proteins and include the viral intermediate transcription factor-1 or VITF-1 (E4, RNA polymerase subunit) (133), the heterodimeric transcription factor VITF-3 (A8R and A23R) (134) and capping enzyme composed of D1R and D12L with role in early transcription termination (108, 130). Sequence analysis showed that intermediate promoters have TAAATG motif as the initiator element and an A-T-rich upstream element (70). Among the well-characterized intermediate products are A1L, A2L, I1L and G8R (135). Using high-throughput deep RNA sequencing, about 53 genes are estimated to belong to the intermediate class, although some may have a prolonged expression during viral infection (136). Other viral proteins such B1 kinase, an early protein, has been suspected to play role in intermediate transcription (Kovacs et al, 2001). In addition to virus-encoded factors, cellular proteins, such as VITF-2 and YinYan1 (YY1) proteins, enhance viral intermediate gene expression. VITF-2, a cellular component identified in HeLa cells, contributes to intermediate transcription (133). The nuclear transcription factor YY1 binds to the initiator element of I1L intermediate promoter and inhibits intermediate gene expression (135).

B.5.3.4. Several late proteins are packaged into the nascent virion

The transcription of late genes requires intermediate proteins, and produces transcription factors required for early gene expression and host response

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modulation, which are packaged into the virions, as well as structural proteins readily mediating morphogenesis (70).

Contrary to early and intermediate gene expressions, late gene expression requires newly synthesized RNA polymerase subunits (137). Phenotypic studies showed that mutations within the 22- and 147-kDa subunits of VACV DNA-dependent RNA polymerase inhibit late gene transcription (137). In addition to newly synthesized RNA polymerase, three intermediate proteins are considered as late transcription factors: G8R (VLTF-1), A1L (VLTF-2), and A2L (VLTF-3) (131, 138). Mutational studies confirmed the requirement of A1L and G8R for late gene expression (104). Another viral factor, H5R (VLTF-4) stimulates late transcription (139), while G2 (140) and J3 (141) enhance late transcription elongation. Further, G8R, A1L, A2L and H5R interact with each other, an indication that a transcriptional complex may mediate late gene expression (142).

B.5.3.5. Two types of infectious particles are produced during morphogenesis

Morphogenesis, a complex and multi-step process involving several viral proteins, takes place within the viral factories (143). The first visible structures are distinctive crescent-shaped structures made of lipids and proteins. These crescents expand in length to become closed spherical structures filled with viroplasmic material, called immature virions, or IV (85). Numerous viral proteins were shown to structurally and enzymatically regulate this process (85, 144, 145).

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A key step in the mature virion formation is the encapsidation of viral DNA within the IVs, which results in the formation of IVs with nucleoid, or IVNs (146, 147). Abnormal genome encapsidation is associated with accumulation of spherical particles without viral DNA (148), (149), (150). The IVNs, undergo internal changes to become intracellular mature virions or IMVs (85). The IMVs exhibit the barrel-shaped structure of poxvirus virions with an internal core (85). The IMVs leave the viral factories either by trafficking through the endosomes and the trans-Golgi to acquire a double membrane and become intracellular enveloped viruses (IEV), or exist directly by budding through the plasma membrane (151). To exit the infected cells, these IEV are transported to the cell border via a microtubule-based mechanism (Rietdorf J, Ploubidou A, Reckmann I, Holmström A, Frischknecht F, Zettl M, Zimmermann T, Way M., 2001; Ward & Moss, 2001)(152, 153). Upon release, vaccinia virus spreads from cell-to-cell by infect adjacent cells or by inducing actin polymerization to infect distal cells or increasing cell motility (154, 155).

B.6. A complex web of signaling pathways mediates antiviral responses

The discovery of the refractory nature of the cytoplasm to foreign agents raises the question of how viruses, and poxviruses in particular, evade or modulate host immune responses in order to sustain their life cycle. Several studies showed that in responses to viral infections, numerous cellular factors and pathways are activated to target viruses (156, 157). In response to a viral infection, two signaling pathways primarily mediate the activation of interferons (IFN): a virus-induced signal activating the transcription of IFNα/β, and a second

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wave of antiviral factors, IFN-stimulated genes or ISGs, are transcriptionally activated by IFN-mediated pathway (158-160).

The recognition of a pathogen-associated molecular pattern (PAMPs) by a cellular pattern recognition receptor (PRRs) is key in the activation of the IFN signaling pathway (161). The PRRs constitute a growing class of cytosolic proteins with essential role in the host's ability to mount effective immune responses. These include membrane-associated Toll-like receptors (TLRs), the retinoic acid inducible gene 1 (RIG-I)-like receptors (RLRs) and several other cytosolic nucleic acid sensors (162). The recognition of PAMPs by PRRs initiates the activation of transcription factors, such as the IFN-regulatory factors (IRF3 and 7) and /or the nuclear factor NFkβ and their translocation into the nucleus where they promote transcription of IFN-α/β and proinflammatory cytokines (158, 159). These ISGs strengthen the antiviral response by enhancing defense mechanisms, targeting specific viral processes and factors, inducing apoptosis, and conferring resistance for uninfected cells to viral infection (163).

B.6.1. Poxviruses express homologues of cellular immune responses

The early step of poxvirus infection is critical since several factors targeting host immune responses are released (93). The uncoating of the viral genome is simultaneously associated with the release of virion-encapsidated proteins that modulate host immune responses by targeting primary immune responses mediators, inhibiting apoptosis and interfering with signaling pathways (156, 157). In addition, poxviruses have apparently "captured" and integrated into

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their genome several homologues of cellular immune genes (164-166). Genomic analysis showed that these viral genes exhibit a significant amino acid sequence and/or conserved amino acid motifs similarity to cellular proteins associated with immune responses (165). Clustered at both ends of the viral genome, these viral genes are not essential for viral replication in cell culture (167); although they may still be essential in strain- and tissue-dependent manner. For instance, the E3 protein is a required protein for vaccinia replication in HeLa cells, but not in BHK cells, while the opposite is true for K3 protein (168). The inactivation of BAF by B1 may account for the VACV strategie to modulate host responses.

B.6.4. Apoptosis is used as an active antiviral arm to remove viral infection

In attempt to definitively block viral infection after virus modulates and evades the coordinated webs of immune responses, infected cells induce apoptosis (169), making apoptosis on of the arms of the host immune response to a viral infection (170). The virus-induced cytophatic effects (CPE) can trigger apoptosis, and early apoptosis during infection may prevent the completion of virus life cycle. Poxviruses express anti-apoptotic proteins to modulate both apoptotic pathways as a survival strategy (171, 172).

B.7. Conditional lethal mutants are instrumental in poxvirus studies

Most genetic studies on specific viral genes during of poxvirus life cycle employ temperature-sensitive (ts) or conditionally lethal mutant viruses, and to some extent inducible viruses. The ts viruses have been instrumental in the analysis of the VACV life cycle as well as the role of key viral proteins. As a

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consequence of mutation within a specific ORF, the resulting virus produces a thermolabile or nonfunctional protein at nonpermissive temperatures (173-177).

Under a nonpermissive temperature, usually 39.5ºC, these ts viruses exhibit a growth defect (such as small plaques) that may indicate the functional implication of a specific gene on specific stages and processes of the viral life cycle (70, 85).

Several ts viruses are available to us, and many of them have been well studied for their in a particular viral process and during virus-host interactions (176, 177). In this work, I will use three ts mutant VACV (ts2, ts24 and ts42) to assess the absence of B1 and the effect of BAF during viral infection with them. These ts mutants have been well charatcerized for their role in DNA replication.

B.8. Poxviruses express serine/threonine kinases

Post-translational modifications, such as phosphorylation, are regulatory switches employed by cells and viruses for the regulation of biological processes. Phosphorylation, or the transfer of a phosphate group from a nucleoside (Adenosine or guanosine-5'-triposphate) to a substrate by a protein kinase, has wide biological implications by regulating enzymatic activity, cell cycle, interaction between proteins or with nucleic acids (178). Dependent on the substrate residues to be phosphorylated, kinases are grouped into serine/threonine and tyrosine kinases. Like other DNA viruses (179), poxviruses encode and express two protein kinases: a Ser/Thr B1 (177, 180, 181) and F10 (182, 183).

The B1R gene encodes a 30kDa protein, and is expressed early during infection, although a few copies of B1 are also virion-encapsidated (180, 184).

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With the exception of *molluscum contagiosum* virus, B1 gene is found in all poxviruses known to date with some degree of variation in their sequence identity (185). It plays an essential role in viral DNA replication and at least in one post-DNA replication events (11, 105). Among the substrates of B1 are the cellular BAF (11, 12), two viral proteins, H5 (186) and possibly A30 (187), and the two ribosomal proteins, Sa and S2 (188).

Structural studies showed that B1 harbors conserved catalytic domains with strong homology with those found in serine/threonine kinases such as kinases regulating cell cycle progression, CDC7 and CDC28 (180, 189). These domains, critical for kinase activity, include the ATP binding site, the phosphorylation receptor site, and substrate recognition domain (180, 184, 190). Interestingly, a group of nuclear protein kinases, vaccinia virus-related kinases (VRKs), expressed by many organisms (animals, fruit flies to nematodes), display a 37-40% identity with B1 (191, 192).

B.8.1. B1 kinase is required for viral DNA replication

One of regulatory switches employed for the control of biological processes by cells and viruses is the phosphorylation/desphosphorylation of specific proteins. This makes kinases a major regulator of cellular processes. Their presence in large DNA viruses is an indication of a key viral need for them. This is exemplified by the finding that VACV B1 kinase is essential for DNA replication (11, 181, 193).

Early studies on the biological significance of B1 during vaccinia virus infection used ts2 and ts25 mutant viruses, both exhibit a DNA-negative

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phenotype (177, 180). Using marker rescue analysis and homologous recombination it was shown that these ts viruses harbor each a single mutation mapped to a distinct and non-overlapping section of the HindIII B fragment (B1R) (180, 181).

Studies using ts2 and ts25 under permissive and nonpermissive temperatures showed the 30 kDa protein expressed from these mutants is labile, with little to no detectable kinase activity (181, 184). Further, using a dot blot filter hybridization technique, Rempel et al (1990) showed that in L and BSC40 cells infected with ts2 or ts25 and incubated at 39.5ºC, the accumulation of viral DNA as well as viral yied was severely inhibited compared to similar conditions but at 32ºC (181). At 39.5ºC, viral yield in L cells was only 0.11% in ts2 (MOI of 15) and 0.68% in ts25 (MOI of 15) at compared to those produced in L cells at 32ºC. In BSC40 cells, there was only 15% in ts2 (MOI of 15) and 13% in ts25 (MOI of 15) virus produced at 39.5ºC compared to those produced in BSC40 at 32ºC (181). These data are the first evidence that B1 stimulates viral DNA replication. Further, the severity of the ts phenotype of these mutant viruses may be cell type specific. Taking in consideration the severity of ts2 phenotypes in L cells, these cells were employed in our investigation of B1's role during VACV lifecycle.

B.8.2. Uninfected cells express kinases similar to viral B1

Structural studies revealed several similarities between B1 and with other serine/threonine kinases (180). There was a high sequence similarity between B1 and a mammalian family of cellular vaccinia virus-related kinases (VRKs),

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especially their catalytic domains (191, 192). This suggest that VRK and B1 phosphorylate similar substrates. VRKs belong to the casein kinase family (178, 194), and are expressed in mammalian cells, *Caenorhabditis elegans* (Vrk1) and *Drosophila melanogaster* (nucleosomal histone kinase 1 or nhk1), but not in *S. cerevisiae* (191, 192).

The human genome encodes three VRKs proteins, VRK1 to 3 (195, 196), and chromosomal mapping showed that human VRKs have different genomic loci, size and exons numbers (196, 197). VRK1 has been detected in different cellular locations, but because it has a canonical nuclear localization signal, it is predominantly nuclear (192, 196). Because of interactions with the endoplasmic reticulum and the mitochondria through the hydrophobic region of VRK2, VRK2 is mostly cytoplasmic, however the loss of these interactions gives VRK2 a cytoplasmic and nuclear presence (196). Both VRK1 and VRK2 have conserved kinase activity, but VRK3 has no kinase activity due to amino acid substitutions (196-198).

B.8.3. Key cellular biological processes are regulated by VRKs

The role of VRKs in many cellular processes is essentially associated with the phosphorylation of, and the modulation of interacting partners of their substrates. The VRK signaling pathway regulates gene expression, and several mitotic phases and related processes (Golgi defragmentation, chromatin condensation; DNA damage) and developmental processes (embryonic development, fertility) (199, 200).

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In *D. melanogaster,* mutations within the nhk-1 are associated with defect in the formation of spindles during mitosis and meiosis, and embryonic lethality and sterility in both sexes (199, 200). Similar phenotypes were observed in *C. elegans* with mutations of vrk-1, in the addition to slow growth phenotype in adults (201). In humans, mutations within the VRK1 is associated with a muscular atrophy syndrome (202), and no viable VRK1-knockout mice can be generated, because defects during gamete formation lead to sterility (203).

DNA synthesis is an essential event during mitosis, and VRKs may play regulates it because of their functional relationship with BAF. During mitosis, VRK1 is highly expressed at G_1/S transition (204), and is necessary for cell cycle entry (205). VRK1 and VRK2 allows these kinases to regulate chromatin structure by phosphorylating proteins such as BAF and Rb (26). In addition, VRKs regulate gene expression through the phosphorylation of c-Jun, ATF2 and CREB transcription factors and factors directly associated with the MAPK (mitogen-associated protein kinases) signaling (206-209).

The role of VRKs in the regulation of gene expression is of interest for this study. Because of the functional similarity between VRKs and B1, it is likely that B1 may functionally perform as VRKs during VACV infection. For example, VRK1 does not directly regulate cellular DNA replication (206), but both VRK1 and VRK2 enhance poxviral DNA replication in the absence of active B1 (193).

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B.8.4. Studies on cellular VRKs led to uncover the cellular substrate of B1

The sequence similarity between the viral B1 kinase and the mammalian VRKs (191, 192), renewed interests in understanding the mechanism of action of B1 as well as determining its substrates in relationship to its role in viral DNA replication. In a signature study, Boyle and Traktman (2004) showed that both human and mouse VRKs (hVRK1, and mVRK1) can functionally replace B1 during VACV infection (193). These studies employed hVRK1 (ts2/hVRK1) or mVRK1 (ts2/mVRK1). It is not known whether B1 can complement the cellular functions of VRKs. The rescue of viral DNA replication in the absence of active B1 by VRKs proteins showed VRKs target BAF, their cellular substrate during cell cycle. Further studies showed that both VRKs and B1 phosphorylate residues Thr3/Thr2/Ser4 at the N'terminus of BAF, and abrogate the interaction of BAF with cellular DNA as well as with LEM-domain proteins (7, 11, 12, 26).

The phosphorylation status of BAF during vaccinia virus infection is thus essential for successful viral DNA replication. Phosphoamino-acid analysis showed that Ser4 is preferentially phosphorylated by B1 and VRKs before Thr2 and Thr3 (12). During a nonpermissive ts2 infection, there is no indication of BAF's phosphorylation and BAF colocalizes with the viral I3 (a single stranded DNA binding protein) to viral DNA replication sites (11). These data showed that phosphorylation of BAF, thus its inactivation, is essential for viral DNA replication.

B.8.5. B1 regulates viral transcription via an unknown mechanism

In eukaryotic systems, the phosphorylation of transcription factors and transcriptional coregulators is one of the posttranslational modifications

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regulating transcription. In viral system, phosphorylation/desphosphorylation is essential for viral gene transcription (210, 211). For example, VACV VH1, a virion-encapsidated protein and a dual-specific phosphatase, serves as an early transcription factor for viral transcription (210) as well as an immunomodulatory effector role (93).

Like VH1, B1 is also reported to regulate viral intermediate transcription but the mechanism is not known (181). To study the role of B1 in intermediate gene expression, a plasmid pG8R-CAT (expressing the chloramphenicol acetyltransferase (CAT) ORF under G8R (a viral intermediate promoter) was transfected and G8R promoter activity measured during a VACV infection (105). The transfected plasmid provides a way to bypass the requirement of viral DNA replication for postreplicative events; and cytosine β-D-arabinoside (AraC) was added to block viral DNA replication, so that B1's role can be investigated independent of its role in DNA replication. This experimental approach will be used to investigate the role of B1 in postreplicative events in function of BAF level of expression.

The promoter activity measured as the expression of CAT during ts25 (a B1-defective virus) showed that viral intermediate gene expression required B1, and this is independent from viral DNA replication (105). Further, in ts25-infected cells, there was a pronounced decrease in mRNA transcripts-specific to pG8R-CAT compared to WT-infected cells, indicating a block at the transcriptional level (105). Although, this study established the role of B1 during intermediate gene transcription, the mechanism by which this is done is not known. It is also

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unknown whether B1 targets a viral or cellular substrate or B1 is directly involved with the viral transcriptional machinery. Further, it is not known whether this activity of B1 is specific to G8 promoter or B1 is a general transcriptional regulator during intermediate gene transcription. This work will investigate the mechanism by which B1 acts as a transcriptional regulator as well as the role of B1 during viral DNA replication.

C. OVERALL GOALS OF THIS STUDY

For my studies, I chose to investigate the role of the BAF-B1 axis during VACV infection as well as the potential of BAF to recognize cytosolic dsDNA. Although, BAF is shown to relocalize to VACV replication sites and inhibit viral DNA replication, many questions remain to be answered. For example, it is not known whether BAF's recruitment to these viral sites requires its cellular interacting partners such as Lap2α, or a viral protein? Further, if any BAF-interacting partner, such as LEM-domain proteins, is involved, what is its contribution to BAF's activity? Likewise, because of the temporal order of events during the life cycle of vaccinia virus, and the early inactivation of BAF by B1, it is reasonable to hypothesize that BAF has additional effects on viral processes beyond viral DNA replication, such as viral intermediate gene expression and morphogenesis. The requirement of viral DNA replication for viral post-replicative events make it complex to study B1's potential role in these events because B1 is itself required for viral DNA replication.

Because of BAF's dsDNA-binding properties, I investigated the capacity of BAF to respond to cytosolic dsDNA. I also examined the potential role of BAF in

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the regulation of gene expression from transfected plasmid. I assessed whether known cellular proteins interacting with BAF and unknown proteins (viral and cellular) are present at BAF-DNA complexes, and examined the potential contributions of these proteins to BAF's activity.

The viral kinase B1 has been shown to play key role in viral intermediate gene expression through unknown mechanism (105). Considering the finding that BAF is a substrate of B1, I investigated the BAF-B1 axis to identify the mechanism by which B1 is involved with viral gene expression. Finally, in our attempt to understand the importance of the BAF-B1 axis during infection in different cell lines, we uncovered a potential but novel role of B1 in viral morphogenesis in U2OS human osteosarcoma cells.

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CHAPTER II

MATERIALS AND METHODS

2.1. Antibodies

Anti-Flag M2 (mouse monoclonal, F1804) antibody was purchased from Sigma Aldrich. Mouse monoclonal anti-BrdU (G3G4) was developed by Dr. Stephen J. Kaufman and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242. Anti-Ku86 (FL-254 rabbit polyclonal, #2753) antibody was purchased from Cell Signaling Technology. Anti-Lap2alpha (rabbit polyclonal, ab5162) antibody was purchased from Abcam. Anti-emerin (FL-254 rabbit polyclonal, sc-15378), FL-89 (anti-BAF, rabbit polyclonal, sc-33787), Ku70 (rabbit monoclonal, sc-5309), GFP (FL rabbit polyclonal, sc-8334), GAPDH (FL-335 rabbit polyclonal, sc-25778) antibodies were purchased from Santa Cruz Biotechnology, Inc. Alexa Fluor-488 goat anti-rabbit (A11034) and alexa Fluor-594 goat anti-mouse (A11032) were purchased from Life Technologies. Goat anti-mouse IgG (H+L) # NK 179841 and rabbit anti-goat IgG (H+L) HRP conjugate #172-1034 were purchased from Bio-Rad laboratories, Inc. RPA32 (p34 Ab-1 (9H8) mouse monoclonal, MS-691-P1) antibody was purchased from NeoMarkers.

2.2. Chemicals

All chemicals were purchased from Fisher Scientific or Sigma-Aldrich unless otherwise stated. Puromycin (100 mg/mL), hygromycin (50 mg/mL),

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zeocin (100 mg/mL), blasticidin (50 mg) were purchased from Invitrogen. Penicillin-Streptomycin (Ref 15140-122) and 0.5% Trypsin-EDTA-10X (Ref 15400-054) were purchased from Gibco Life Technologies. Fetal Bovine Serum was purchased from Atlanta Biologicals, and Arac (Cytosine b-D-arabinosidase) (50 mM) was from Sigma.

2.3. Cell culture and maintenance

Human thymidine kinase-negative 143B osteosarcoma cells (TK-), human osteosarcoma U2OS cells (a kind gift from Dr. Jones), African green monkey kidney cells, BSC40 (BSC-40 are a continuous line derived from BSC-1 cells (Hruby et al., 1979)) and CV1, mouse fibroblast cells L929, human osteosarcoma cells U2OS, human kidney cells 293 cells were obtained from ATCC and maintained in DMEM supplemented with 10% fetal bovine serum containing penicillin/streptomycin and incubated at 37ºC in a 5% CO_2 atmosphere. Flp-In CV1 cells were purchased from Invitrogen and maintained in DMEM/10% FBS and 100 µg/ml zeocin (Invitrogen) prior to stable transfection, after which the zeocin was replaced with 100 µg/ml hygromycin (Invitrogen). The shRNA-transduced cells were maintained in DMEM supplemented with 10% fetal bovine serum containing penicillin/streptomycin, and additional puromycin or hygromycin (for selection) and incubated at 37ºC in a 5% CO_2 atmosphere.

2.4. Plasmids and other nucleic acids

The following plasmid were used: pUC19, K12 (E. Coli DNA), M13 ssDNA (New England Biolabs), poly I:C dsRNA mimic (Invitrogen), purified Vaccinia

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virus dsDNA and CMV-Luc (expresses luciferase under the CMV immediate early enhancer promoter (pGL4.51[*luc2*/CMV/Neo], Catalog# E1320)), and minP-Luc (expresses luciferase under a minimal promoter containing only a TATA box (pGL4.26[*luc2*/min/Hygro], Catalog# E8441)) and T7-luciferase (Promega). These DNA plasmids were isolated from JM109 cells and purified using an endotoxin-free Qiagen kit. Isolation of vaccinia DNA was performed as described (Sinclair J., 2010)(212). Briefly, viral cores were first treated with β-mercaptoethanol, 1% SDS, and 1 mg/ml proteinase K, and then gently extracted with phenol/chloroform and chloroform prior to ethanol precipitation of the DNA. The pG8-Luciferase plasmid expresses luciferase under the vaccinia virus intermediate G8 promoter and is a generous gift from Dr. Bernard Moss at NIAID, Bethesda, MD (Kovacs et al., 2001).

2.5. Molecular cloning

2.5.1. Construction of viral intermediate promoters

2.5.1.1. Cloning of viral intermediate promoters

Plasmids containing A2, I1 and Consensus Intermediate promoter sequences upstream of the firefly luciferase gene were constructed as follows. PCR was performed using pG8-Luciferase plasmid as a template, and primers designed to include sequences previously published of I1, A2 and Consensus promoters (106, 213, 214). All three promoters (in bold) were placed upstream of 18bp homologous to the 5' end of firefly luciferase gene (in italics). The upstream primers are A2L-Luc (5'ccggaattc**GCAACGTCTAGAAATAAAATGTTTTTATA TAAAA***atggaagatgccaaaaac*-3'), I1L-Luc (5'-ccggaattc**TTTGTATTTAAAAGTTG**

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TTTGGTGAACTTAA*ATGGCagacgccaaaaacataaag*-3') and Consensus-Luc (5'-ccggaattc**TAATATATTTAAAATAAAAATTAATATTATAAA***atgga***a***gatgccaaaa ac*-3'). The downstream primer is common, Universal-Luc-3' (5'-caggaattc*ttacacggcgatctttc*-3'), is homologous to the 3'-end of the firefly luciferase ORF, and contains an EcoRI site (lowercase) as well. PCR products were digested using the EcoRI restriction enzyme (Promega), run on 1% agarose gel and purified using phenol chloroform method, and resuspended in ddH2O. The plasmid serving as vector, the pCRII-TOPO plasmid (Invitrogen), was EcoRI-digested, phosphatase-treated and gel-purified, and resuspended in ddH2O.

2.5.1.2. Ligation

The inserts (A2L-Luc, A1L-Luc and Consensus-Luc) and the vector backbone were ligated using 1 µl (10% of total reaction volume) of T4 DNA ligase (Thermo Scientific). Vector to insert ratio was 1:3 to 1:5. Ligation reactions were incubated overnight at 16ºC.

2.5.1.3. Transformation

For transformation, 5-10 µl of ligation mix was added to 50 µl of competent *Escherichia Coli* JM109 (Promega). The mixtures were incubated on ice for 30 minutes, heat-shocked at 42ºC for 45 seconds, and incubated for 5 minutes on ice. Subsequently, 900 ml of LB broth (10% peptone, 10% NaCl, 10% yeast extract, pH 7.5) at room temperature was added, and incubated further at 37ºC for 45 minutes in a shaker at 200 rpm. The bacteria were briefly centrifuge, and the pellets plated on LB agar (LB + 15 g/l of agar) with kanamycin, and

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incubated at 37ºC overnight for the selection of transformants bacteria containing the expected plasmids.

2.5.1.4. Plasmid screening purification

For screening of transformed bacteria containing the expected plasmids, colony-PCR was performed on individual colony with primers used during early PCR for cloning. Briefly, single bacterial colony was picked, diluted in 10µl ddH2O, and 8 µl used in PCR reaction mix with the appropriate primers. As an approach to determine the PCR feasibility, primers specific to plasmid pG8-Luc were used with pG8-Luc DNA as a positive control, and with single colony from each cloned plate. PCR products were run on 1% agarose gel, and for colony samples showing the appropriate band, the remaining 2 µl of single-colony mixed with ddH2O were grown 1.5 ml BL broth containing kanamycin, and plated on LB agar plate with kanamycin.

For DNA extraction, large-scale plasmid preparations were prepared by gravity flow column using QIAGEN plasmid purification kit (QUIAGEN).

2.5.2. Construction of BAF expression vectors

2.5.2.1. Cloning of BAF Expression Vectors

pcDNA5/FRT/TO/CAT, pcDNA5/FRT/TO/3XFLAG-BAF, pJS4-RFP-BAF and pJS4-RFP-BAF-MAAAQ vectors have been described previously (11). To construct vectors introducing the single amino acid mutations K6A, G47E, and K53E, wild-type BAF or BAF-MAAAQ sequence was used as a template for overlap PCR mutagenesis using outside primers and one set of the following internal mutagenesis primers: WTBAF-K6AUP (5'-

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GACAACCTCCCAAGCGCACCGAGACTTCGTG-3') and WTBAF-K6ADN (5'-CACGAAGTCTCGGTGCGCTTGGGAGGTTGTC-3'), or MAAAQBAF-K6AUP (5'-GCAGCCGCCCAAGCGCACCGAGACTTCGTG-3') and MAAAQBAF-K6ADN (5'-CACGAAGTCTCGGTGCGCTTGGGCGGCTGC-3'), or BAF-G47EUP (5'GACAAGGCCTATGTTGTCCTTGAACAGTTTCTGGTGCTAAAGAAAG -3') and BAF-G47EDN (5'-CTTTGTTTAGCACCAGAAACTGTTCAAGGACAACATAG GCCTTGTC -3'), or BAF-K53EUP (5'-GTTTCTGGTGCTAGAGAAAGATGAAGA CC-3') and BAF-K53EDN (5'-GGTCTTCATCTTTCTCTAGCACCAGAAAC-3'). Outside primers were specific for the expression vector and included KpnBamFlag (5'-GAGGGTACCGGATCCGCCACCATGGACTACAAAGACC-3') and BAF-DNBam (5'-GCTGAATTCGGATCCTCACAAGAAGGCG-3') for pcDNA5/FRT/TO insertion at the BamHI site (underlined in this primer set). Alternatively, FLAG-BAF-UPXho (5'-CAG*CTCGAG*GCCACCATGGACTACAAAG ACC-3') and BAF-DNBam were used, which places an XhoI site (italics) upstream and a BamHI site downstream of the ORF for pJS4-RFP insertion at these sites. The introduction of each of these BAF mutations into selected clones was verified by DNA sequencing.

2.6. Production of Stable Cell Lines.

2.6.1. Stable overexpression of BAF in CV1 cells

The stable integration of chloramphenicol acetyltransferase (CAT), 3XFlag-BAF or BAF mutants was performed using the Flp-In system (Invitrogen) using methods described by the manufacturer. The FLAG tag is a short, hydrophilic 8-amino acid peptide (GACTACAAAGACGATGACGACAAG), and

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3XFlag were fused to the N'terminus of BAF. This system employs CV1 Flp cells containing a single integrated copy of the pFRT/lacZeo plasmid, which possesses a FRT recombination site. Briefly, these cells were co-transfected with the pcDNA5/FRT/TO/3XFLAG-BAF wild-type or mutant vector of choice and pOG44, a vector expressing the Flp recombinase. Stable cell lines were selected by growth in 200µg/ml hygromycin for 3 weeks and 100µg/ml hygromycin thereafter.

2.6.2. Stable overexpression of BAF in L929 cells

The stable overexpression of BAF in L929 cells was performed by using a lentivirus expressing 3XFlag-BAF (plasmids were a kind gift from Dr. Paula Traktman (215). Specifically, 293T cells were transfected with pHM-3XFlag-BAF or pHM-MCS plasmid (216, 217) with a combination of viral packaging plasmids pVSV-G, pTat, pREV and pGag/Pol. The next day, media was replaced with fresh media containing 5mM Sodium Butyrate. Eight hours later fresh media containing 10mM HEPES pH7.4 was added for additional 12h. Next, the media-containing lentivirus was filtered through a 0.45µm sterile filter, and polybrene (8µg/ml) was added and stored at -80ºC. For transduction, L929 cells were seeded in 35 mm dishes at $1X10^6$ per well. The next day, medium was replaced with 1 mL of lentivirus supernatant. After 24hr, medium was replaced with fresh media for additional 24h. Cells were then grown in media containing 100 µg/ml of hygromycin to select for stable lentiviral integration.

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2.7. Plasmids DNA Transfection

All experiments involving transfection of DNA or RNA were performed using respectively Lipofectamine2000 or RNAimax (Invitrogen) as per manufacturer's instructions.

2.8. Viruses

2.8.1. Viruses used

The following viruses were used: wild-type vaccinia virus (Western Reserve or WR strain), the B1-deficient ts2 virus (175, 181, 184), and vTF7.3 (218). Recombinant viruses, ts2/B1 and ts2/VRK1 (193), were kind gifts from Dr. Traktman (Medical College of Wisconsin). Both viruses ts42 (E9 mutant) and ts24 (D5 mutant) were gifts from Dr. Rich Condit (University of Florida). Stocks of all viruses were purified from cytoplasmic lysates of infected BSC40 cells by ultracentrifugation through 36% sucrose; and quantified by plaque assay titration on BSC40 cells.

2.8.2. Preparation of viral stocks and viral yield determination

Wild-type vaccinia virus (WR strain) and *ts* viruses (ts2, ts2/B1, ts2/VRK1, ts24 and ts42) were propagated in BSC40 cells at 37°C or 31.5°C, respectively. Viral stocks were prepared from cytoplasmic lysates of BSC40 infected cells by ultracentrifugation through 36% sucrose cushions at 18,000rpm in a SW41 rotor for 90 minutes and resuspension of the viral pellet in 1 mM Tris pH 9, and freeze/thawed three times and stored at -80ºC.

The day prior to infection equal numbers of each cell line was plated at confluence in 6- well tissue culture plates. Viral stocks are sonicated, and 10-

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and 9-fold serial dilutions of cytoplasmic lysates of cells infected respectively with Wt and ts viruses were prepared in DMEM and 500 µl of the diluted virus were plated per well and incubated respectively at 37ºC and 31.5ºC. For ts viruses, a 5-, 6-, 7-fold dilutions were incubated at 39.7ºC to identify revertant viruses. Dishes were rocked every 10 minutes for 1hr, and feed with 1.5 ml fresh DMEM-10% FBS for 48h for wild type at 37ºC, 72hr for ts at 31.5ºC and 48hr for ts at 39.7ºC. Inoculums were aspirated in the appropriate waste container containing bleach, and to stain 1.5 ml of crystal violet/formaldehyde solution was added for staining at room temperature. 1-2 hr later, stain was washed off with H2O in the appropriate waste container, and let dry. The number of plaques is reported as measure of pfu/ml.

2.8.3 Plaque assay

The day prior to infection equal numbers of appropriate cells were plated at confluence in a 6- well tissue culture plates, and were infected with Wt and ts viruses at an MOI of 0.01 or 3 for 24 or 48hr at indicated temperatures. At the time points given, cells were harvested into 100 µl of 10 mM Tris (pH 9) and freeze/thawed three times prior to titration on BSC40 cells or stored at -80°C.

Plaque assays were performed on equal number of BSC40 cells in a 12-well tissue culture plates. A 9- and 8-fold serial dilution of cytoplasmic lysates of cells infected respectively with Wt and ts viruses were prepared in DMEM and 250 µl of the diluted virus were plated per well. Incubate the dishes at the indicated temperatures (31.5ºC, 37ºC and 39.7ºC) and rock the dishes every 10 minutes for 1 hr, then dishes were feed with 1 ml DMEM-10% FBS, and

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incubated for 48 hour for WT and or 72 hours for ts viruses. Inoculums were aspirated in the appropriate waste container containing bleach, and to stain 1 ml of crystal violet/formaldehyde solution was added for staining at room temperature for 2 hr, then washed with ddH2O in the appropriate waste container, and let dry. The number of plaques is reported as measure of pfu/ml.

2.9. Production of lentivirus for stable depletion

2.9.1. Production of shRNA Lentivirus

Primers specific to human BAF mRNA shBAF-UP (5'-TGGCCTATGTTGTCCTTGGCTTCAAGAGAGCCAAGGACAACATAGGCCTTTTTGGAAAC-3') and shBAF-DN (5'-TCGAGTTTCCAAAAAGGCCTATGTTGTCCTTGGCTCTCTTGAAGCCAAGGACAACATAGGCCA-3') were annealed and the product was cloned into the HpaI and XhoI sites of pLL3.6/Puro to construct pLL3.6-shRNA plasmid expressing shRNA(11). The pLL3.6-Scram expressing a scrambled RNA was constructed by annealing primers shScram-UP (5'-TCAGTCGCGTTTGCGACTGGTTCAAGAGACCAGTCGCAAACGCGACTGTTTTTGGAAAC-3') and shScram-DN (5'TCGAGTTTCCAAAAACAGTCGCGTTTGCGACTGGTCTCTTGAACCAGTCGCAAACGCGACTGA-3'), and the cloning the product was cloned into the HpaI and XhoI sites of pLL3.6/Puro plasmid. For control, GFP expressing lentivirus was also produced using the same protocol with the exception of using GFP sequence instead of a short hairpin, thus the resulting lentivirus expresses GFP. These shRNA-pLL3.6 plasmids were transfected in 293T cells in combination with pMD2.G, pRSV-REV, and pRRE encoding VSV-G, Rev and Gag/Pol genes respectively. 16hr

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posttransfection, fresh media containing 5mM NaButyrate was added for additional 8hr, then cells were washed with 1XPBS and media containing 10mM Hepes pH7.4 was added for additional 24hr. The media containing recombinant lentiviruses were harvested and filtered through a 0.45um sterile filter, and 8ug/ml polybrene added and stored at -80ºC. These recombinant viruses produced a short hairpin RNA that mediate the degradation of BAF-specific RNA though the cellular RNA-induced silencing complex (RISC).

Primers specific to mouse BAF mRNA shBAF-sense (5'TGGCTTATGTGGTCCTTGGCTTCAAGAGAGCCAAGGACCACATAAGCCTTTTTGGAAAC-3') and mBAF-antisense (5'TCGAGTTTCCAAAAAGGCTTATGTGGTCCTTGGCTCTCTTGAAGCCAAGGACCACATAAGCCA -3') were annealed and cloned into the pLL3.6 as previously described.

2.9.2. Production of Stable Cell Lines

The recombinant viruses produce a short hairpin RNA that mediate the degradation of BAF-specific RNA though the cellular RNA-induced silencing complex (RISC).

2.9.3. Stable depletion of BAF in cells

To stable depletion the expression of BAF, CV1 were infected with pLL3.6-shBAF, GFP, shmBAF or -Scram for 24hr. At the exception of pLL3.6-GFP cells, all cells were selected with 15ug/mL of puromycin.

2.9.4. Stable depletion of emerin in CV1 cells

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To deplete emerin from cells, lentiviruses were produced as previously described (43), using vector pLL3.6 expressing emerin-specific (5'-GACCUGUCCUAUUAUCCUA-3') shRNA.

2.10. Immunofluorescence

Specific cells were plated on chamber slides (Lab-Tek) or on 6-wellplate 24 hours prior to the transfection. Transfection was performed using 1 ug nucleic acid per milliliter of media and 2uL of Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's protocol. At 7 hours post transfection, cells were fixed for 15 minutes at room temperature using 4% paraformaldehyde in phosphate buffered saline (PBS; 10mM Na_2HPO_4-$7H_2O$, 1mM KH_2PO_4, 2mM KCl, 140 mM NaCl, pH 7.4). Cells were permeabilized using 0.1% saponin or 0.2% Triton X-100, as indicated in the figure legend, in PBS for 5 minutes at room temperature. Cells were then incubated with mouse α-FLAG M2 antibody (Sigma) at a dilution of 1:400 in PBS +0.05% saponin. This was followed by Alexa-fluor 594-conjugated goat α-mouse (Molecular Probes) at 1:500 in PBS+0.05% saponin. DNA was stained with DAPI. Proteins were observed by indirect fluorescence on an inverted (Olympus IX 81) confocal microscope. Images were pseudocolored using ImageJ software. The same immunofluorescence protocol was used for lap2α, emerin, Ku86, RPA32.

2.11. Immunoblot Analysis

L929 and L929 stably expressing specific shRNA were freshly collected in 300 µl of SDS sample buffer (100 mM Tris pH6.8, 2% β-mercaptoethanol, 2% SDS, 32.5% glycerol, bromophenol blue) supplemented with 10 units of

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Benzonase. Lysate volume equivalent to 10^5 cells were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 18% gel, transferred to PVDF, and incubated with primary against BAF (11), and rabbit secondary antibodies. Blots were developed with chemiluminescent reagents, and quantified by a Bio-Rad Chemidoc XRS instrument to verify that BAF expression had been depleted >85%.

2.12. B1 siRNA Transfection

B1-specific and control siRNAs were designed and ordered from Dharmacon. The B1-1siRNA sense sequence is 5'-caauaugcaccuagagaauuu-3' and the B1-2 siRNA sense sequence is 5'-GCCCAAAGCUAACGGAUCAUU-3'. The siControl sense strand sequence is 5'-CAGUCGCGUUUGCGACUGGUU-3'. L929 or U2OS cells ($3.6X10^5$ per well in a 12-well tissue culture plate) were transfected with 100 nM of siRNA Control, siB1R-1, or siB1R-2 using RNAimax (Life Technologies) as per manufacture's protocol. 16-24 hours post transfection cells were infected with WT virus at an MOI of 3 and harvested for B1R mRNA at 4 hpi. RNA was extracted using TRIzol reagent, purified with Aurum Total RNA mini kit (Biorad), reverse transcribed to generate cDNA (Applied Biosystems, High Capacity cDNA Reverse Transcription Kit), and treated with 0.5 µg/ml RNase A prior to qPCR analysis.

2.12.1 Quantification of viral mRNA, Reverse transcriptase and qPCR

Extracted viral RNA were reverse-transcribed in cDNA using High Capacity cDNA Reverse transcription kit (Applied Biosystems), and treated with

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0.5 µg/ml RNase A prior to qPCR analysis. Using TaqMan master mix (Applied Biosystems) and 900 nM of primers B1R F (5'-AATCAATGGGTCGTTGGACCAT -3') and B1R R (5'-AATACATCATTTTTATCTCGGGTTTCGATTGC-3'), and 250 nM B1R probe (5'-56-FAM/AG GTG CAG ATC TAG ATG CGG TGA TCA /3IABkFQ-3'), qPCR performed on B1R cDNA to determine the degree of depletion of mRNA specific to the vaccinia virus B1R gene.

2.12.2. Viral DNA extraction and qPCR

To assess viral DNA replication, total viral DNA was extracted from a 6-well tissue culture plate previously infected using the QIAamp DNA Blood Mini Kit (Qiagen) and treated with 5 µg/ml RNase A as per manufacturer's instructions. To quantify viral DNA replication, SYBR green PCR mix (Applied Biosystems) with primers specific to the vaccinia virus HA gene at a concentration of 900 nM each (HA-F: 5'-CATCATCTGGAATTGTCACTACTAAA-3', and HA-R: 5'-ACGGCCGACAATATAATTAATGC-3') was used.

2.13. Flow cytometry

L929 cells were seeded at 6.0 × 10^5 per well of a 6-well plate, and the next day they were transfected with 0.5 µg pInt-mCherry per well for 7 hours, then infected with WT or ts2 at MOI of 3 in the presence of 50 µM AraC at 37ºC for 18-20 hours. Cells were harvested with trypsin, washed with 1xPBS, and fixed with 4% paraformaldehyde for 15 minutes at room temperature and suspended in 1 ml of 1xPBS. For FACS analysis (FACSort, Becton Dickinson Biosciences), 10,000 events were captured for every sample, and cells were sorted based on the expression of mCherry using the SSC (side scatter for internal complexity)

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and FSC (forward scatter for related size). Data were analyzed with FlowJo 7.6.1 software (Tree Star Inc).

2.14. Luciferase assay

2.14.1. Transfection experiments alone

L929 cells were seeded at 2 × 10^5 per well of a 12-well plate, and the next day they were transfected using Lipofectamine 2000 (2 µl per µg DNA; Invitrogen) with CMV-Luc or minP-luciferase for additional 12h. Cells were washed twice with PBS, and then lysed with 300 µl of 1X Reporter Lysis buffer (Promega), and freeze-thaw twice. The luciferase activity was measured using 50 µl of lysate and 100 µl of luciferase assay substrate buffer in a Berthold multiwell Luminometer. Firefly luciferase expression was normalized to relative total protein level in each lysate. Protein level in each lysate was quantified by BCA protein assay (Thermo Scientific).

2.14.2. Transfection-Infection experiments

Indicated cells seeded at 2 × 10^5 per well of a 12-well plate were transfected using Lipofectamine 2000 (Invitrogen) with 10 ng of pG8-Luc DNA per well. The plasmid-Lipofectamine transfection complexes were prepared as follows:130ng of pG8-luciferase and 1 µl of Lipofectamine 2000 were combined in 2.3 mL of DMEM; and 200 µl of the transfection mixture was used per well. Cells were incubated at 37ºC in a 5% CO_2 atmosphere for 7h to allow for plasmid introduction into the cells, which were then infected with vaccinia virus at a multiplicity of infection (MOI) of 3 in media containing 50 µM AraC and placed back in the incubator for additional 18h. Cells were then washed twice with

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phosphate-buffered saline (PBS) (10 mM Na_2HPO_4-$7H_2O$, 1 mM KH_2PO_4, 2mM KCl, 140 mM NaCl [pH 7.4]), then lysed in 300 µl of 1X Reporter Lysis buffer (Promega) by two freeze-thaw cycles. The luciferase activity was measured using 50 µl of lysate for 100 µl of luciferase assay substrate buffer in a Berthold multiwell Luminometer.

2.14.3. Quantification of firefly-specific mRNA and RT-qPCR

L929-shControl and -shBAF cells seeded at 2 × 10^5 per well of a 12-well plate were transfected using Lipofectamine 2000 (Invitrogen) with 10, 100 and 500 ng of pG8-Luc DNA per well as previously reported (see transfection-infection section) and incubated at 37ºC in a 5% CO_2 atmosphere for 7h. Cells were infected with vaccinia virus at a multiplicity of infection (MOI) of 3 for 4 hours. Cells lysates were collected and RNA extracted for RT- qPCR analysis using a luciferase-specific primer/probe set.

Extracted viral RNA were reverse-transcribed in cDNA using High Capacity cDNA Reverse transcription kit (Applied Biosystems), and treated with 0.5 µg/ml RNase A prior to qPCR analysis. Using TaqMan master mix (Applied Biosystems) and 900 nM of primers G8Pro Fwd (5'- CTTCGTGGATCCTGTAGA ACG-3') and G8Pro Rev (5'- CCATCTTCCAGCGGATAGAATG-3') which flank the pG8-Luc DNA, and 250 nM B1R probe (5'-56-FAM/AG GTG CAG ATC TAG ATG CGG TGA TCA /3IABkFQ-3'), qPCR performed on pG8R-Luc cDNA to determine the degree of depletion of mRNA specific to firefly luciferase gene.

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CHAPTER III

MOLECULAR MECHANISMS ASSOCIATED WITH BAF DNA-BINDING PROPERTIES

Part of the work described in this chapter was published in Journal of Virology, 2011

Nouhou Ibrahim, April Wicklund and Matthew S. Wiebe. Molecular characterization of the host defense activity of the Barrier-to-Autointegration Factor against Vaccinia Virus Journal of Virology 85 (22):11588-11600

Despite several studies on BAF and its biological functions, much remains to be determined about the role of the cytosolic pool of BAF. A recent report showed that the cytosolic pool of BAF acts as a host defense protein against vaccinia virus (11). The antiviral activity of BAF correlates with its relocalization to vaccinia virus factories. The ability of BAF to relocalize to VACV factories prompted us to assess whether BAF acts in a similar manner in the presence of cytosolic dsDNA delivered by transfection. Further, the contributions of intrinsic molecular features of BAF (DNA-binding, dimerization and LEM-domain interaction) on its activities within the cytoplasm have not been studied in the context of BAF as a host defense protein. In this chapter, the molecular features and mechanisms associated with this novel function of the cytosolic pool of BAF were examined.

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3.1. Stably overexpressed BAF retains its normal cellular distribution

To study the role of the cellular BAF, the Flp-In T-REx system was used to stably integrate a single copy of epitope-tagged 3xFlag-BAF or chloramphenicol acetyltransferase (CAT) in the genome of CV1-flp cells (11). The Flp-In T-REx system has the advantage of permitting the stable integration of a gene of interest in a single copy into the cellular genome. Further, because of the poor sensitivity of existing anti-BAF antibodies (for immunofluorescence for instance), the stable overexpression of 3XFlag-BAF allows efficient and sensitive detection of BAF. The overexpression of 3xFlag-BAF brings the total BAF to ~500% of the endogenous BAF protein (Figure 3.1.1). As shown, both 3xFlag-BAF and endogenous BAF migrate as two bands: the arrowhead indicates the phosphorylated form of BAF, mostly visible for the 3xFlag-BAF because of its greater expression level, while the arrow indicates the endogenous BAF. In addition, two additional CV1 cell lines were transduced with replication-incompetent lentiviral vectors to stably express either a BAF-specific or scrambled (control) shRNA to stably deplete BAF. In CV1-shBAF cells, the expression of the BAF protein is depleted to 15% of control levels, while in CV1-shScram, the control shRNA sequence has no impact on BAF levels (Figure 3.1.1).

The stable overexpression of a protein may have a wide range of effects on the cell and the cellular distribution of the target protein. Therefore, to assess the cellular distribution of 3xFlag-BAF vis-à-vis the endogenous BAF, CV1-CAT cells were permeabilized with 0.1% Triton X-100, then 0.5% SDS; while for CV1-

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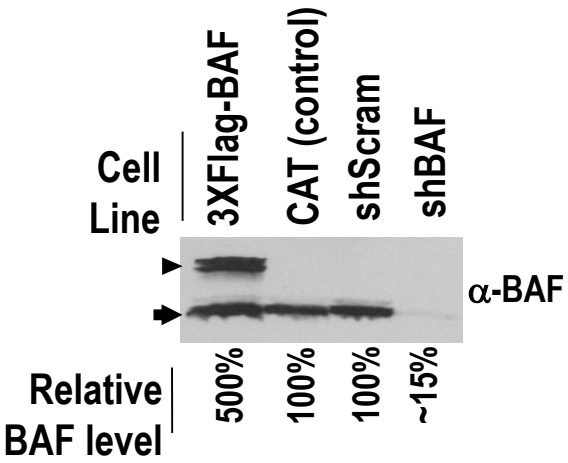

Figure 3.1.1. Immunoblot analysis of BAF expression in model cell lines. The migration of endogenous BAF is indicated with the arrow at the left, and that of 3xFlag-BAF by an arrowhead. Total amounts of BAF in each cell line were quantified using a Bio-Rad Chemidoc XRS instrument, and are shown relative to the control at the bottom of the blot. Equivalent lysates of CV-CAT, CV1-3xFlag-BAF, CV1-shBAF and CV1-shScram were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 18% gel, transferred to PVDF, and incubated with a primary against BAF, and a rabbit secondary antibodies. Blots were developed with chemiluminescent reagents on a Bio-Rad Chemidoc XRS instrument.

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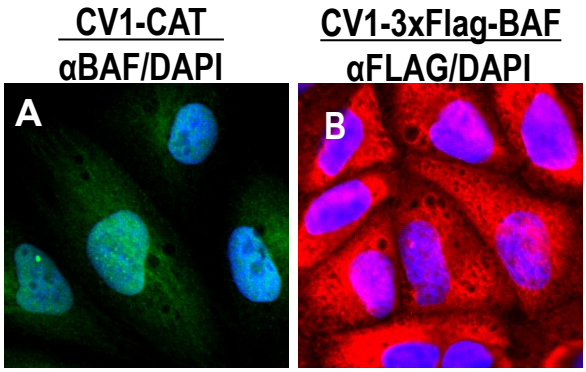

Figure 3.1.2. Localization of 3X-Flag-BAF is consistent with endogenous BAF. CV1-CAT cells (A) or CV1-3xFlag-BAF (B) were fixed with 4% PFA. CV1CAT cells and CV1-3xFlag-BAF were permeabilized with 0.1% saponin. CV1-CAT cells were also treated with 0.5% SDS to unmask the BAF antigen. For immunofluorescence imaging, a primary antibody against BAF was used with AlexaFluor 488 secondary antibody for CV1-CAT cells; and for CV1-3xFlag-BAF, an M2 αFlag primary antibody and AlexaFluor 594 secondary antibody were used, and DAPI was used for both cells. Representative images shown were taken using a confocal microscopy at 60X magnification.

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3xFlag were permeabilized with 0.1% saponin. For immunofluorescence, a primary antibody against the endogenous BAF was used in CV1-CAT cells, and the anti-Flag M2 antibody in CV1-3xFlag-BAF cells. As shown in figure 3.1.2.A and 3.1.2.B, like the endogenous BAF in CV-1-CAT cells, 3xFlag-BAF is present in both the nucleus and cytoplasm. There is no difference in the location of BAF in either cell lines. Further, the doubling rate of CV1-CAT and CV-3xFlag-BAF was similar (data not shown). Together, these data suggest that the overexpression of 3xFlag-BAF did not affect cell growth and 3xFlag-BAF is likely to behave as the endogenous one.

3.2. The cytoplasmic presence of dsDNA is sufficient for BAF relocalization

Previously, BAF has been shown to relocalize to VACV DNA replication sites, and this is one of the reasons BAF is considered as a host antipoxviral factor (11). Considering the immune responses resulting from the detection of cytosolic nucleic acids, the ability of BAF to relocalize to viral DNA replication has an important implications in term of innate responses. Thus, determining the ability of BAF to recognize/relocalize to cytosolic nucleic acids as well as the molecular features associated with it is of interest. To assess whether BAF can relocalize to cytosolic dsDNA in the absence of VACV infection, CV1-CAT and CV1-3xFlag-BAF cells were transfected with Lipofectamine 2000 alone or with 1 µg DNA pUC19 DNA for 7 hr at 37ºC. For immunofluorescence imaging, a primary antibody against the endogenous BAF was used in CV1-CAT cells (figure 3.2.1), and the M2 anti-Flag antibody in CV1-3xFlag-BAF (figure 3.2.2).

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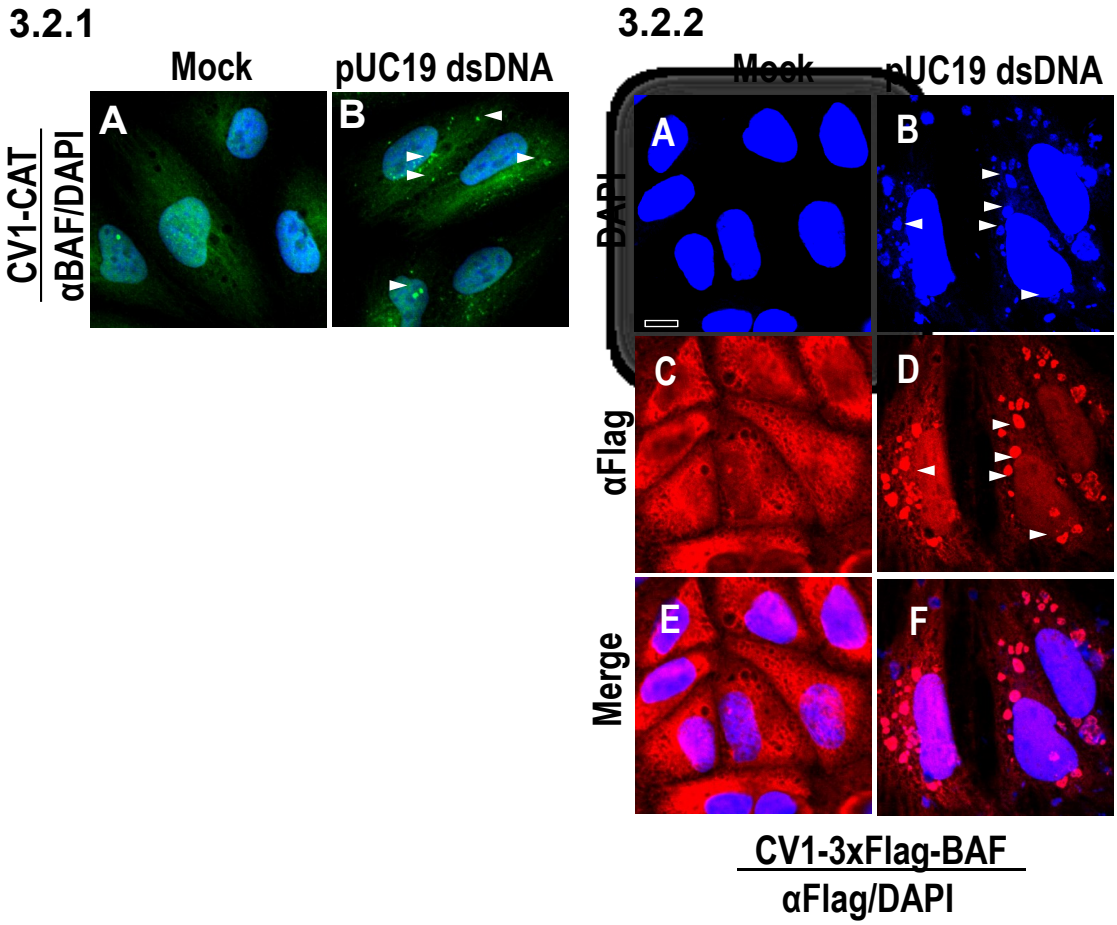

Figure 3.2. BAF relocalizes to discrete puncta during plasmid transfection. CV1-CAT (3.2.1) and CV1-3xFlag-BAF (3.2.2) were mock transfected with Lipofectamine 2000 (3.2.1A and 3.2.2 A) alone or with 1 µg pUC19 DNA per milliliter of media (3.2.1B and 3.2.2B) and incubated at 37ºC. Cells were fixed 7 hours later. Both cell lines were fixed and antigens unmasked as in figure 3.1.2. Representative images shown were taken using a confocal microscopy at 60X magnification. Arrowheads mark some of the sites of BAF and DAPI colocalization. Scale bars = 10 µm.

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The reason for using an anti-BAF primary antibody is to show that both the tagged and endogenous BAF recognize the transfected dsDNA in similar fashion. As shown in figure 3.2.1A, in CV1-CAT mock-transfected, there is no change in the cellular distribution of BAF; the same observation was made in mock-transfected CV1-3xFlag-BAF cells (figure 3.2.2C & E).

The transfection of dsDNA triggered the relocalization of BAF to cytoplasmic puncta in CV1-CAT (arrowheads in Fig 3.2.1B), these puncta are also easily visible in CV1-3xFlag-BAF cells (arrowheads in Fig 3.2.2D). These cytoplasmic puncta are visible as little as 2-3 hr after transfection (data not shown), but easily visible by 6-7 hr post transfection. Strikingly, these puncta were observed in more than 90% of all cells examined. It is noteworthy to say that the antibody against the endogenous BAF was able to detect these cytosolic puncta, although they are not very visible compared to ones in overexpressing BAF cells. In figure 3.2.2B (arrowheads), DAPI staining showed cytoplasmic transfected DNA that colocalize with cytoplasmic BAF as illustrated by the merge figure 3.2.2F. Based on these results, cytoplasmic dsDNA is sufficient to trigger BAF's relocalization to cytoplasmic puncta, consistent with its DNA-binding properties.

3.3. BAF does not relocalize to ssDNA and RNA

In vitro studies showed that BAF exhibits dsDNA-binding in a sequence independent manner, but little or no affinity for single stranded (ss) DNA or dsRNA (18, 64). This specificity has not been tested in live cells. Therefore, the specificity of BAF for several types of nucleic acid was examined. CV1-CAT and

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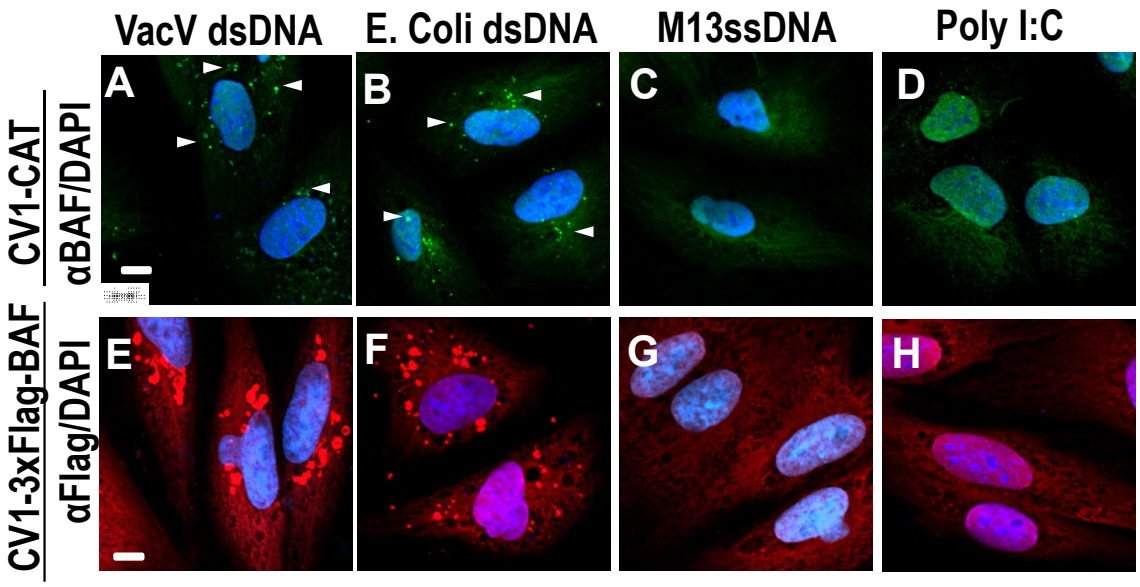

Figure 3.3.1. BAF relocalizes to dsDNA, but not ssDNA or dsRNA. CV1-CAT (A to D) and CV1-3xFlag-BAF (E to H) cells were transfected with identical amounts (1 μg/mL) of purified vaccinia DNA, E. Coli dsDNA, M13 single stranded DNA, or poly I:C (a synthetic double stranded RNA) and incubated at 37ºC. Cells were fixed 7 hr later, permeabilized, and processed for immunofluorescence imaging using against BAF (A to D), or an M2 αFlag (E to H) primary antibodies, Alexa Fluor 488 (A to D) and 594 (E to H) secondary antibodies, and DAPI. Representative images shown were taken using a confocal microscopy at 60X magnification. Scale bars = 10 μm.

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CV1-3xFlag-BAF were transfected with equal amounts of bacterial DNA (E. coli), purified vaccinia virus dsDNA, M13 ssDNA, or the dsRNA mimic poly I-C. After 7hr at 37ºC, cells were fixed and treated for immunofluorescence imaging as reported above using antibodies against endogenous BAF (Fig 3.3.1A to D) and the FLAG epitope (Fig 3.3.1E to H). The transfection of dsDNA, from either bacteria or vaccinia virus origin, readily triggered the formation of cytosolic puncta in both CV1-CAT and CV1-3xFlag-BAF. These puncta, regardless of the type of dsDNA, were indistinguishable in size in CV1-CAT or CV1-3xFlag-BAF (compare Fig 3.3.1A against B, and E against F). The size of these cytoplasmic puncta was smaller when detected with anti-antibody against the endogenous BAF compared to those detected with anti-flag antibody. The relocalization of BAF was observed with other dsDNAs including plasmids and synthetic DNA oligos as well (data not shown). The transfection of either M13 ssDNA or poly I-C did not trigger the formation of cytosolic puncta, suggesting BAF did not relocalize (Fig 3.3.1C, D, G & H).

Together, these data demonstrate that dsDNA devoid of proteins and independently of its source (viral or bacterial) is sufficient to cause BAF's relocalization. Furthermore, this is the first evidence that BAF's relocalization behavior in live cells is consistent with BAF's nucleic acid binding specificity characterized in vitro. From now forward, these cytoplasmic puncta will be called BAF:DNA complexes.

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3.4. Emerin and Lap2α co-localize with BAF-DNA complexes

Mass spectrometry data from our laboratory showed that other cellular proteins, such as Ku86 and RPA32, are also present at these BAF:DNA complexes (Wiebe and Wicklund data). In addition to these proteins, two LEM-domain proteins, emerin and Lap2α were selected because they interact with BAF in late mitosis or during retroviral infection (54, 55, 67). These four proteins were investigated for their localization during transfection of dsDNA. CV1-CAT, CV1-3xFlag-BAF and CV1-shBAF cells were transfected with Lipofectamine 2000 alone or with 1 µg DNA pUC19 DNA for 7 hr at 37ºC. Then cells were processed for immunofluorescence imaging using anti-emerin antibody (figure 3.4.1 A to D), anti-Lap2α antibody (figure 3.4.1 F and I) and M2 anti-Flag antibody (figure 3.4.1 E and J).

In untreated CV1-CAT cells, emerin is found at the nuclear rim and in the endoplasmic reticulum (ER) (Fig. 3.4.1A), while Lap2α exhibits diffuse staining in the nucleus but weak in the cytoplasm (Fig. 3.4.1F). In transfected CV1-CAT cells, both Lap2α and emerin relocalize to cytoplasmic depots (Fig. 3.4.1 B and G), but emerin forms a ring-shaped structure surrounding the cytoplasmic puncta (Fig. 3.4.1 B). In CV1-shBAF, where BAF is stably depleted, emerin does not relocalize to cytoplasmic puncta contrary to Lap2α which relocalizes to cytoplasmic puncta (compare Fig. 3.4.1C and H). In dsDNA-transfected CV1-3xFlag-BAF cells both emerin (Fig. 3.4.1D) and Lap2α (Fig. 3.4.1I) colocalize very well with the cytoplasmic puncta, and also with BAF (Fig. 3.4.1E and J). A comparison of Lap2α relocalization in the 3 cells lines revealed little difference in

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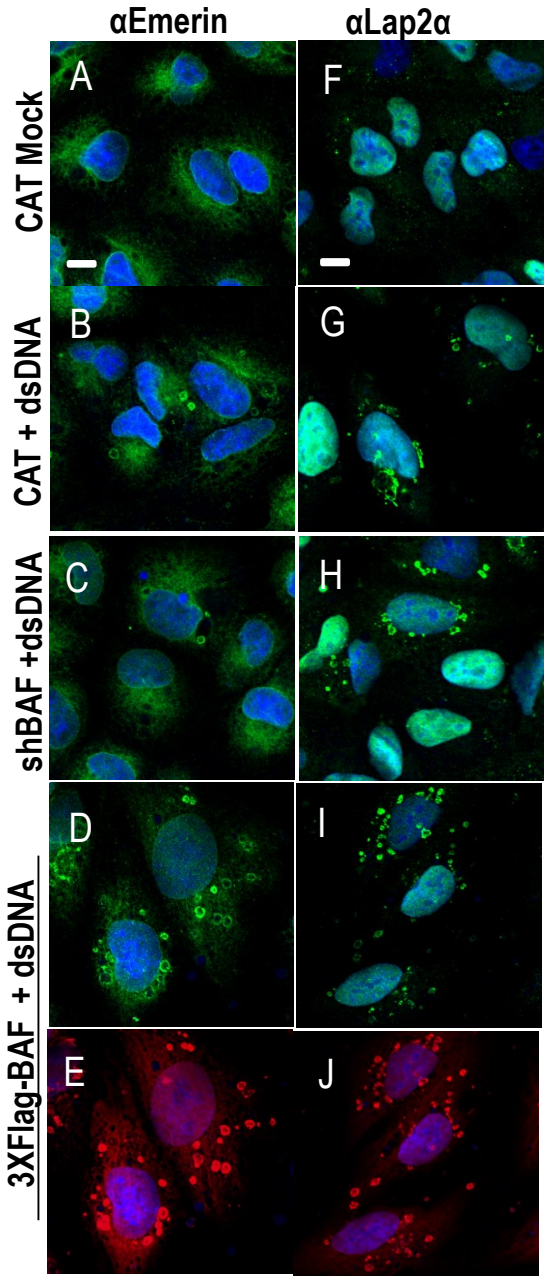

Figure 3.4. Emerin and Lap2α co-localize with BAF-DNA complexes. CV1-CAT (A, B, F and G), CV1-shBAF (C and H) or CV1-3xFlag-BAF (D, E, I and J) cells were mock transfected with Lipofectamine 2000 alone, or with 1 µg/mL of pUC19 DNA for 7 hr at 37ºC. Cells were permeabilized using 0.1% saponin, and immunofluorescence imaging using against M2 αFlag (E and J), anti-emerin (A to D) or anti-Lap2α (F to I) primary antibodies, Alexa Fluor 488 (A to D and F to I) and 594 (E and J) secondary antibodies, and DAPI. Representative images shown were taken using a confocal microscopy at 60X magnification. Scale bars = 10 µm.

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the number of puncta (compare panels figure 3.4.1 G, H and I), suggested that while Lap2α localizes to the same sites as BAF3.4.1 G, H and I), it does so in a BAF-independent manner. In contrast, emerin 'rings' were more prevalent in cells overexpressing BAF and rare in the BAF-depleted cells (compare panels figure 3.4 B, C, and D). This indicates that emerin is likely recruited to cytoplasmic DNA by BAF, as it is to cellular DNA during telophase (8, 54)

3.5. RPA32 and Ku86 co-localize with cytoplasmic DNA independent of BAF

RPA32 and Ku86, both identified by mass spectrometry to co-purify with BAF-DNA complexes, are not known BAF-interacting proteins. Therefore, they were also tested for their potential dependence on BAF to relocalize to BAF:DNA complexes during transfection.

Ku is a heterodimeric DNA end-binding complex composed of ku70 and Ku86 kDa subunits in humans (219). The Ku70/80 heterodimer and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) constitutes a holo-enzyme, part of the core nonhomologous recombination DNA end joining (NHEJ) pathway, one of the double strand break (DSB) repair pathway (219). The Ku complex binds to dsDNA (with 5' and 3' overhangs as well as blunt or stem-loop structures at the ends of dsDNA) in sequence independent manner (220). Mutational studies showed that the inactivation of Ku86 in human somatic cell lines results in a defective cell proliferation, severe growth defect, polyploidy leading to apoptosis after few cell doublings (221). The replication protein A32 or RPA32 is a component of a stable complex of three subunits RPA70, RPA32 and RPA14 (222). These RPAs binds to ssDNA, and are essential for chromosomal

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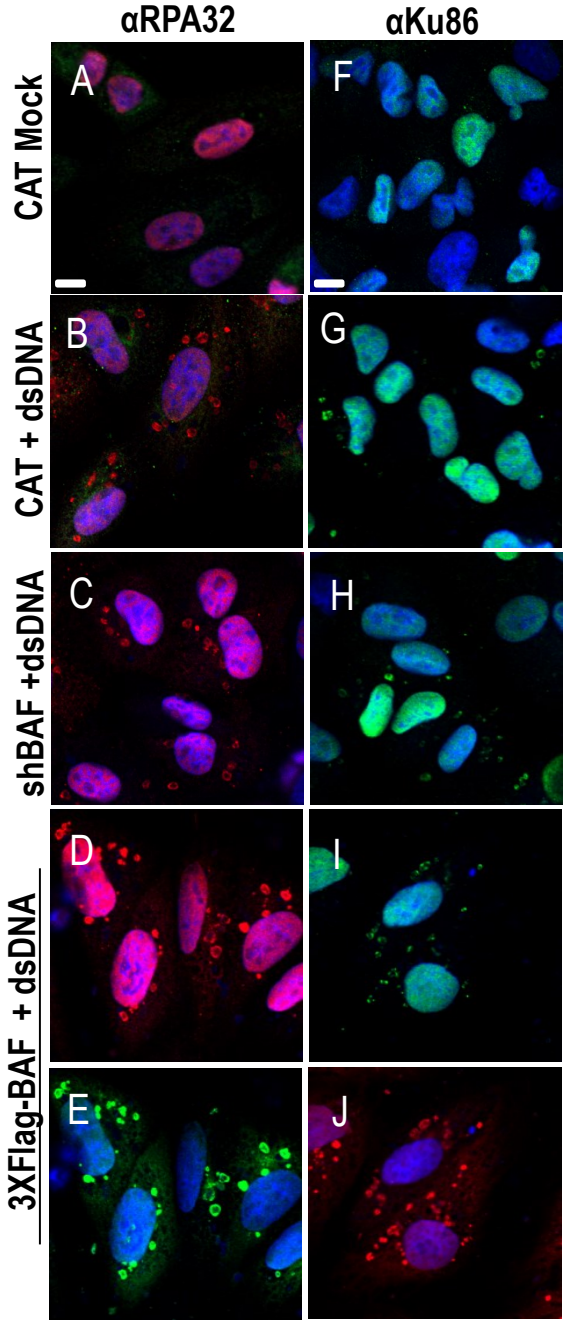

Figure 3.5. RPA32 and Ku86 co-localize with cytoplasmic DNA in a BAF-independent manner. CV1-CAT (A, B, F and G), CV1-shBAF (C and H) or CV1-3xFlag-BAF (D, E, I and J) cells were mock transfected with Lipofectamine 2000 alone, or with 1 µg/mL of pUC19 DNA for 7 hr at 37ºC. Cells were permeabilized using 0.1% saponin, and processed for immunofluorescence imaging using against M2 αFlag (E and J), anti-RPA32 (A to D) or anti-Ku86 (F to I) primary antibodies, Alexa Fluor 488 (F to I, and E) and 594 (A to D, and J) secondary antibodies, and DAPI. Representative images shown were taken using a confocal microscopy at 60X magnification. Scale bars = 10 µm.

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DNA replication, repair and recombination processes and DNA damage responses in eukaryotic cells (223, 224). Both RPA32 and Ku86, because of their role in DNA metabolism, may generally exhibit a nuclear presence. Therefore, their relocalization to the cytoplasm may suggest an additional function yet to be determined.

To determine RPA32 and Ku86's distribution during a dsDNA transfection, CV1-CAT, CV1-3xFlag-BAF and CV1-shBAF cells were transfected with Lipofectamine 2000 alone or with 1 µg DNA pUC19 DNA for 7 hr at 37ºC. Then cells were processed for immunofluorescence imaging using anti-RPA32 antibody (figure 3.5.1A to D), anti-Ku86 antibody (figure 3.5.F to I) and M2 anti-Flag antibody (figure 3.5.1 E & J). In mock-transfected CV1-CAT cells, RPA32 and Ku86 are found within the nucleus (Fig. 3.5.1 A & F), however, 7 hr after dsDNA transfection in CV1-CAT as well as in CV1-shBAF cells, both proteins relocalize to cytoplasmic puncta (Fig. 3.5.1B & G). In dsDNA-transfected CV1-shBAF cells, both proteins relocalize to BAF:DNA complexes (Fig. 3.5.1 C & H). In CV1-3xFlag-BAF cells, RPA32 colocalizes to these BAF-DNA cytoplasmic puncta (Fig. 3.5.1D & E), as did Ku86 (Fig. 3.5.1I & J). Regardless of BAF's level of expression, both RPA32 and Ku86 relocalize to transfected DNA in BAF-independent manner, suggesting these proteins may be recruited to the cytoplasm through a cellular mechanism not required BAF. In the mock-transfected cells, these proteins are most nuclear, and their relocalization to the cytoplasm may suggest a novel activity associated with cellular responses to cytoplasmic nucleic acids.

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3.6. DNA Binding and Dimerization Properties are essential for BAF's activity

To examine the importance of DNA-binding, homodimerization, or LEM-domain interaction of BAF for its ability to relocalize to foreign cytoplasmic DNA, BAF mutants that are deficient in each of these three properties were used. Previous biochemical and structural studies of BAF had identified lysine-6 (K6), glycine-47 (G47), and lysine-53 as critical for DNA-binding, dimerization and interaction with LEM-domain proteins respectively (5, 7, 16, 64).

In figure 3.6.A, a schematic representation of BAF-dsDNA complexes depicts the estimated position of each residue within BAF dimer. The K6 residue is located in helix-1, and mediates multiple contacts with the phosphate backbone of DNA, and its mutation to alanine sharply reduces BAF's affinity for DNA (7, 64). The G47 residue is at the center of the dimerization helix-3, and increases the rate at which BAF homodimers exchange monomers (7). K53 is also in helix-3, and its mutation abolishes binding to the LEM-domain protein emerin, while leaving dimerization largely unaffected (7). April Wicklund constructed these BAF mutant proteins. The lysine-6 was replaced with alanine (K6A), G47 was replaced with glutamic acid (G47E) and lysine-53 was replaced with glutamic acid (K53E). These BAF mutants were epitope-tagged with 3xFlag, and stably expressed in CV1 cells.

The stable expression of these mutants was assessed by western blot using M2 anti-Flag antibodies. Both 3xFlag-BAF-K6A and -G47E proteins were

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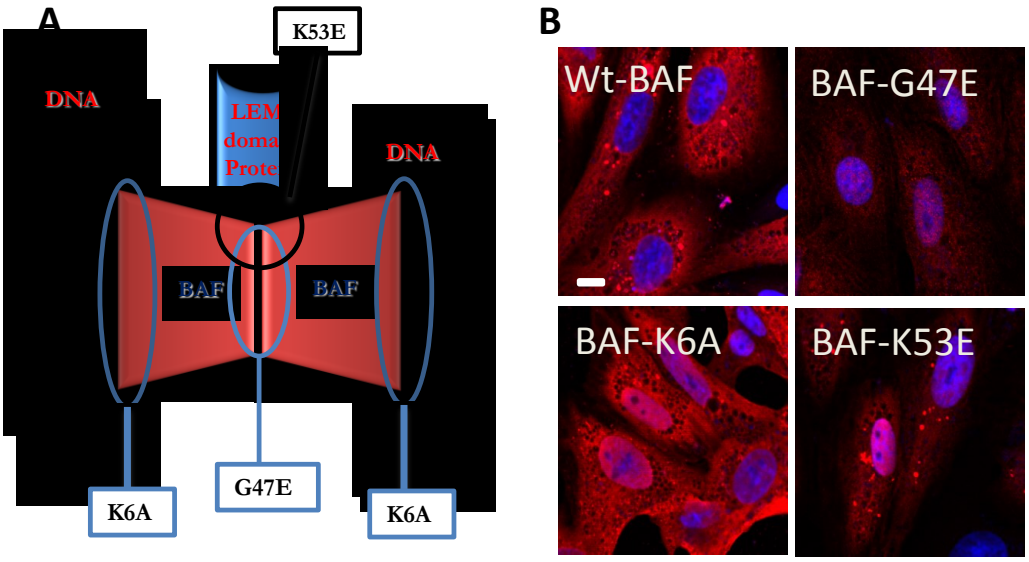

Figure 3.6. Puncta Formation Requires BAF's DNA Binding and Dimerization Properties. A) A schematic representation of BAF-dsDNA complexes showing the points mutations and their estimated positions within BAF dimer. **B)** CV1 cells expressing 3XFlag-BAF or BAF mutants transfected with 1 µg pUC19 DNA per milliliter of media and incubated at 37ºC. Cells were fixed 7 hr later, permeabilized using 0.1% Triton X-100, and processed for immunofluorescence imaging using an M2 αFlag primary antibody and Alexa Fluor 594 secondary antibody. Representative images shown were taken using a confocal microscopy at 60X magnification. Scale bars = 10 µm.

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expressed at similar levels to the wild-type BAF, but BAF-K53E protein was expressed ~10% of wild-type BAF (data not shown). Further, these mutant proteins displayed defect in the activity specifically associated with the mutation (reference Ibrahim 2011).

The stable expression of these mutants was assessed by western blot using M2 anti-Flag antibodies. Both 3xFlag-BAF-K6A and -G47E proteins were expressed at similar levels to the wild-type BAF, but BAF-K53E protein was expressed ~10% of wild-type BAF (data not shown). Further, these mutant proteins displayed defect in the activity specifically associated with the mutation (reference Ibrahim 2011).

To examine the impact of each of these mutations on BAF's ability to localize to cytoplasmic dsDNA, cells expressing 3xFlag-BAF (WT-BAF) or these mutants were transfected with an equal amount of dsDNA. In untransfected cells, the overall localization of the 3xFlag-BAF-K6A and -K53E proteins was evenly distributed between the cytoplasm and nucleus, similar to that of the wild-type 3xFlag-BAF protein (data not shown). In cells expressing 3xFlag-BAF-G47E, the mutant protein was more concentrated in the nucleus, but a small portin was still present in the cytoplasm (data not shown). In regard to puncta formation in the presence of dsDNA, as shown in Fig 3.6.B, the formation of BAF-DNA complexes was greatest in cells expressing WT-BAF and BAF-K53E proteins. However, in cells expressing BAF-K6A, these puncta were reduced, and absent in BAF-G47E expressing cells. Based on these mutational studies, it

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is likely that DNA-bind and homodimerization are necessary for BAF's ability to relocalize and form cytoplasmic BAF-DNA complexes.

3.7. DISCUSSION

Based on its dsDNA-binding properties, BAF relocalizes to VACV DNA replication sites and inhibits DNA replication in the absence of an active viral B1 kinase (Wiebe & Traktman, 2007). Although BAF binds dsDNA with high affinity, poxviral replication factories are comprised of DNA, RNA, and viral and cellular proteins (225, 226), and any of these factors may play a role in BAF's relocalization. For example, the viral matrix protein appears to participate in BAF recruitment to retroviral PIC during HIV infection (9). While these studies provide evidences of the role of the cytoplasmic pool of BAF during viral infection, it is not known whether BAF can relocalize to cytoplasmic nucleic acids. Little is known about the molecular mechanism and intrinsic properties of BAF associated with this activity as well as the contribution of cellular or viral factors to BAF's activity. Therefore, this study was designed to understand the role and molecular mechanisms associated with the activity of the cytoplasmic BAF in the presence of cytoplasmic nucleic acids.

In attempt to study the function of BAF, the Flp-In TREx system was used to stably integrate a single copy of epitope- tagged 3xFlag-BAF in the genome of CV1-flp cells. This approach permitted stable overexpression of 3xFlag-BAF without affecting its apparent normal cellular distribution (Fig.3.1.2). The previous observation that BAF relocalizes to VACV DNA replication prompted me to determine whether BAF colocalizes to transfected nucleic acid. I show here that

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in both endogenous (CV1-CAT cells) and epitope-tagged BAF (CV1-3xFlag-BAF cells), many types of transfected dsDNA trigger BAF relocalization to form a cytoplasmic puncta (Fig. 3.2.2 and 3.3.1). However, ssDNA or RNA (ss and ds) are not sufficient to trigger the relocalization of BAF. Remarkably, the puncta formed by 3XFlag-BAF appear larger than that formed by endogenous BAF in our immunofluorescence studies (see figure 3.3.1 A, B, E and F). It is unclear whether this is a bona fide difference in complex size, a difference in sensitivity of the two antibodies employed or the size of 3XFlag tagged. These data showed that DNA alone is sufficient to trigger BAF's relocalization in accordance with BAF's DNA-binding specificity observed using purified protein in vitro (18, 64). Further, these data provide the first evidence that BAF's recruitment to dsDNA is mediated through direct interaction between BAF and DNA in vivo.

Further, I investigated whether other proteins are also present at these cytoplasmic puncta, as well as their dependentce on BAF's activity. I used RPA32 and Ku86, two proteins identified by IP pull down assay using BAF to identify proteins interacting with BAF-DNA cytoplasmic puncta. In addition, two LEM-domain proteins, Lap2α and emerin were selected because they are both recruited by BAF to host DNA during post-mitotic nuclear reassembly (54, 55, 67). By immunofluorescence assay, both proteins, Lap2α and emerin co-localize with BAF at cytoplasmic depots during transfection (Fig. 3.4.B, D and G, I). The relocalization of emerin appears to be BAF-dependent (Fig. 3.4C), while Lap2α is independent of BAF (Fig. 3.4H). One possible explanation for this difference between these two LEM-domain proteins is that Lap2α has been observed to

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bind DNA on its own (67), while no DNA binding function has been ascribed to emerin. In the case of RPA32 and Ku86, although they are present at cytoplasmic BAF-DNA depots during transfection (Fig. 3.5.B, D and G, I), these proteins colocalize with dsDNA in BAF-independent manner (Fig. 3.5. C and H). Both proteins are involved with DNA metabolism within the nucleus, they are able to bind to DNA. These data suggest that the presence of dsDNA leads to BAF's relocalization but also other cellular proteins to dsDNA to BAF-dependent and independent manners.

The biological significance of these events on the cytoplasmic DNA remains to be elucidated. The relocalization of BAF to dsDNA and the formation of BAF-DNA complexes within the cytoplasm may suggest a cellular response mechanism to respond to cytoplasmic DNA as a foreign element. It is not known whether the presence of other proteins at these cytoplasmic puncta enhance whatever biological activity BAF has.

The relocalization of BAF to cytoplasmic DNA depends on intrinsic properties of BAF such as DNA-binding and dimerization. Three cells stably expressing BAF mutants were employed. These mutations, a single amino acid changes, affect individually DNA-binding, dimerization and LEM-domain interactions, which have been previously characterized in multiple studies using recombinant protein (5, 7, 16, 64). Immunoblot analysis of these mutant proteins showed that the BAF-K53E protein was present at a much lower level in stable cells. This reduction in protein level of expression of BAF-K53E protein was highly reproducible, and also found when the protein was stably expressed in

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293 HEK Flp-In cells (data not shown). The low level of BAF-K53E may indicate that this mutation affects the stability of BAF, suggestive of the importance of LEM-domain interaction as an essential feature for BAF's stability. The location of this mutation may also suggest its role in the stable dimerization of BAF.

Using cytoplasmic puncta formation as a marker of BAF's activity after the transfection of dsDNA, cells stably expressing BAF-K6A (mutation affecting DNA-binding) and G47E (mutation affecting dimerization) mutations were far less effective at forming puncta than cells expressing BAF-WT and -K53E (mutation affecting LEM-domain interaction) (Fig. 3.6) (5, 7, 64). However, the reduced stability of the BAF-K53E protein could also lead to impaired puncta formation in our model system (Fig. 3.6). Based on these results, it is likely that DNA-binding and dimerization properties of BAF are necessary for BAF to form nucleoprotein complexes in the presence of cytoplasmic dsDNA.

Further, DNA cellulose-binding data from our laboratory confirmed DNA-binding and dimerization as essential. The DNA-binding efficiency of both BAF-K6A and -G47E proteins was about 10% of that of the wild-type BAF. The amount of BAF-K53E protein bound to dsDNA was ~80% of that of wild-type BAF, which correlates with previous studies showing that the K53E mutation has little effect on DNA binding of BAF (7). While it was expected that BAF-K6A to not bind DNA, the lack of DNA-binding and puncta formation in the dimerization mutant BAF-G47E provide evidence that BAF monomers synergistically cooperate in DNA binding as a dimeric unit. It is possible that the lack of

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dimerization of BAF-G47E protein blocks such synergism, and leads to the decrease in DNA binding that we observed with this mutant.

Based on these data, the ability of BAF to relocalize to a transfected dsDNA is associated to its intrinsic molecular features, and that BAF interacts directly with the transfected dsDNA. Further, these data showed that the binding of DNA by BAF is an essential step in the formation of cytoplasmic puncta, while the recruitment of other proteins such as emerin plays a nonessential, but potentially substantive role in stabilizing BAF:DNA cytoplasmic complexes. Our working model is that in the presence of cytoplasmic dsDNA triggers BAF's recruitment and the formation of puncta. These puncta become a platform for the recruitment of other cellular proteins in BAF-dependent or independent manner.

The presence of nuclear proteins (RPA32, Lap2α and Ku86) at these cytoplasmic puncta suggests these proteins like BAF may be part of a cellular response mechanism to cytoplasmic dsDNA. More studies on the role these proteins in response to foreign DNA are needed to elucidate the significance of these cytoplasmic puncta.

In summary, this study shows that the relocalization of BAF during dsDNA transfection depends on its intrinsic molecular features. However, more studies are needed to determine whether the sole presence of dsDNA in the cytoplasm is enough to trigger BAF's recruitment or a cellular mechanism exists to regulate this event. Further, I provide evidence of the presence of other proteins at the BAF-DNA complexes, providing an indication of potential existence of cellular

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mechanism of response to cytoplasmic dsDNA involving proteins associated with DNA repair and replication.

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CHAPTER IV

BAF RECRUITS EMERIN TO TS2-REPLICATION SITES.

Part of the work described in this chapter was published in Journal of Virology, 2011

Nouhou Ibrahim, April Wicklund and Matthew S. Wiebe. Molecular characterization of the host defense activity of the Barrier-to-Autointegration Factor against Vaccinia Virus Journal of Virology 85 (22): 11588-11600.

Early studies of the viral function of BAF showed that BAF is a component of the pre-integration complexes of MoMLV and HIV-1, and enhances the integration of viral cDNA into the host genome. The recent discovery of antiviral role of BAF during vaccinia virus infection (11) provided evidence of the role of the cytosolic pool of BAF. However, much remains to be understood about the molecular mechanisms and features governing BAF's function, as well as the role of other cellular proteins in assisting BAF in its antiviral function.

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4.1. BAF inhibits the growth of B1-deficient ts2 Virus (by April Wicklund)

The goal of this study was to further understand the molecular mechanisms through which BAF relocalizes to VACV replication factories and inhibits viral growth of the B1-deficient, temperature sensitive ts2 virus as previously observed in 293-HEK cells (11). In comparison to the 293 HEK cell line, CV1 cells exhibit a superior ability to form plaques when infected with VACV and are highly amenable to immunofluorescence imaging. Therefore, we utilized them as our primary cell model system in this study.

The impacts of BAF on viral yield and DNA replication of ts2 vaccinia virus were tested in CV1-CAT, CV1-3xFlag-BAF, CV1-shBAF and CV1-Scram at 37ºC and various MOIs (see figure 4.1). The viral yield in CV1-shScram and CV1-CAT cells were indistinguishable, thus the results for the CV1-shScram cells were not shown and discussed. As shown in figure 4.1A, regardless of BAF's level of expression, WT virus has better viral yield than ts2 virus at 37ºC. As expected, since WT virus expresses an active B1, no difference in viral yield was observed in the three cell lines infected with WT virus. In contrast, while ts2 could still replicate albeit more weakly in all 3 cell lines at 37ºC, there was an inverse correlation between viral yield and BAF expression.

In CV1-CAT cells at 48 hr post infection (hpi), there was 29.6-fold less ts2 virus than in CV1-shBAF cells, and the viral yield in CV1-3xFlag-BAF was 1230-fold less than that in CV1-shBAFcells. However, when tested with a range of MOIs (0.1, 0.3, 1, 3 and 5), ts2 viral yield in CV1-shBAF cells plateaued; while the gap in ts2 viral yield between CV1-shBAF and CV1-3XFlag-BAF cells shrunk

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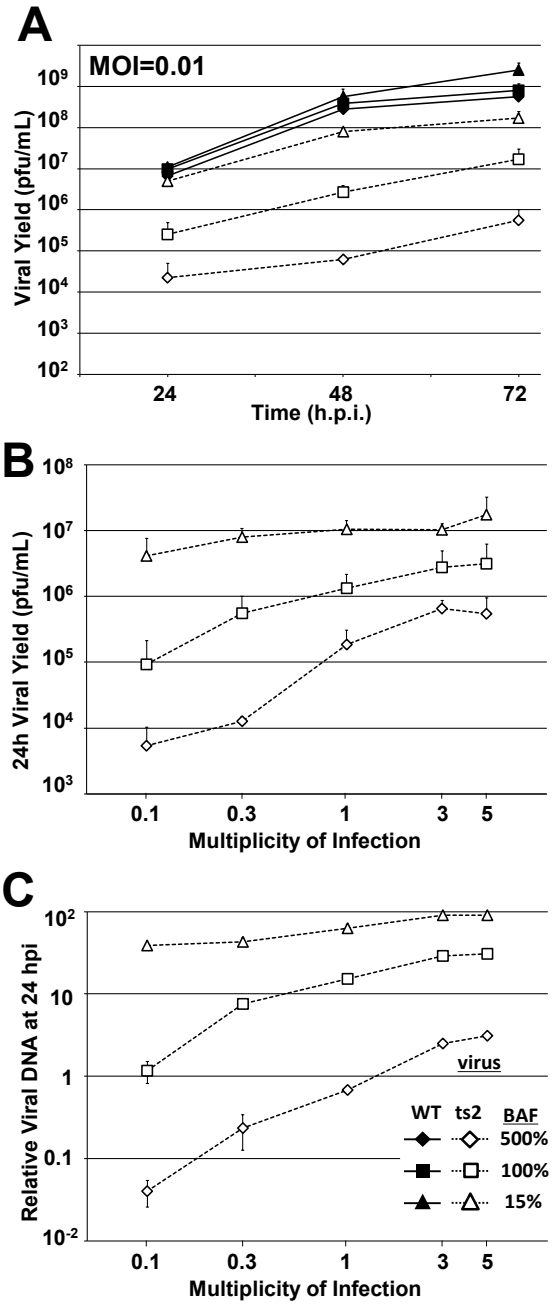

Figure 4.1. BAF inhibits the growth of B1-deficient ts2 virus. A) Viral yield obtained following the infection of CV1-CAT, CV1-3xFlag-BAF and CV1-shBAF cells with WT or ts2 virus at a MOI=0.01 for 24, 48 and 72 hr at 37ºC. **B)** Viral yield obtained following CV1-CAT, CV1-3xFlag-BAF and CV1-shBAF cells with indicated MOIs of ts2 virus for 24hr at 37ºC. BSC40 cells were used for virus production at 32ºC for results shown in panels A and B. **C**) Viral DNA accumulation in cells infected as in A). Total DNA was isolated 24hr after infection with ts2 virus at 37ºC at the indicated MOIs and quantified by qPCR. All data were obtained from two independent experiments, errors bars represent the standard errors of the means.

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from 770-fold at an MOI of 0.1 to 32-fold at an MOI of 5 (Figure 4.1B).

The same trend is observed when the amount of viral DNA produced in ts2 infection at different MOIs was isolated 24 hr post infection (hpi) at 37ºC, and quantified by qPCR (Figure 4.1C). Specifically, at an MOI of 0.1 the DNA yield in ts2 differs by 3 orders of magnitude between CV1-3xFlag-BAF and CV1-shBAF, but at an MOI of 5, the difference is 50-fold. These data provide a more thorough characterization of BAF's impact on ts2 viral DNA yield and progeny virus than was initially described (43). Further, these data demonstrate that BAF possesses potent antiviral activity at a range of infectious doses, but is most effective at low MOIs. The fact that 3XFlag-BAF overexpression reduced viral growth even further than endogenous BAF in control cells also indicates that BAF remains capable of acting as a host defense against vaccinia infection when epitope-tagged.

4.2. Emerin Co-localizes to ts2 replication sites in a BAF-dependent Manner

During transfection of dsDNA, other cellular proteins were recruited to BAF-DNA complexes, and we wanted to investigate whether these proteins are also recruited to VACV replication sites. Because BAF interacts with LEM-domain proteins (54, 55, 67), we tested whether emerin and Lap2α are also recruited to ts2 DNA replication sites utilizing a variation of the temperature shift protocol initially used to observe BAF at these sites (11). CV1-CAT cells were infected with wild-type or ts2 virus at 32ºC for 9 hr to allow DNA replication factories to efficiently form. At 4 hpi, BrdU was added to the infected cells to

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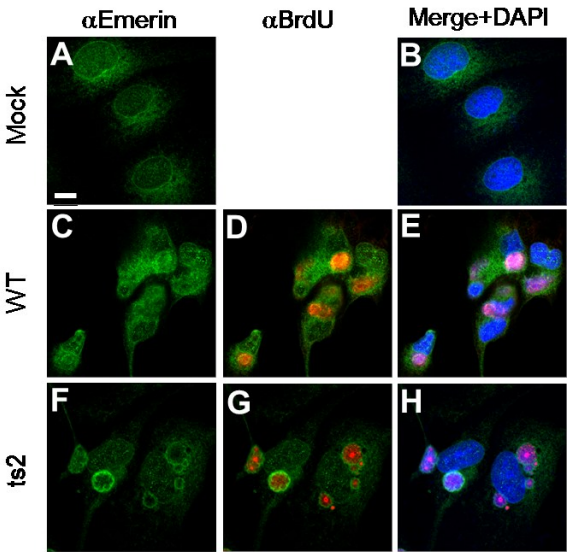

Figure 4.2. Emerin Colocalizes to ts2 replication sites in a BAF-dependent Manner. CV1-CAT cells were left untreated or infected with wild-type or ts2 virus at 32°C. At 4 hpi, cells were treated with 25 μg/ml BrdU to label replicating DNA, and at 9 hpi, cells were shifted to 40°C. Cells were fixed 7 h later (16 hpi), and permeabilized by using 0.1% saponin–PBS. Cells were processed for immunofluorescence imaging using M2 anti-emerin and anti-BrdU primary antibodies, Alexa Fluor secondary antibody, and DAPI. The representative independent and overlaid images shown were taken by using a confocal microscope at a 60X magnification. Scale bars, 10 μm.

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label replicating DNA for later detection of viral factories using a BrdU-specific antibody.

At 9 hpi, cultures were then shifted to non-permissive temperature (40ºC) for an additional 7 hr prior to fixation and downstream processing for immunofluorescence imaging of BrdU, and emerin localization. In mock-infected CV1-CAT cells, emerin exhibits perinuclear localization (figure 4.2A). Strikingly, during ts2 infection, emerin localized and surrounded ts2-replication factories in a ring-like structure (Fig. 4.2F, G and H). However, in WT-infected cells, the perinuclear presence of emerin is lost, but emerin did not form these distinctive rings around wild-type replication factories (Fig. 4.2C, D, and E). As BAF is also known to relocalize to ts2 replication sites, but not wild-type sites, these data further support a model in which the LEM-domain protein emerin can be recruited by BAF to cytoplasmic nucleoprotein complexes, as seen during dsDNA transfection where emerin relocalizes to BAF-DNA complexes in a BAF-dependent manner (Chapter 3).

4.3. Emerin has no effect on viral yield

Since emerin is recruited to ts2-replication sites, its impact on the ts2 viral yield was tested. To this end, emerin was stably depleted by the expression of a shRNA-specific to emerin or shScram (control non-targeting emerin) in CV1-CAT cells. As shown in the immunoblot in Fig. 4.3A, CV1-shEmerin cells exhibit undetectable levels of emerin in comparison to both CV1-CAT and CV1-shScram cells. CV1-shEmerin and CV1-shScram were infected with ts2 virus at MOI of 0.01 for 48 hours at both 37°C and 40°C. Lysates were collected and assayed for

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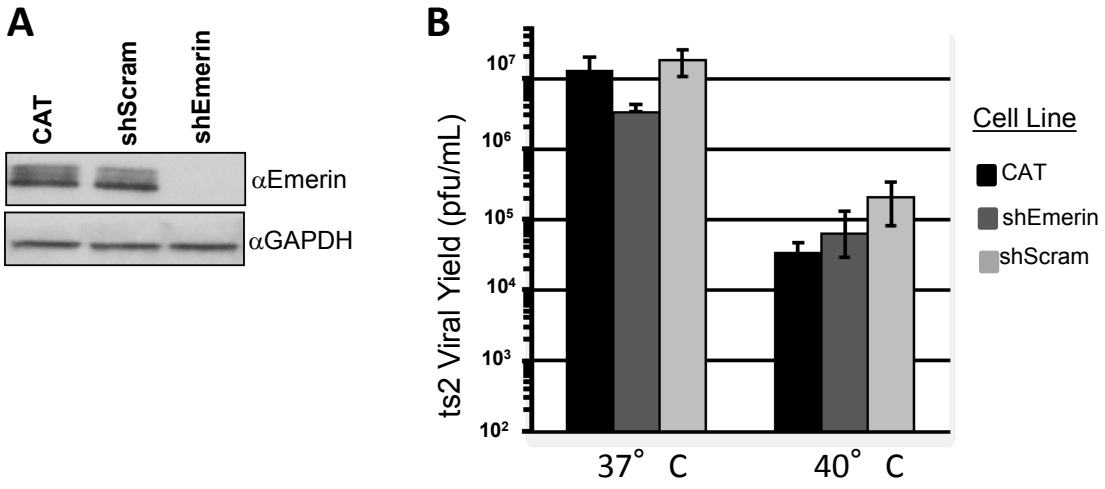

Figure 4.3. Emerin no effect on viral yield. A) Immunoblot analysis of emerin expression in CV1-CAT cells stably depleted of emerin using specific shEmerin. Lysates from equivalent numbers of cells were collected and analyzed using antibody against emerin. The total amounts of emerin in each lane were quantified using a Bio-Rad Chemidoc XRS instrument. GAPDH level was used as loading control. B) Equal numbers of cells of the indicated CV1-CAT and stably shRNA-expressing cell lines were infected with ts2 virus (MOI = 0.01) and incubated at 37°C or 40°C. Cells were harvested at 48 hpi, and viral yield was assessed. Bars represent average yields from at least three independent experiments, titrated in independent duplicates. Error bars represent standard deviations.

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virus production. As shown in figure 4.3B, at 37ºC in CV1-shEmerin cells, there is a minor decrease in viral yield, while the CV1-shScram produce similar amount of virus as CV1-CAT cells. At 40ºC, there is no significant increase in ts2 yield between CV1-shEmerin and C1-CAT and CV1-shScram. These data indicate that while emerin is recruited to viral replication sites (Fig. 4.2), its presence is not critical for BAF's ability to inhibit ts2 life cycle. However, this does not exclude the possibility that other LEM-domain proteins may affect ts2 infection.

4.4. Lap2α does not colocalize to viral replications sites

Lap2α, a well-known LEM-domain protein interacting with BAF (55), was also tested for its potential recruitment to ts2-replication sites. CV1-CAT cells were infected with ts2 virus at 32ºC for 9 hr to allow DNA replication factories to efficiently form. At 4 hpi, BrdU was added to the infected cultures to label replicating DNA. At 9 hpi, cultures were then shifted to non-permissive temperature (40ºC) for additional 7 hr prior to fixation and downstream processing for immunofluorescence imaging of BrdU, and Lap2α localization.

Contrary to emerin (figure 4.2), Lap2α did not relocalize to ts2-replication sites rather it stayed mostly nuclear both in mock and infected cells (compare figure 4.4.C, D and G, H). These data indicate that Lap2α is not recruited to viral replication although BAF is recruited to ts2-replication sites at 40ºC (11). Further, the sequestration (virally mediated or not) of Lap2α to the nucleus, is opposite to

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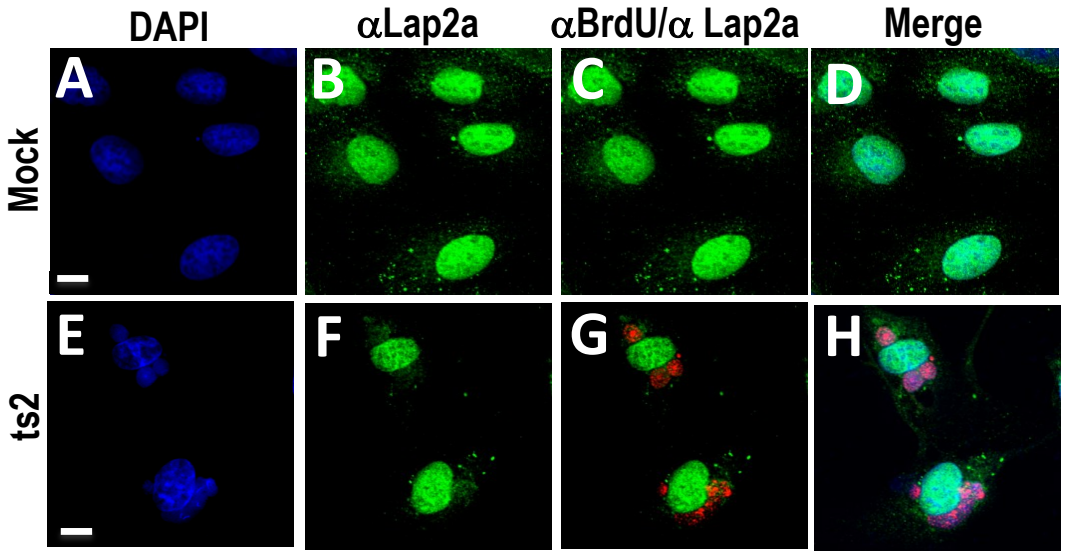

Figure 4.4. Lap2α does not colocalize to viral replications sites. CV1-CAT cells were left untreated (A to D) or infected with ts2 virus at 32°C (E to H). At 4 hpi, cells were treated with 25 μg/ml BrdU to label replicating DNA, and at 9 hpi, cells were shifted to 40°C. Cells were fixed 7 hr later (16 hpi), and permeabilized by using 0.1% saponin–PBS. Cells were processed for immunofluorescence imaging using anti-Lap2α and anti-BrdU primary antibodies, Alexa Fluor secondary antibody, and DAPI. The representative independent and overlaid images shown were taken by using a confocal microscope at a 60X magnification. Scale bars, 10 μm.

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its ability to relocalize to a transfected dsDNA, and this may suggest a potential avoidance or manipulation of Lap2α by VACV.

4.5. The presence of active B1 inhibits the formation of BAF-DNA complexes

The relocalization of BAF to viral replication sites during ts2 infection is related to the absence of active B1, while BAF readily relocalizes to a transfected dsDNA to form puncta visible by immunofluorescence. It is known that during the cell cycle, BAF is motile and may be phosphorylated by the cellular VRKs (26). However, it is not clear during infection, whether B1 inactivates BAF prior to its localization to viral factories, or B1 inactivates BAF even when BAF-DNA complexes were formed.

To determine whether the inhibitory activity of B1 against BAF requires the presence of BAF at viral replication sites, the formation of BAF-DNA complexes was assessed during viral infection. To address this question, 293 cells stably expressing GFP-BAF were transfected with 1 µg DNA (purified from E. coli) for 12 hr to allow the formation of BAF-DNA complexes, then cells were infected with WT or ts2 at MOI of 5 in the presence and absence of 50 µM AraC to block viral DNA replication, thus the formation of viral replication sites. Ara-C (1-β-D-arabinofuranosylcytosine, cytosine arabinoside, cytarabine) is a structural analogue of deoxycytidine used for the treatment of cancers. AraC differes from deoxycytidine by the presence of a hydroxyl group in the β-configuration at the 2'-position of the sugar moiety. The incorporation of AraC into replicating DNA causes chain termination and a block in DNA replication. Since during a viral

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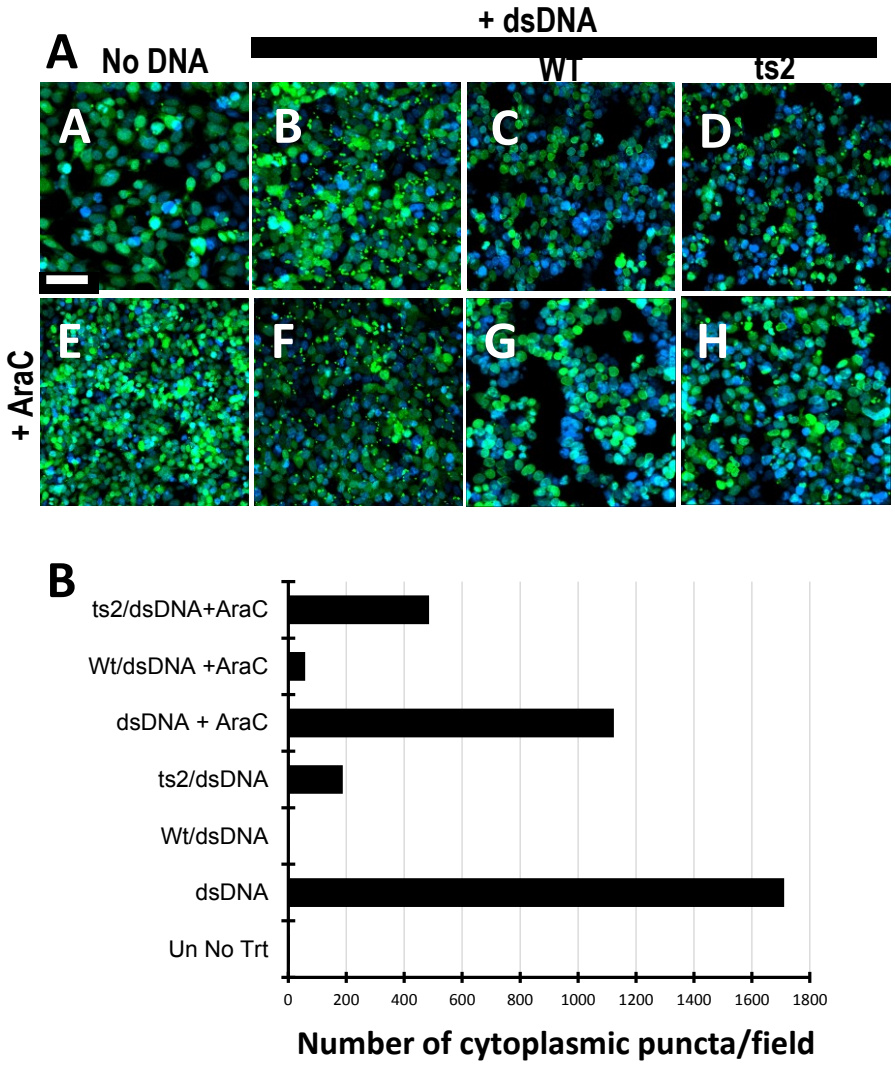

Figure 4.5. The presence of active B1 interferes with the formation of BAF-DNA complexes. A) 293 cells stably expressing GFP-BAF were untreated or transfected with 1 µg K12 DNA (purified from E. coli) for 12 hr to allow the formation of BAF-DNA complexes, then cells were infected with WT or ts2 at MOI of 5 in the presence (E to H) and absence (A to D) of 50 µM AraC to block viral DNA replication. Cells were incubated for additional 6 hr at 37ºC, then cells were fixed and processed for immunofluorescence imaging after DAPI staining. The representative images shown were taken by using a confocal microscope at a 20X magnification. Scale bars, 10 µm. **B)** Quantitation of cytoplasmic puncta/field using the same cells was done using ImageJ.

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infection, there is an exponential viral DNA replication compared to cellular DNA, AraC is incorporated more into viral DNA resulting in a block in DNA replication. In addition, AraC is only used for less than 24hr on the infected cells. Cells were incubated for 6 hr at 37ºC, then processed for immunofluorescence imaging after DAPI staining. In the absence as well as in the presence of AraC, transfection of dsDNA triggered the formation of BAF-DNA complexes in the cytoplasm (compare figure 4.5.1A and B & E and F). However, in cells pre-transfecfected with dsDNA, WT infection likely disrupted BAF-DNA complexes regardless of thethe presence and absence of AraC (compare figure 4.5.1B and C, and F and G). This is an indication that the active B1 likely dismantled these complexes and inactivates BAF regardless of its functional involvement. Strikingly, in ts2-infected cells previously transfected with dsDNA, BAF-DNA complexes were still detectable with anti-Flag antibodies, but less numerous than in transfected only cells regardless of AraC treatment (compare figure 4.5.1B and D & F and H). This indicates that the defective B1 expressed during ts2 infection holds some residual activity to inactivate BAF. The figure 4.5.2, showed the count of BAF-DNA complexes during transfection alone and transfection/infection. Together, these data showed that regardless of the location of BAF, and its established functional interaction with DNA, the viral B1 kinase is able to inactivate BAF.

4.6. BAF-DNA complexes form independently of viral replication sites

BAF readily localizes to cytoplasmic DNA during transfection, but also to ts2-replication sites. While data in figure 4.5 (D and H) showed that BAF-DNA complexes formed prior to ts2 infection are still maintained, it is not known

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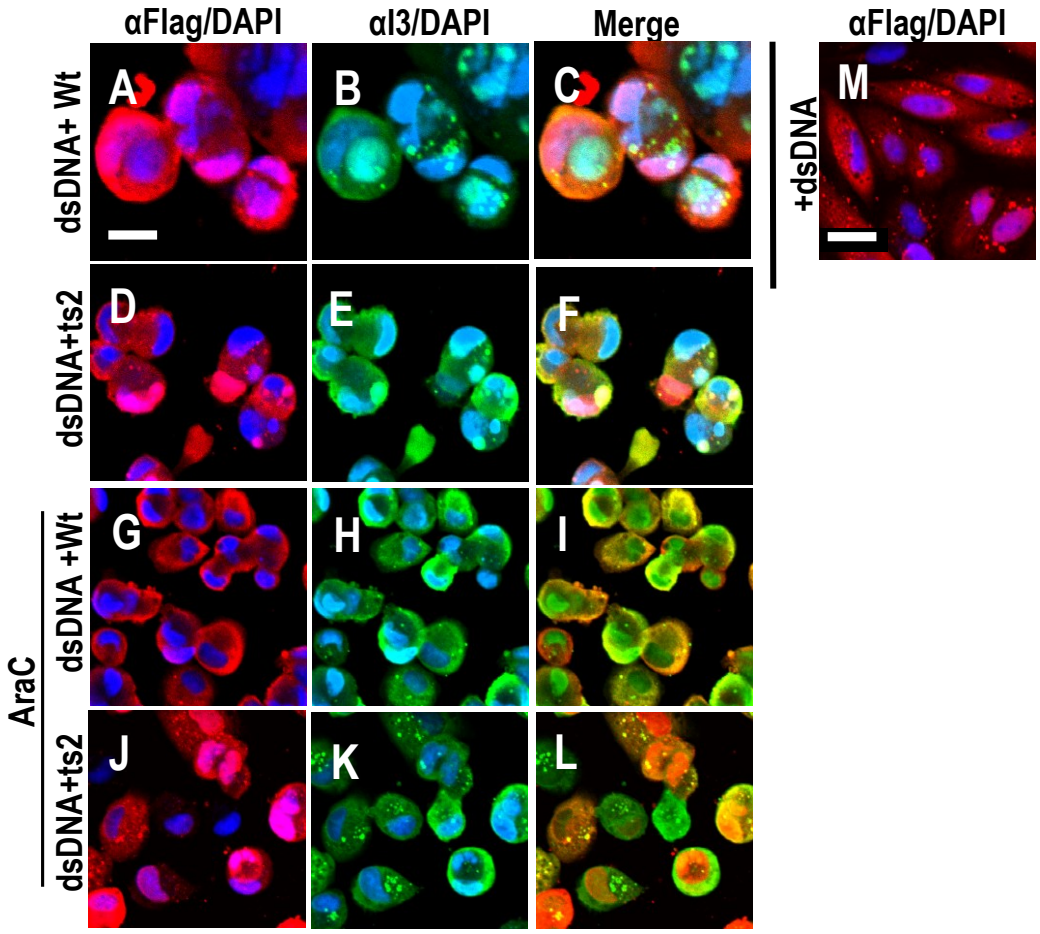

Figure 4.6. BAF-DNA complexes form independently of viral replication sites. CV1-3xFlag-BAF cells were transfected 1 µg K12 DNA for 12 hr to allow the formation of BAF-DNA complexes, then cells were infected with WT (A to C and G to I) or ts2 (D to F and J to L) at MOI of 5 in the presence and absence of 50 µM AraC to block viral DNA replication. The panel M is shown as a control. Cells were incubated for 6 hr at 37ºC, then cells were fixed and processed for immunofluorescence imaging using an anti-Flag M2 and anti-I3 primary antibodies, Alexa Fluor secondary antibody, and DAPI. The representative independent and overlaid images shown were taken by using a confocal microscope at a 60X magnification. Scale bars, 10 µm.

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whether these BAF-DNA complexes colocalize with ts2 replication sites or not. The quantification in figure 4.5B showed a 4- to 8-fold decrease in cytoplasmic puncta after ts2-infection respectively in the absence or presence of AraC, it is not known whether the transfected DNA relocalize to viral DNA replication sites. To assess whether the transfected DNA is present at ts2 DNA replication sites, CV1-3xFlag-BAF cells were transfected with 1 µg DNA for 12 hr to allow the formation of BAF-DNA complexes, then cells were infected with WT or ts2 at MOI of 5 in the presence or absence of 50 µM AraC to block viral DNA replication and the formation of viral replication sites. Cells were incubated for 6 hr at 37ºC, then fixed and processed for immunofluorescence imaging using an anti-Flag M2, and anti-I3 (I3 is an early viral ssDNA-binding protein) antibodies. In WT-infected cells regardless of AraC treatment (figure 4.6 A and G), BAF had a diffuse distribution, consistent with previously published report (11), while cytoplasmic puncta were noticeable in ts2-infected cells (figure 4.6 D and J). These puncta are evocative of BAF-DNA complexes (compare figure 4.6D & J to M). Using I3 antibody, replications sites are present in both WT and ts2-infections in the absence of AraC (figure 4.6B and E), but these replication sites are much bigger than those detected in the presence of AraC (compare figure 4.6B and H; and E and K). It is not known whether the viral I3 bind to the transfected dsDNA.

In ts2-infected cells in the presence of AraC, more cytoplasmic puncta were detected using anti-Flag or anti-I3 antibodies than in WT-infected cells (compare figure 4.6G against J (anti-Flag); and H against K (anti-I3)). While there is no evidence to suggest that I3 binds to the transfected dsDNA, both BAF and

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I3 mostly localize to the same cytoplasmic puncta regardless of AraC treatment (compare merged figures 4.6C,F, I and L). However, in the presence of AraC, in ts2-infected cells, few cytoplasmic puncta detected by I3 antibody do not colocalize with BAF (figure 4.6L). These data primarily indicate that BAF-DNA complexes formed independently of viral replication sites. Further studies and better antibodies are needed to resolve and understand whether BAF-transfected DNA complexes are associated with viral replication sites.

4.7. Ku86 does not co-localize to viral replications sites

Another protein identified by mass spectrometry to relocalize to BAF-DNA complexes, Ku86 was tested for its ability to relocalize to viral replications. CV1-CAT cells were infected with WT or ts2 virus at 32ºC for 9 hr to allow DNA replication factories to efficiently form and cultures were then shifted to 40ºC for 7 additional hr prior to fixation and processing for immunofluorescence imaging using an anti-Ku86. Ku86 is mostly nuclear in mock control cells (figure 4.7A to C), and stayed nuclear both during WT and ts2 infections (figure 4.7D to I). Because Ku86 relocalizes to transfected dsDNA in a BAF-independent manner (see figure 3.5H), these data indicate that Ku86 may not be responding to the presence of viral DNA in the cytoplasmic as it does to transfected dsDNA, or it may be avoided or inhibited by the virus. The absence of Ku86 at the viral replication sites is consistent with recent report that VACV C16 protein binds Ku heterodimer, and blocks the DNA-sensing activity of DNA-PK, a heterodimeric complex containing Ku70/80 heterodimer (227). The DNA-PK is a DNA-sensing factor that activate IRF3-dependent innate immunity (228). It is likely that by

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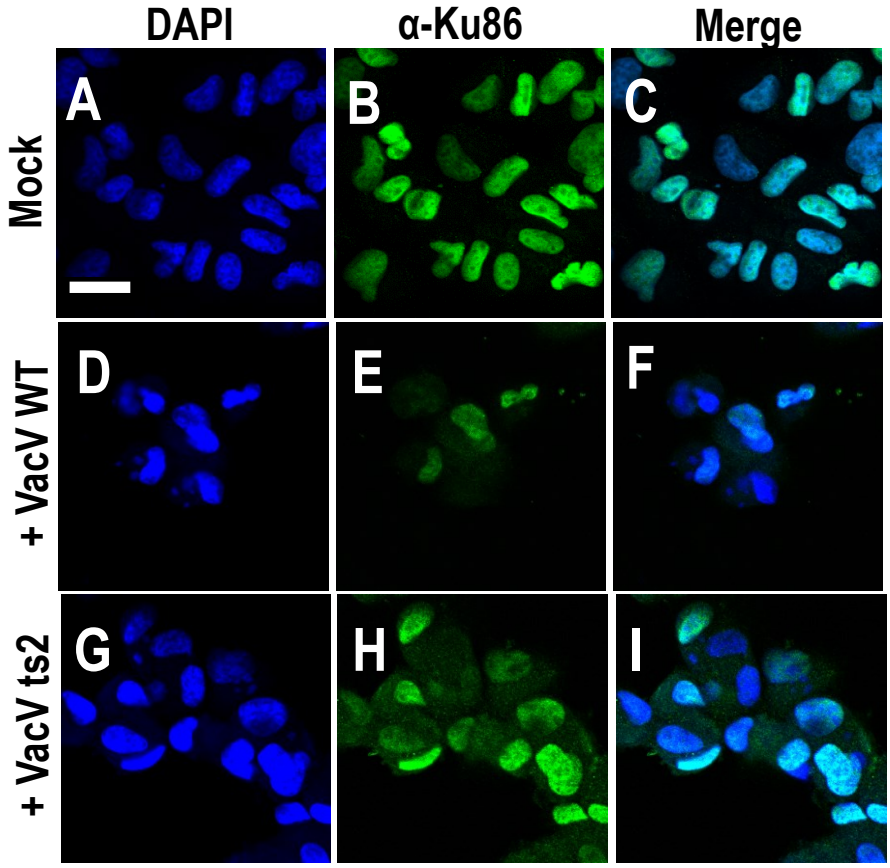

Figure 4.7. Ku86 does not co-localize to viral replications sites. CV1-CAT cells were left untreated (A to C) or infected with wild-type (D to F) or ts2 (G to I) virus at 32°C. At 9 hpi, cells shifted to 40°C. Cells were fixed 7 hr later (16 hpi), and permeabilized by using 0.1% saponin–PBS. Cells were processed for immunofluorescence imaging using anti-Ku86 primary antibody, Alexa Fluor secondary antibody, and DAPI. The representative independent and overlaid images shown were taken by using a confocal microscope at a 60X magnification. Scale bars, 10 μm.

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binding to Ku heterodimer, C16 affects the functional integrity of DNA-PK complex.

4.8. RPA32 maintains a nuclear presence during vaccinia virus infection

The RPA32 protein was also tested for its localization during viral replications, as it was identified by mass spectrometry to be part of BAF-DNA complexes. CV1-CAT cells were infected with ts2 virus at 32ºC for 9 hr to allow DNA replication factories to efficiently form. Cultures were then shifted to non-permissive temperature (40ºC) for additional 7 hr prior to fixation and downstream processing for immunofluorescence imaging of I3 and RPA32 localization. As shown in figure 4.8, in mock cells, RPA32 is mostly nuclear (see figure 4.8A and C) and stayed nuclear during WT and ts2 infections (see figure 4.8). These data indicate that RPA32 maintained a nuclear presence during viral infection, although it relocalizes to cytoplasmic dsDNA. This may suggest either RPA32 is involved in cellular responses to cytoplasmic dsDNA through a mechanism specific to transfected naked dsDNA or RPA32 is virally manipulated to stay nuclear. As in the case of Ku86, it is likely that VACV may target it to inhibit a potential antiviral activity associated with RPA.

4.9. Discussion

The barrier-to-autointegration factor (BAF) was first characterized as a host component of the retroviral PIC (6), but its role is controversial (69, 229). In contrast, BAF is a potent host antiviral factor during VACV infection (11). Specifically, during infection with vaccinia virus, BAF is capable of localizing to

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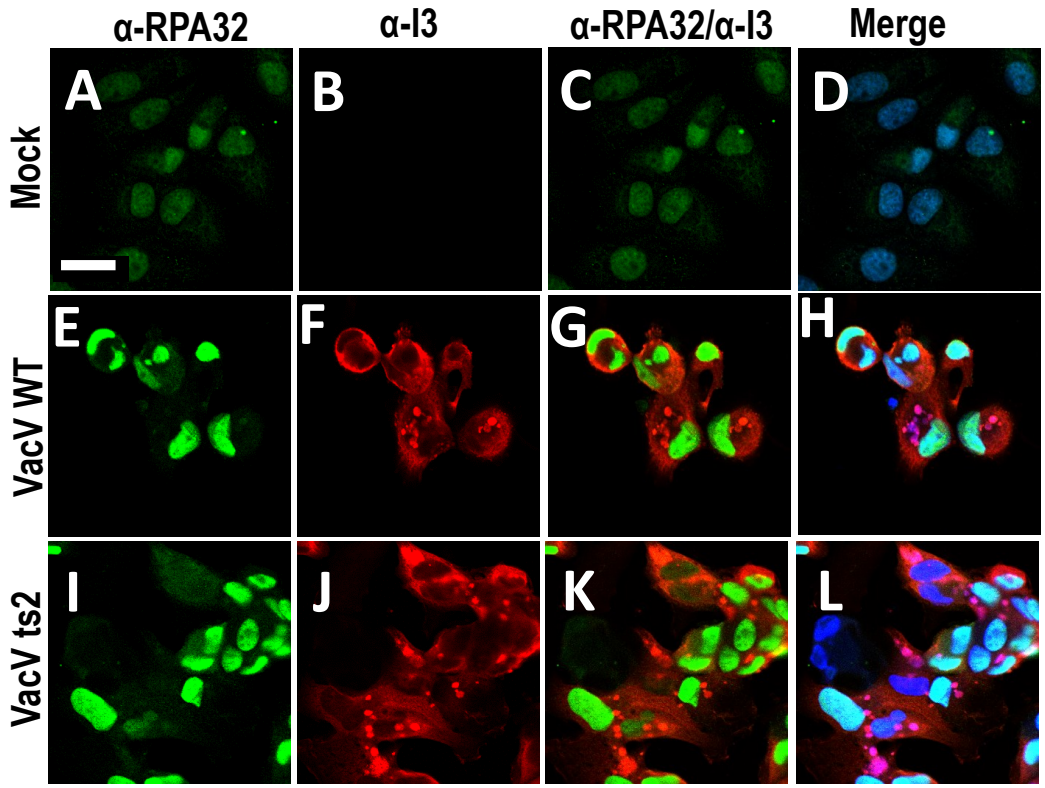

Figure 4.8. RPA32 does not co-localize to viral replications sites. CV1-CAT cells were left untreated (A to D) or infected with wild-type (E to H) or ts2 (I to L) virus at 32°C (E to H). At 9 hpi, cells shifted to 40°C. Cells were fixed 7 hr later (16 hpi), and permeabilized by using 0.1% saponin–PBS. Cells were processed for immunofluorescence imaging using anti-RPA32α and anti-I3 primary antibodies, Alexa Fluor secondary antibody, and DAPI. The representative independent and overlaid images shown were taken by using a confocal microscope at a 60X magnification. Scale bars, 10 μm.

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cytoplasmic DNA replication factories and strongly inhibiting viral genome replication (11). However, VACV expresses B1 kinase, with a high level of similarity to cellular VRKs (192, 193), and which is capable of phosphorylating the N-terminus of BAF and inactivating BAF's DNA-binding ability (12). Poxviruses encode numerous homologs of cellular proteins as part of their strategy to evade or inactivate the host immune system (156, 230-233). These studies showed that the cytoplasmic pool of BAF is or a part of cellular mechanism to block viral DNA replication. However, much remains to be determined regarding the function and regulatory mechanism associated with BAF as a host defense protein during VACV infection.

Based on our studies on the molecular determinants associated with BAF's ability to relocalize to cytoplasmic dsDNA and the recruitment of other cellular proteins (Chapter III), I wanted to understand whether these factors are also necessary for BAF's antiviral activity during ts2 infection. Viral yield and DNA replication assessed in cells expressing different level of BAF (Figure 4.1) showed that regardless of the level of BAF expression, viral yield in ts2 infection is persistently compared to that in WT-infected cells (Figure 4.1A). However, when ts2 infections were considered alone, the stable depletion of BAF consistently rescues both viral yield and DNA replication compared to the normal cellular level of BAF (Figure 4.1B and C). The stable overexpression of BAF inhibits ts2 viral events regardless of the MOIs (Figure 4.1B and C). While BAF inhibits both viral production and DNA replication, the contribution of cellular

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proteins (LEM-domain proteins, RPA32, Ku86) identified at BAF-DNA puncta is not known.

Taking into account our results in chapter 3 regarding on the relocalization of proteins to transfected dsDNA, the distribution of Lap2α, emerin, RPA32 and Ku86 were assessed during infection with ts2 virus. During a ts2 infection, emerin relocalizes and forms a ring surrounding the DNA replication sites, while emerin was not found at viral factories during a wild-type infection in this (Figure 4.2) and previous studies (112). However, the stable depletion of emerin has no effect on viral yield suggesting a minor role for its presence at these viral sites, and in assisting with BAF's antiviral activity (Figure 4.3).

While Lap2α, Ku86 and RPA32 relocalize to a transfected dsDNA in BAF-independent manner (Figure 3.4 and 3.5), these proteins were not found at the ts2-viral replication sites rather sequestered within the nucleus (Lap2α (Figure 4.4), Ku86 (Figure 4.7) and RPA32 (Figure 4.8)). These results suggest the potential existence of a viral mechanism to sequester or block these proteins from relocalizing to viral DNA replication sites. Further, the absence of these proteins at the viral replication sites is independent of B1. Indeed, a recent report showed that VACV expresses an early protein, C16 that target Ku70/80 heterodimer (227), a component of the DNA-PK, a heterodimeric complex with antiviral activity (228).

The presence of BAF at the viral replication sites is essential for its antiviral activity (11), however the molecular determinants fundamental to BAF's activity were not studied in vivo, as well as the ability of viral B1 to inactivate BAF

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already interacting with DNA or other cellular proteins. Data from our laboratory showed when cells stably overexpressing BAF-K6A and BAF-G47E are far less effective in inhibiting ts2 infection than the wild-type BAF, while the mutant BAF–K53E inhibits ts2 growth with reduced efficiency compared to wild type BAF. The level of expression of BAF-K53E mutant was only 10% of that of BAF, and BAF-K53 still retains some BAF's antiviral activity. Based on these data, BAF-K6A and BAF-G47E mutants provide an indication that DNA-binding and dimerization are essential for BAF's antiviral activity.

Because the phosphorylation of BAF by B1 inactivates BAF's DNA-binding properties, the ability of B1 to inactivate BAF already engaged in BAF-DNA interactions was investigated. Our data showed that when pre-transfected cells, which contain preformed BAF-DNA complexes, were infected with WT there were less BAF-DNA complexes. Conversely, when these cells were infected with ts2 virus, there were still a substantial number of cytoplasmic puncta (Figures 4.5 and 4.6). While more sensitive methods are needed to elucidate the ability of B1 to inactivate BAF alreading interacting with DNA, these data suggest that B1 inactivates BAF regardless of its interaction with DNA. These results showed the activity of BAF during vaccinia virus infection is both regulated by its intrinsic molecular features as well as its phosphorylation by B1.

In summary, this study reveals BAF as a potent antiviral factor that blocks viral production and DNA replication in the absence of B1. The anti-VACV activity of BAF requires DNA-binding and dimerization, while interaction with emerin has a minor effect. Although preliminary, this study provides evidence of B1

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inactivates BAF regardless of its interaction's status. The absence of Lap2α and RPA32 is likely regulates through a viral mechansims as the one reported in the case of Ku86. Further studies may help understand the implications of BAF and B1 on other viral processes.

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CHAPTER V

BAF IS A TRANSCRIPTIONAL REGULATOR OF VACCINIA VIRUS INTERMEDIATE GENE TRANSCRIPTION

Part of the work described in this chapter was published in Virology, 2013

Nouhou Ibrahim, April Wicklund, Augusta Jamin, and Matthew S. Wiebe. Barrier-to-Autointegration Factor (BAF) Inhibits Vaccinia Virus Intermediate Transcription in the Absence of the Viral B1 Kinase. Virology 444:363-373

Analysis of the temperature sensitive mutant virus, ts2, which contains a point mutation within the B1 ORF provided the first evidence of the essential role played by B1 during the vaccinia virus DNA replication. Subsequent studies showed that B1 is essential for viral DNA replication as well as for optimal intermediate transcription. While it is known that B1 targets BAF to permit genome replication, its role in intermediate gene expression is not well understood. Considering BAF's ability to relocate to ts2 DNA replication sites and cross-bridge dsDNA, it is likely that BAF affects viral transcription. In an attempt to identify the mechanism of action of B1 during intermediate gene expression, the importance of the B1-BAF signaling axis during poxviral intermediate gene expression was explored.

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5.1 Luciferase assay is the most sensitive method to measure intermediate gene expression during vaccinia virus

To study vaccinia virus intermediate gene expression, Kovacs et al. used a combination of a plasmid transfection followed by an infection with vaccinia virus in the presence of AraC, an inhibitor of viral DNA replication (105). In this study, fluorescence activated cell sorting (FACS) analysis was employed to sort cells expressing mCherry protein regulated by the well-characterized VACV intermediate promoter, pG8R. Prior to FACS analysis, mouse fibroblast L929 cells were transfected for 7 hr at 37ºC with 1 µg of pInt-mCherry (a plasmid expressing mCherry under G8R promoter); then cells were infected with WT or ts2 virus at an MOI=3 incubated at 37ºC overnight. During the duration of both WT and ts2 infections, AraC was added at a final concentration of 50 µM so as to examine viral intermediate gene expression independent of DNA replication. This approach is based on previous studies that showed that the expression of vaccinia genes from a plasmid introduced into infected cells does not require viral DNA replication (130, 131). Cells were collected, processed for FACS analysis, and sorted for 10,000 events based on SSC (side scatter) by FSC (forward scatter) using FlowJo 7.6.1 Software. As shown in Figure 5.1A, the fluorescence intensity of mCherry in ts2-infected was about 60% less than that in WT-infected cells.

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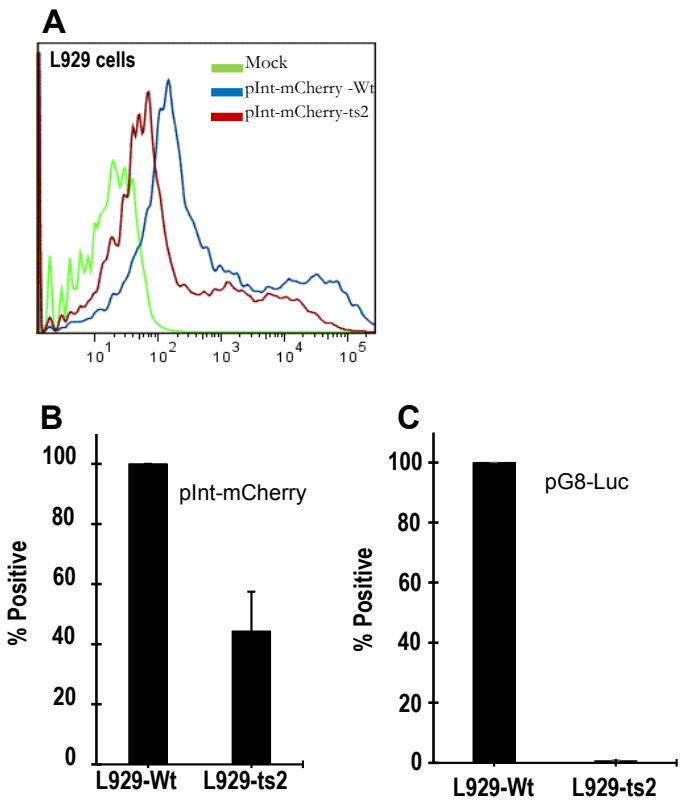

Figure 5.1. Luciferase assay is the most sensitive method to measure intermediate gene expression during vaccinia virus. Mouse fibroblasts, L929 cells were transfected with 1 µg of (A) pInt-mCherry or (C) 10ng pG8-Luc for 7 hr at 37ºC. Cells were then infected with WT or ts2 (MOI=3) in the presence of 50 µM AraC at 37ºC overnight. **A)** Histogram plot representation of mCherry fluorescence intensity vs. cell count positive cells sorted for 10,000 events. Cells were washed with 1xPBS, collected and fixed in 4% paraformaldehyde; cells were sorted based on the expression of mCherry using the SSC by FSC using FlowJo 7.6.1 software (Tree Star Inc). **B)** Graph presentation of the fluorescence of mCherry. **C)** Cells were lysed in 300 µl of 1X Reporter Lysis buffer, and the activity of reporter gene was measured using a Berthold multiwell Luminometer. Error bars rerepresent the standard deviation calculated from triplicate experiments.

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However, during the optimization process, the difference in mCherry expressing cells between WT and ts2-infected cells was not consistent between replicates. The variation and irreproducibility of FACS's data, and the large amount of plasmid to transfect prompted us to use luciferase assay.

For the luciferase assay, a plasmid expressing firefly luciferase under G8R promoter or pG8-Luciferase (pG8R-Luc) (a kind gift from Dr. Moss, NIH) was tested in L929 cells during infection. L929 were transfected with 10ng pG8R-Luc for 7 hr at 37ºC; then cells were infected with WT or ts2 virus at an MOI=3 and incubated at 37ºC overnight in the presence of 50 μM AraC. Cells were washed with 1xPBS, and processed for measurement of luciferase activity. As shown in figure 5.1C, the expression from pG8-Luc in ts2-infected cells is about 1% of that in WT-infected cells. The difference in intermediate gene expression between WT and ts2 is consistent between replicates and is reproducible. The advantage of the luciferase assay is its sensitivity. Throughout this study, pG8R-Luc plasmid was used to measure the activity of pG8R promoter by quantifying luciferase activity during vaccinia virus infection in the presence of AraC.

5.2. Viral intermediate gene expression requires B1 independently of its role in DNA replication

Temperature sensitive vaccinia viruses with lesions in the B1 kinase (ts2 and ts25) display a primary block at the stage of DNA replication at non-permissive temperature (175, 181, 184), because defective B1 is unable to inactivate BAF via phosphorylation (11, 12, 234). Prior to the discovery of the

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B1-BAF signaling axis, Kovacs et al demonstrated that B1 is also required for at least one post replicative stage in the viral life cycle (105).

In light of the recent discovery of the BAF-B1 axis, the goal of this study was to further examine the role of B1 during vaccinia intermediate gene expression in relationship to BAF. As shown in figure 5.1C, expression from pG8-Luc in ts2-infected cells is about 1% of that in WT-infected cells, and this is line with what was previously reported (105). Thus the presence of a functional B1 kinase is necessary for the viral intermediate gene expression.

5.3. The viral B1 is a general regulator of viral intermediate gene expression

The observation that B1 is required for intermediate gene expression mediated by the G8R promoter prompted me to examine whether B1 regulates other viral intermediate promoters in general. To this end, a plasmid expressing firefly luciferase under two well-characterization poxvirus intermediate promoters, A2L and I1L, and a generated consensus intermediate promoter or Consensus were constructed. These plasmids were then used to determine whether the viral B1 affects their activation during transfection/infection in the presence of AraC. L929 cells were transfected with these plasmids following the transfection/infection +AraC treatment previously described. As shown in Figure 5.3, the activation of each of these promoters was significantly decreased in the ts2-infected L929 cells compared to the WT-infected cells.

However, the pG8R-Luc construct exhibited the greatest fold difference between WT and ts2 infections compared to other constructs, therefore it was employed.

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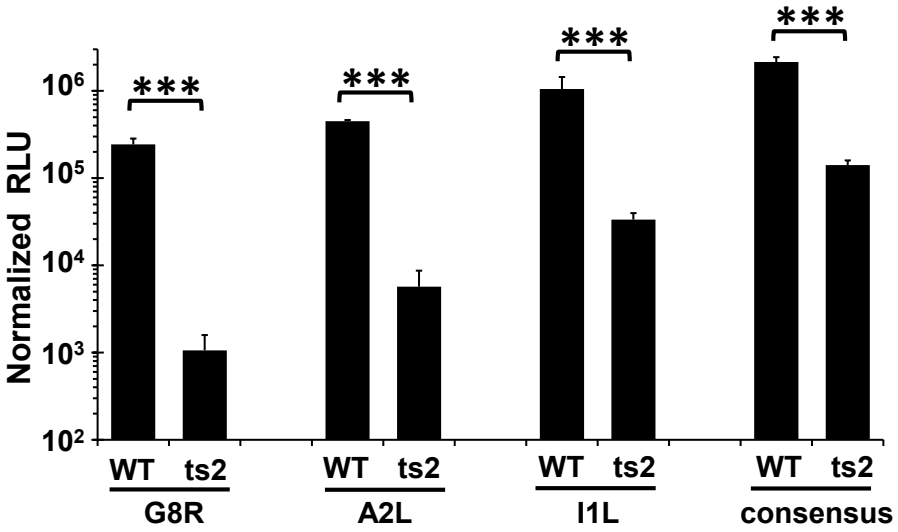

Figure 5.3. The viral B1 is a general regulator of viral intermediate gene expression. L929 cells were transfected with 10 ng of pG8-, A2L-, I1L, and Consensus-Luc for 7 hr at 37ºC. Cells were then infected with WT or ts2 (MOI=3) in the presence of 50 µM AraC at 37ºC overnight. Cells were lysed in 300 µl of 1X Reporter Lysis buffer, and the activity of reporter gene was measured using a Berthold multiwell Luminometer. RLU shown was normalized to the total protein measured by BCA assay. Data were obtained from triplicate experiments, and the error bars represent standard deviation. (***indicates a p-value <0.05).

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for the remainder of this study. Based on these results, B1 is likely a general regulator of intermediate gene expression.

5.4. The role of B1 in viral intermediate gene expression is cell-dependent

To assess whether the role of B1 during intermediate gene expression is not cell-type specific, CV1 and BSC40 (both fibroblast from green monkey kidney), U2OS (human osteosarcoma cells) were transfected with 10ng of pG8R-Luc for 7hr at 37ºC; then infected with WT or ts2 viruses at an MOI of 3 and incubated at 37ºC overnight in the presence of AraC. As shown in Figure 5.4A, the magnitude of fold reduction in ts2-driven reporter activation as compared to WT virus was 2 to 2.5 in CV1, BSC40 and U2OS. However, in L929 cells, the difference in the activation of pG8R promoter is around 300-fold higher in WT-infected compared to ts2-infected cells. For this reason, L929 cells were employed for the remainder of this study. Considering the variation in promoter activity in cell-type dependent, the level of expression of BAF in these cells was assessed by western blotting. As shown in figure 5.4B, there is no apparent difference in BAF expression level of BAF in these cells (Figure 5.4B). However, the possibility of cellular factors contributing to viral intermediate viral gene expression has to be considered.

5.5. B1 or VRK1 expressed from the ts2 genome rescues viral yield

Previous studies have shown that the re-expression of B1 or the cellular kinase VRK1 from the viral TK locus of the ts2 virus rescues viral DNA replication even at non-permissive temperatures (193). To assess whether the defect in

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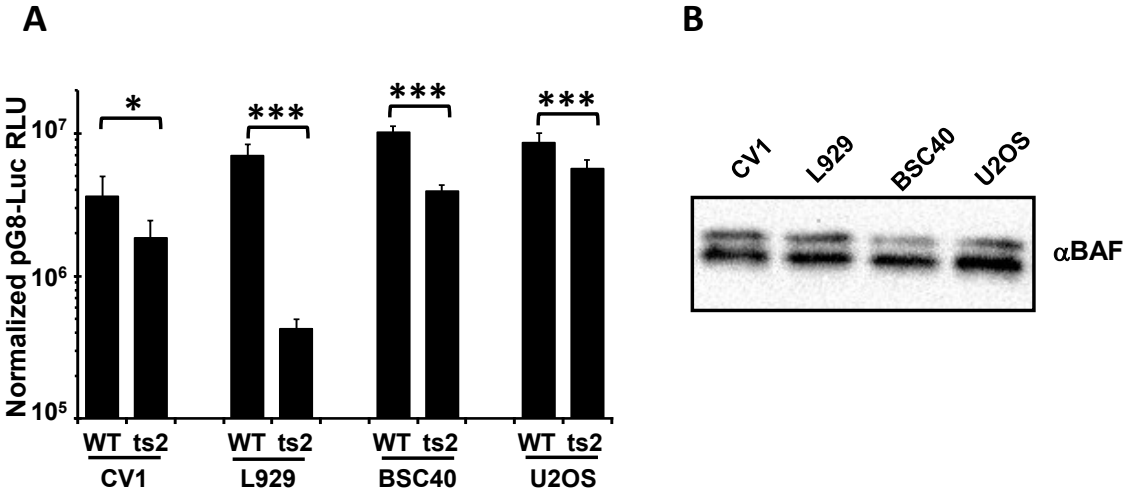

Figure 5.4. The role of B1 in viral intermediate gene expression is cell-independent. A) CV1, L929, BSC40 and U2OS cells were transfected with 10ng of pG8R-Luc for 7hr at 37ºC; then infected with WT or ts2 viruses (MOI = 3), and incubated at 37ºC overnight in the presence of 50 μM AraC. Lysates were prepared at 16hpi and assayed for luciferase activity. RLU shown is normalized to total protein measured by BCA assay. **B)** Immunoblot analysis of BAF expression in CV1, L929, BSC40 and U2OS cells. Lysates from equivalent numbers of cells were collected and analyzed using antibody against BAF. The total amounts of BAF in each lane were quantified using a Bio-Rad Chemidoc XRS instrument. GAPDH level was used as loading control. Error bars represent the standard deviation calculated from triplicate experiments. (***indicates a p-value <0.05).

intermediate gene expression of ts2 can be rescued by the reexpression of either B1 or the cellular VRK1 protein from the ts2 genome, ts2/B1 or ts2/VRK1

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respectively. As a reminder, the cellular VRK1, a member of kinases family with high homology to B1, phosphorylates BAF in similar fashion B1 (192, 193,195). Because all previous studies with ts2/B1 and ts2/VRK1 were performed on BSC40 (193), these recombinant viruses were tested in L929 cells for their potential to rescue viral yield. L929 cells were infected with WT, ts2, ts2/B1 or ts2/VRK1 at MOI of 0.1 or MOI of 5, and incubated at 37ºC for 24 hr. Both ts2/B1 and ts2/VRK1 rescued viral yield compared to ts2 at either low or high MOI (Figure 5.5), and this was similar to previous data observed in BSC40 cells (193). These data indicate the re-expression of B1 or VRK1 from the viral TK locus of the ts2 virus rescues viral yield, and the functional role of B1 in viral DNA replication is likely restored by VRK1 in L929 cells.

5.6. The expression of B1 or VRK1 enhances viral intermediate gene

The observation that the viral yield was rescued by the expression of B1 or VRK1 from the ts2 virus prompted us to test whether the restored activity of B1 can affect intermediate gene expression independently of its role in VACV DNA replication. Using the transfection/infection +AraC protocol, the activation of pG8R promoter following ts2/B1 or ts2/VRK1 infection was respectively 50-fold and 10-fold higher than that measured in ts2-infected cells (Figure 5.6). These results confirm the role of B1 kinase during viral intermediate gene expression independently of its role in DNA replication, and demonstrated that expression of B1 or VRK1 from the ts2 genome can enhance viral intermediate gene expression in L929 cells at 37ºC.

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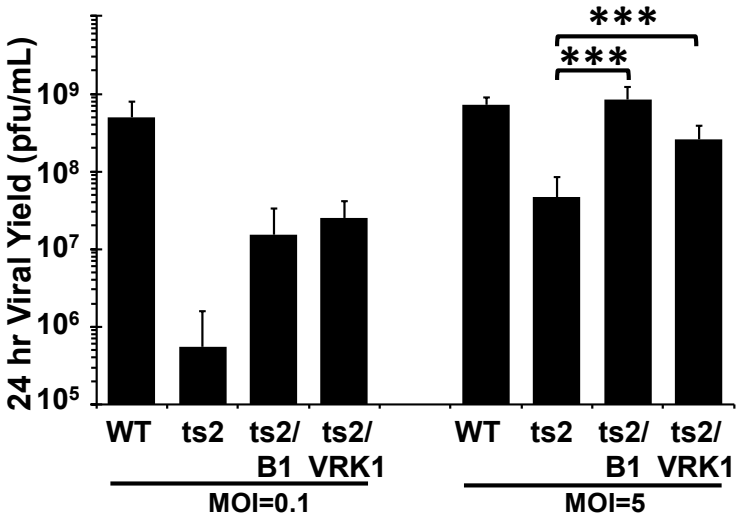

Figure 5.5. B1 or VRK1 expressed from the ts2 genome rescues viral yield. L929 cells were infected with WT, ts2, ts2/B1 or ts2/VRK1 at MOI=0.1 or MOI=5 for 24hr at 37ºC. After lysates were collected virus yield was determined by a plaque titration on monolayers of BSC40 cells at 32ºC. Data were obtained from triplicate experiments, and the error bars represent standard deviation. (*** Indicates a p-value <0.05).

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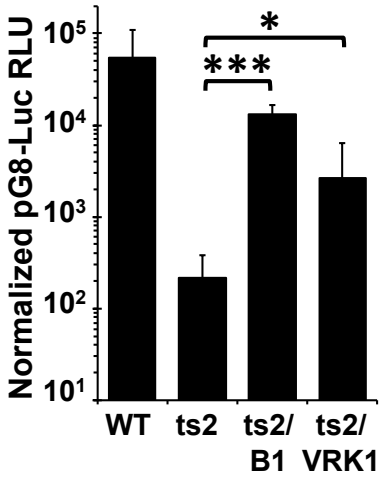

Figure 5.6. B1 or VRK1 expressed from the ts2 genome enhances viral intermediate gene. L929 cells were transfected with 10ng of pG8R-Luc for 7hr at 37ºC; then infected with WT, ts2, ts2/B1 or ts2/VRK1 viruses (MOI = 3), and incubated at 37ºC overnight in the presence of 50 μM AraC. Lysates were prepared at 16h after infection and assayed for luciferase activity. RLU shown is normalized to total protein measured by BCA assay. Error bars represent the standard deviation calculated from triplicate experiments. (***indicates a p-value <0.05).

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5.7. B1 is unique in regulating viral intermediate gene expression

The observation that the B1 kinase is required not only for DNA replication, but intermediate gene expression as well, prompted us to determine whether other vaccinia virus proteins involved with DNA replication performed at both these stages of the viral life cycle. Two ts mutants, ts42 and ts24 were tested. The ts42 virus carries a mutation within E9, the viral catalytic core of the trimeric DNA polymerase complex (117, 176, 180, 235, 236) and ts24 carries a mutation in D5, a DNA-independent nuclease triphosphatase (121, 174, 237).

These ts viruses were tested for viral DNA replication in L929 at 37ºC, a non-permissive temperature. L929 cells were infected (MOI=3) with WT, ts2, ts42, or ts24 and lysates were collected at 0.5 and 24hpi for DNA quantification using quantitative PCR. As shown in Figure 5.7A, viral DNA increased more than 150-fold in 24 hr in WT-infected cells, but in ts2-infected cells, there was only a modest ~3-fold increase compared to the input and no increase in viral DNA was detected in both ts42 and ts24 infections. These data confirm the block in DNA replication during infection with ts42 and ts24 in L929 cells at 37ºC.

Because of previous reports showing BAF inhibits viral DNA replication in the absence of active B1 (11), both the role of the BAF-B1 axis was tested during viral intermediate gene expression. To assess whether B1 is unique in regulating both viral DNA replication and intermediate gene expression, the expression of BAF was stably depleted in L929 cells by transducing them with replication-incompetent lentiviral vectors expressing either a BAF-specific (shBAF) or control

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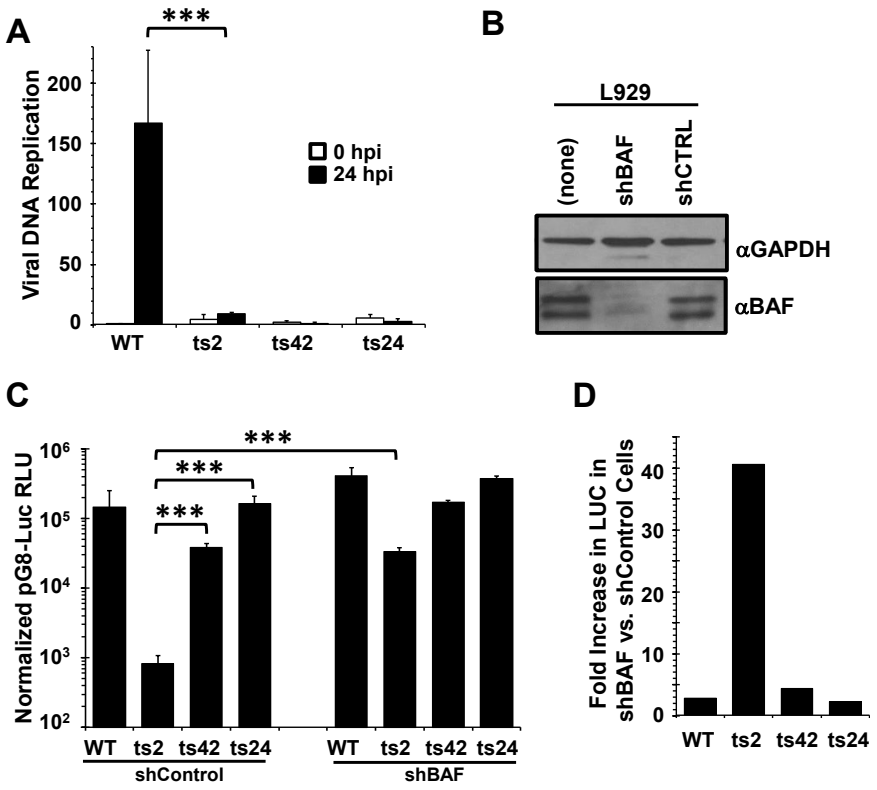

Figure 5.7. B1 is unique in regulating viral intermediate gene expression. **A).** DNA replication assay. L929 cells were infected with WT, ts2, ts42 or ts24 at MOI=3 for 30 min or 24hr at 37ºC. DNA was isolated and quantified by qPCR. Data is shown as a fold difference compared to the WT sample at 0.5hpi. Data were obtained from triplicate experiments, and the error bars present standard deviation. **B**). Immunoblot analysis of BAF expression in L929 cells stably depleted of BAF or Control using specific shRNA. Lysates from equivalent numbers of cells were collected and analyzed using antibody against BAF. The total amounts of BAF in each lane were quantified using a Bio-Rad Chemidoc XRS instrument. GAPDH level was used as loading control. **C)** L929 cells stably

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expressing shBAF or shControl were transfected with pG8-Luciferase for 7hr, then infected at 37ºC with WT, ts2, ts42 or ts24 at MOI=3 in the presence of 50 μM AraC. Lysates were prepared at 16hr after infection and assayed for luciferase activity. RLU shown is normalized to total protein measured by BCA assay. Data were obtained from three independent experiments performed in triplicate wells. Data from a representative experiment is shown. Error bars represent standard deviation. **D)** Data from the luciferase expression shown in (B) was replotted as a fold difference between the shControl and shBAF cell lines for each virus. (*** indicates a p-value <0.05).

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(CTRL). Immunoblot analysis of lysates from these cells revealed that expression of BAF in L929-shBAF cells is decreased to 15-20% compared to that from the non-transduced L929 cells, while no impact on BAF expression was detectable in the shCTRL cells (Fig. 5.7B).

To investigate whether the role of B1 in intermediate gene expression is independent of its role in DNA replication, L929-shControl cells were transfected with pG8-Luc, then infected with WT, ts2, ts42 and ts24 viruses at MOI=3 in the presence of AraC. 16-20 hpi, cells were harvested and luciferase activity quantified. As shown in Fig. 5.7C left, the pG8-Luc reporter activity in ts24-infected cells was identical to WT-infected cells while in ts42-infected cells, it is slightly 2.5-fold lower compared to WT-infected cells. In clear contrast, the pG8-Luc activity in ts2-infected was more than two orders of magnitude lower compared to other viruses. These data indicates that E9 and D5 do not affect intermediate gene expression, and that the dual role of B1 at this stage is not common among viral DNA replication proteins.

5.8. BAF affects viral intermediate gene expression in a B1 dependent manner

Based on the previous studies establishing the role of the axis B1-BAF in viral DNA replication, the main hypothesis is that without an active B1, BAF is not only able to impede DNA replication, but viral transcription as well. The same transfection/infection protocol was performed in L929-shBAF cells where the expression of BAF is stably depleted. As shown in Fig. 5.7C right, the expression

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of reporter gene expression in these cells was enhanced to at least some degree regardless of the virus used. Luciferase activity in WT, ts42, and ts24 infected L929-shBAF cells was 2-3 folds greater than that observed for each of those viruses in L929-shControl cells (Fig. 5.7C left). However, luciferase activity in ts2 infected L929-shBAF cells was close to 40-fold greater than that in L929-shControl cells (Fig. 5.7D). This specific rescue of ts2-mediated intermediate gene expression in BAF depleted cells suggests that BAF affects viral intermediate gene expression, and this is B1-dependent as during viral DNA replication.

5.9. Depletion of B1 impedes transcription in a BAF-dependent manner

To further characterize the role of B1 during intermediate gene regulation, siRNA specific to B1 was used to deplete B1 during a WT infection, and to assess whether siRNA depletion of B1 would yield results similar to those obtained in ts2-infected L929-shControl and –shBAF cells. This approach was adopted to exclude any potential contribution of other viral factors to B1 activity in intermediate gene expression. To establish a method for depletion B1 mRNA, L929 cells were transfected with 100nM of B1-specific or control siRNA for 24 hr, followed by an infection with WT virus at MOI=3 at 37ºC for 4 hr. Two B1-specific siRNA were tested, B1-1 and B1-2. Cell lysates were collected and RNA extracted for subsequent qPCR using primers specific to the B1 ORF. As shown in Fig. 5.9A, B1-specific transcripts were substantially reduced in siB1 pretreated cells compared non-treated cells, and the decrease in B1-specific transcripts was

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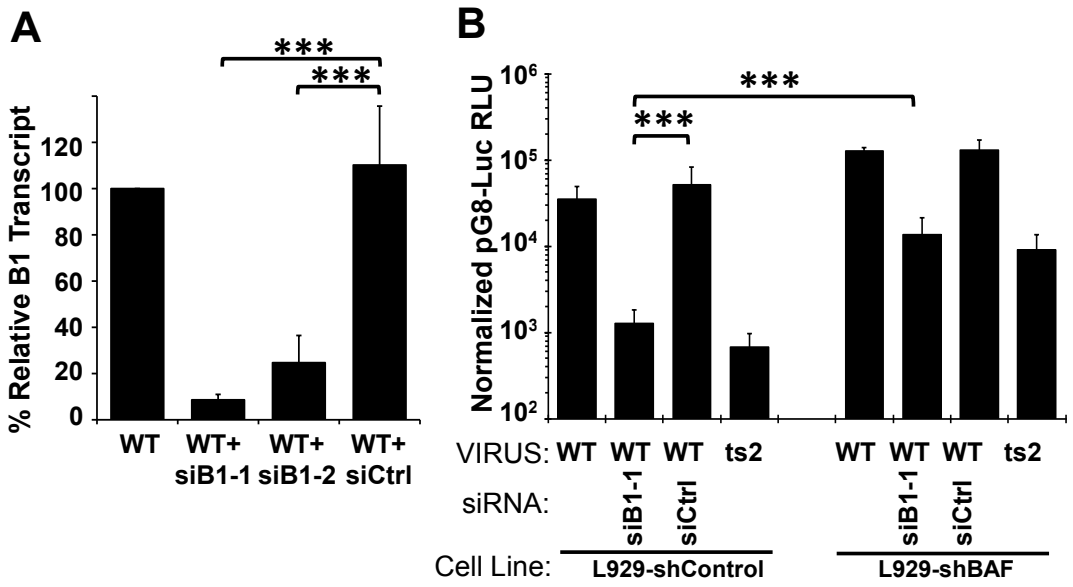

Figure 5.9. Depletion of B1 impedes transcription in a BAF-dependent manner. A) Transient depletion of B1. L929 cells were transfected with 100 nM of siRNA siControl, siB1R-1, or siB1R-2 at 37ºC. At 24 hptransfection, cells were infected with WT virus at a MOI of 3 and total RNA harvested at 4hpi. Following reverse transcription, cDNA was quantified by qPCR. **B)** L929 stably expressing shBAF or shControl were transfected with 100nM siRNA specific to B1 kinase (siB1-1) or siControl for 12hr, and then transfected with pG8-Luciferase for 7hr, then infected at 37ºC with WT virus at MOI=3 in the presence of 50 uM AraC. An infection of L929 with ts2 at MOI=3 in the presence of AraC at 37ºC was also performed for comparison purposes. Lysates were prepared at 12hr after infection and assayed for luciferase activity, and RLU normalized to protein level. Data were obtained from three independent experiments performed in triplicate wells. Data from a representative experiment is shown. Error bars represent standard deviation. (*** indicates a p-value <0.05).

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90% with siB1-1 and 70% with siB1-2. Due to the greater depletion by siB1-1, this siRNA was selected for use in subsequent studies.

To determine whether depletion of B1 by siB1 would diminish intermediate gene expression, L929 were transfected with siB1-1 or siControl for 12hr, then transfected with pG8-luc for 7hr before infecting with WT vaccinia in the presence of AraC. Cells transfected only with pG8-Luc and infected with ts2 + AraC were included for comparison purposes. To simultaneously investigate the involvement of BAF in these studies, both L929-shControl and L929-shBAF cells were employed. As shown in Fig. 5.9B left, the activity of the reporter gene was substantially decreased during the transient depletion of B1 in WT-infected L929-shControl than in siControl-transfected cells (not treated with B1-specific siRNA). Specifically in siB1-depleted cells, the activity of the reporter gene decreases to ~40-fold, bringing the reporter activity to a level very similar to that obtained during the ts2 infection. However, in L929-shBAF cells, the activity of the reporter gene was substantially enhanced both in siB1-treated and ts2-infected cells (Fig. 5.9B right).

The depletion of BAF was able to rescue the loss of intermediate gene expression caused by the absence of B1 due to siRNA treatment or mutation affecting the stability of B1 in ts2 virus. Together, these data indicate that the BAF-B1 axis regulates intermediate gene expression, and further supports our model that B1 is needed to repress the inhibitory activity of BAF.

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5.10. Depletion of BAF rescues viral intermediate gene expression at the transcriptional level (Performed by April Wicklund)

Kovacs et al have previously shown that B1 impacts intermediate gene expression at the level of transcription (105). To confirm and extend those results, the impact of B1 on transcript levels was investigated using the pG8R-Luc in BAF-dependent manner. L929-shControl and shBAF cells were transfected with increasing amounts of pG8-Luc plasmid (10, 100 and 500 ng), infected with WT or ts2 virus (MOI=3) and incubated at 37ºC in the presence of AraC. At 4 hpi, lysates were collected and RNA extracted, and a set of luciferase-specific primer/probe was used for RT-qPCR analysis. Based on the results of the RT-qPCR, there is less accumulation of pG8R-Luc-specific transcripts in ts2-infected L929-shControl cells compared to WT-infected cells (Fig. 5.10). This difference between ts2 and WT transcription was observed at all plasmid concentrations, but was greatest at lower plasmid amounts. As higher amount of plasmids were used, the difference in transcripts between WT and ts2 infection shrinks. However, the depletion of BAF greatly enhanced the accumulation of pG8R-Luc-specific transcripts during ts2 infection.

Most significantly, in ts2-infected L929-shBAF cells, the level of transcripts specific to pG8R-Luc was more than 10-fold higher than in ts2-infected L929-shControl cells when 10 ng DNA was used. Together, these data are consistent with our model that BAF can be a repressor of viral gene expression at the transcriptional level if B1 is not present to phosphorylate it.

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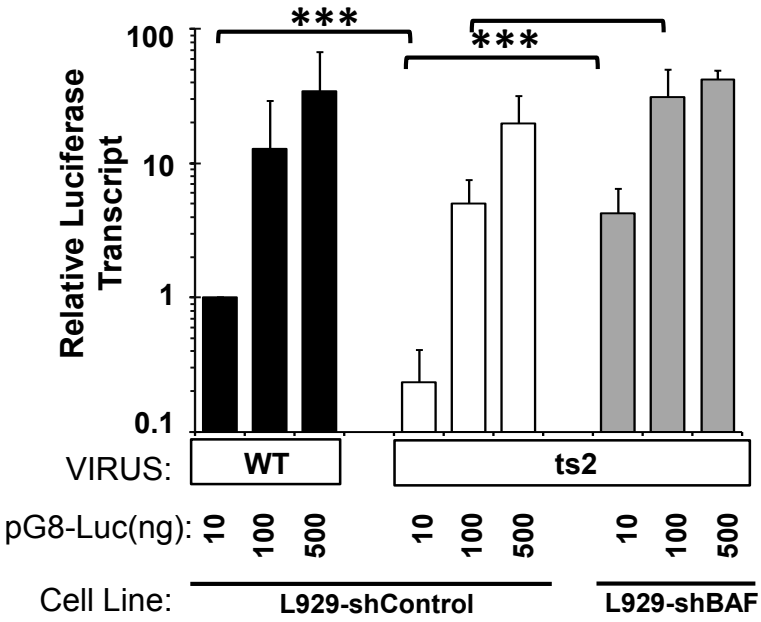

Figure 5.10. The depletion of BAF rescues viral intermediate gene expression at the transcriptional level. L929 cells stably expressing shBAF or shControl were transfected with 10, 100, and 500ng of pG8-luciferase for 7hr, then infected with WT or ts2 at MOI=3 in the presence of AraC at 37ºC. Lysates were collected for RNA extraction and luciferase transcripts quantified by RT-qPCR. (*** indicates a p-value <0.05).

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5.11. The BAF-B1 axis regulates viral gene expression in a promoter-dependent manner

Considering the role of the BAF-B1 axis in regulating viral intermediate gene expression, the potential of the BAF-B1 axis to regulate the activity of T7 promoter when expressed from the virus was investigated. To this end, T7-Luc plasmid (plasmid expressing firefly luciferase under a T7 promoter) in conjunction with vTF7.3 (a recombinant vaccinia virus expressing a wild-type B1 protein as well as the T7 polymerase) were used. The advantage of the T7-Luc/vTF7.3 system is that it provides a means to assess the impacts of the BAF-B1 axis on the activity of a reporter gene undergoing a cytoplasmic transcription, but in a manner independent of VACV RNA polymerase and transcription factors.

L929-shBAF and -shControl cells were transfected with siB1 or siControl, then transfected with T7-luciferase, and later infected with vTF7.3 virus at MOI=3 in the presence of AraC for an additional 16 hr before harvest. As shown in Figure 5.11, siB1 treatment of L929-shControl cells infected with vTF7.3 resulted in only a 2.5-fold reduction in T7-Luc reporter gene expression, contrary to what was observed for pG8R-Luc expression during WT infection (see Fig. 5.9B).

In L929-shBAF cells, the activity of the T7-Luc reporter rose modestly and was similar in all samples regardless of B1 siRNA treatment. While these data suggest that the BAF-B1 axis contributes somewhat to the level of T7-driven reporter activity observed, the magnitude of their contribution is far less than what was observed when studying the pG8-Luc reporter. Since T7 is not a VACV

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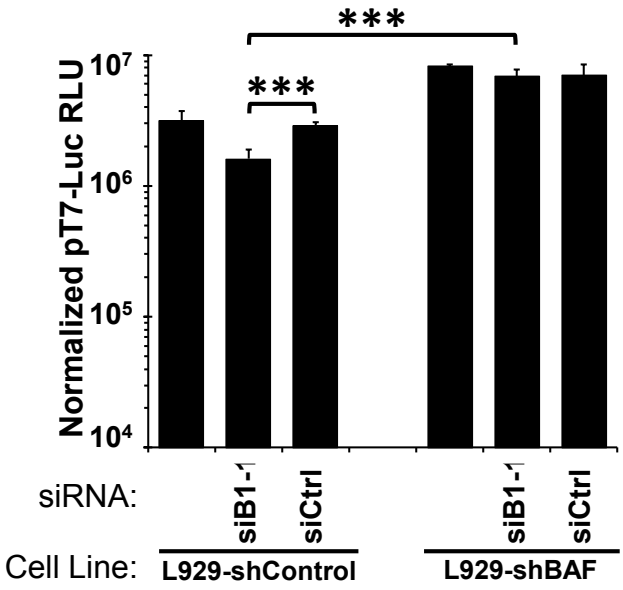

Figure 5.11. The BAF-B1 axis regulates viral gene expression in a promoter-dependent manner. L929 stably expressing shBAF or shControl were transfected with siRNA specific to B1 kinase (siB1-1) or siControl for 12hr, and then transfected with 1 ng of T7-Luciferase for 7hr, then infected with 37ºC with WT-VTF7.3 at MOI=3 in the presence of AraC. Lysates were prepared at 12hr after infection and assayed for luciferase activity, with RLU shown normalized to protein level. Error bars represent standard deviation. (*** indicates a p-value <0.05).

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promoter and is not regulated by BAF, this suggests that that the B1-BAF axis affects transcription in a gene specific manner.

5.12. The BAF-B1 axis affects nuclear promoters in promoter dependent

Considering the ability of BAF to bind to dsDNA in a sequence independent manner and to bind to transfected cytoplasmic dsDNA, the potential of BAF to regulate transcription of reporter genes from transfected plasmids was investigated. The biological relevance of this experiment is to assess the ability of BAF to interfere with gene expression occurring within the nucleus. This would give an understanding of BAF's activity within the nucleus during transfection. To address this, two plasmids expressing firefly luciferase under CMV or a 'minimal' (minP) promoter were used. The plasmid minP contains a weak promoter, as it is primarily constituted of a single TATA box, while the CMV immediate early promoter is a strong promoter. Both promoters undergo nuclear transcription mediated by cellular RNA pol II and transcription factors. Since BAF is present in both the cytoplasm and nucleus, its impact on transcription in both locations is of interest. One set of L929-shControl and –shBAF cells were infected with WT at MOI of 3 + AraC for 1 hr at 37ºC, then transfected with two different amount of CMV-Luc or minP-Luc plasmid for 24hr at 37ºC. Another set of L929-shControl and -shBAF were transfected with two different amount of CMV-Luc or minP-Luc plasmid for 24hr at 37ºC. Then lysates were collected and analyzed for luciferase activity.

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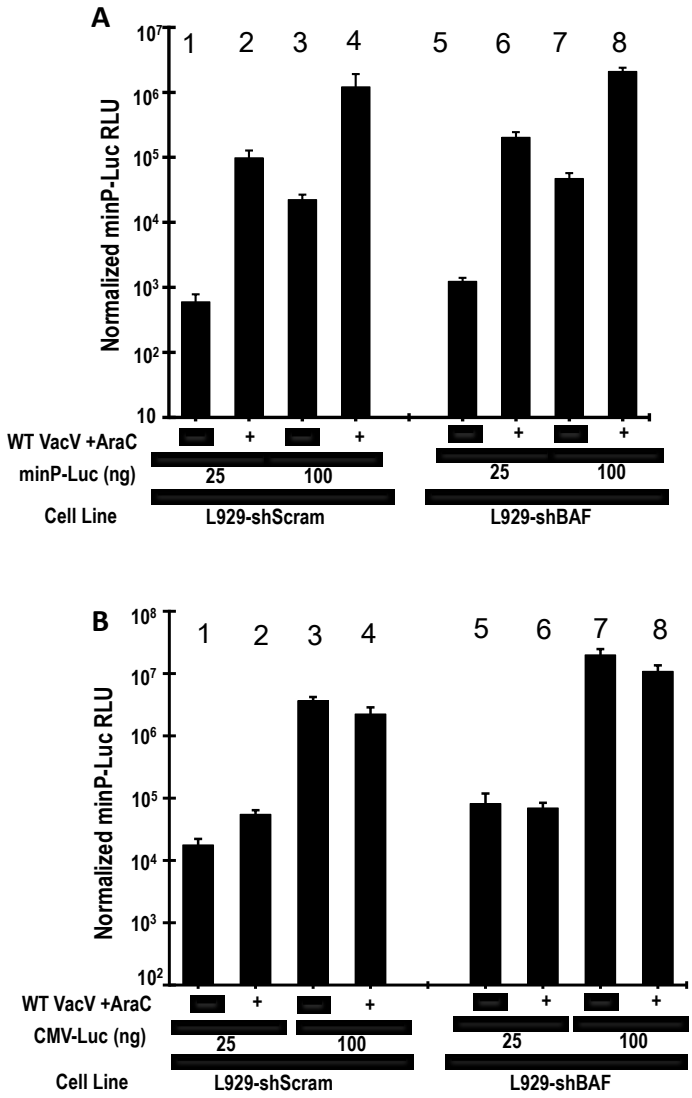

Figure 5.12. The role of B1-BAF in gene expression is promoter dependent. L929 stably expressing shBAF or shControl were left untreated or infected with WT in the presence of AraC at MOI=3 for 1 hr at 37ºC; 24 hr later cells were transfected with 25 or 100ng of **(A)** minP-Luc or **(B)** CMV-Luc at 37ºC for additional 24 hr. Lysates were collected and assayed for luciferase activity, with RLU shown normalized to protein level. The line number is shown on the top. Error bars represent standard deviation.

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As shown in Figure 5.12A, there is a 60-fold increase in luciferase activity between 25ng (lane 1 vs. 3) and 100 ng (lane 5 vs. 7) in both cell lines. However, when considering the level of expression of BAF, there is a minor 2-fold increase in minP promoter activity at both DNA concentrations in L929-shBAF compared to L929-shScram cells (lanes 1 vs. 5, and lanes 3 vs. 7). Strikingly, the pre-infection of these cells with WT virus prior to the transfection of minP-Luc had a remarkable effect on the minP promoter activity. At the lower DNA concentration, there is about 300- and 200-fold increase respectively in L929-shSram and –shBAF cells (lane 1 vs. 2 and lane 5 vs. 6), while an average of 70-fold increase was observed at higher DNA concentration regardless of cell lines (lane 3 vs. 4 and lane 7 vs. 8). The increase in reporter gene activity during infection showed that one or several viral factors have an enhancer effect on the minP promoter activity regardless of the presence or absence of BAF.

Similar experiment was performed using CMV-luc, a strong promoter derived from CMV. As shown in figure 5.12B, while there is a positive correlation between the increase in luciferase activity and that of DNA concentration when each cell lines is separately considered (lane 1 vs. 3 and lane 5 vs. 7), only 4- to 5-fold increase in luciferase activity when the level of expression of BAF is considered (lane 1 vs. 5 and lane 3 vs. 7). The pre-WT-infection of these cells followed by the transfection of CMV-Luc did result in an increase in luciferase activity (lane 1 vs. 2; 3 vs. 4; 5 vs. 6; and 7 vs. 8), contrary to what was observed when the pre-WT-infected cells were transfected with minP-Luc construct (compare figure 5.12 A to 5.12 B).

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The activities of minP and CMV promoters were enhanced when BAF was depleted, similar to what was observed with T7 and G8 promoters. Thus, BAF may be a general transcriptional regulator, although this activity may vary in function of promoters. Whether this specificity is due transcription factors specific to the promoter and / or subcellular localization of the DNA in question will be of significant future interest. Based on these results, the role of the BAF-B1 axis during gene expression from transfected plasmid likely depends on the promoter type, and that the presence of viral factors (from the pre-WT infection) also depends on the promoter type. The functional relation between BAF and B1 is likely to contribute to the expression of these promoters during transfection.

5.13. The inhibitor effect of BAF is dependent on the amount of transfected dsDNA

Considering the potential of BAF to regulate gene expression of transfected plasmid, the effect of BAF on transfected dsDNA was examined in the absence of VACV infection. For this experiment, L929 cells were transfected with 100 ng of pcDNA-3xFlag-BAF plasmid for 24 hr at 37ºC, then re-transfected with 50, 100, 250 or 500ng of CMV-luciferase or minP-luciferase plasmid for additional 24 hr at 37ºC. Another set of L929 cells were transfected only with 50, 100, 250 or 500ng of CMV-luciferase or minP-luciferase plasmid for additional 24 hr at 37ºC. Cells were then processed for measurement of luciferase activity.

As shown in figure 5.13A, in the presence of 100ng of pcDNA-3xFlag-BAF, the minP-mediated luciferase activity was 32-fold less than that in the

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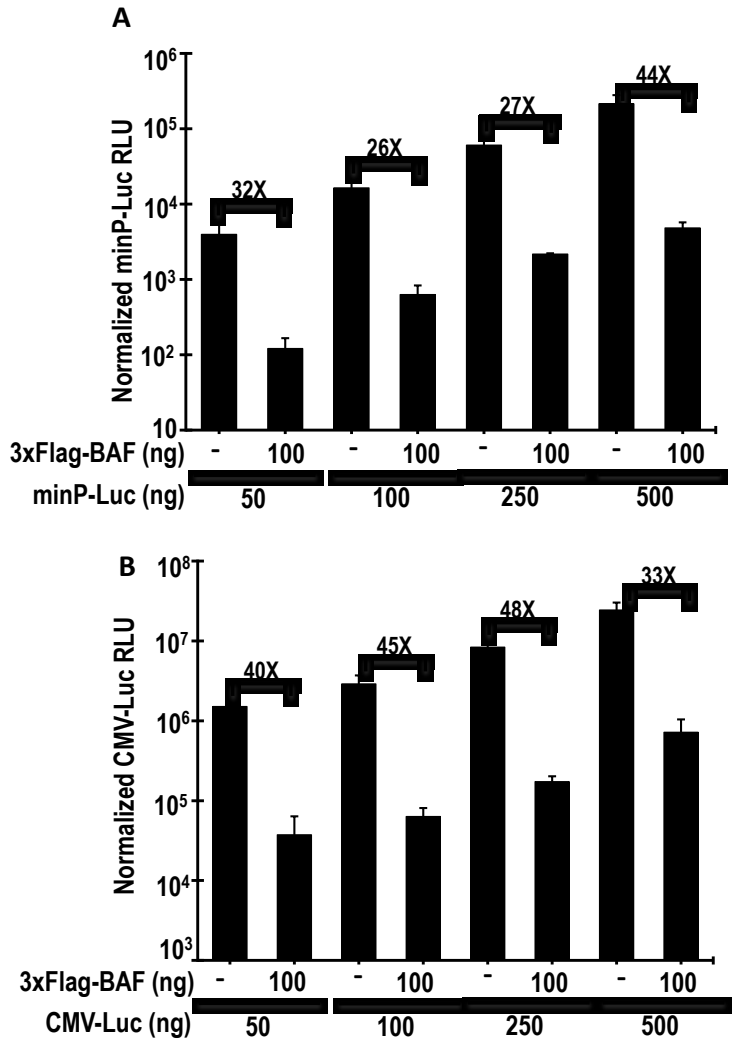

Figure 5.13. The inhibitor effect of BAF is dependent on the amount of transfected dsDNA. L929 were left untreated or transfected with 100 ng of pcDNA-TO-3xFlag-BAF for 24 hr at 37ºC; then transfected with 50, 100, 250 or 500 ng of minP-Luc (A) or CMV-Luc (B) for 24 hr at 37ºC. Lysates were prepared at 24h after transfection and assayed for luciferase activity, with RLU shown normalized to protein level. The number of the bars is the fold difference between them. Error bars represent standard deviation and the p-value is <0.05.

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absence of BAF. There was 26-, 27-, and 44-fold decrease in luciferase activity when BAF and minP-luc (respectively 100, 250 and 500ng) were both transfected. However, the luciferase activity increased 5-, 17- and 40-fold higher than the 50 ng of minP-Luc/100 ng 3xFlag-BAF when the amount of transfected minP-luc construct was increased while the amount of 3xFlag-BAF plasmid is constant. This would indicate BAF is less effective to exert its inhibitory activity in the presence of larger amount of dsDNA.

Similar results were observed when both 3xFlag-BAF and CMV-Luc constructs were expressed together (figure 5.13B); but the decrease in luciferase activity due to BAF expression averaged about 42-fold regardless of the amount of CMV-luc transfectedIn addition, the dual expression of a constant amount of 3xFlag-BAF and increasing amount of CMV-Luc showed an increase of 2-, 4- and 19-fold increase in reporter activity in comparison to 100ng 3xFlag-BAF/50ng CMV-luc.

In conclusion, these data showed that BAF inhibits gene expression from a transfected dsDNA, but the inhibitory effect of BAF becomes less effective in the presence of a higher concentration of dsDNA. These data do not indicate the ability of BAF to intercept transfected DNA within the nucleus.

5.14. Discussion

The requirement of B1 for viral DNA replication, which itself is needed for intermediate and late gene expression, is an obstacle to the study of B1's contribution to processes beyond DNA replication. However, as reported by

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Kovacs et al. (2001), by using plasmid reporters containing viral promoters to avoid the need for genome replication, B1 was needed beyond DNA replication (105). But, the mechanism underlying B1's contribution to gene expression was not fully defined. Therefore, in light of the importance of the B1-BAF axis for DNA replication, herein we examined whether B1 was acting through this same pathway to facilitate intermediate gene expression.

As expected, the B1-deficient ts2 virus exhibits a reduced capability to activate the luciferase reporter gene under several viral intermediate promoters compared to the WT virus in different cell lines tested (Fig. 5.3 and 5.4). Although all these cell lines express similar amounts of BAF protein, the role of B1 in intermediate gene expression varies. This may indicate that either the defective B1 or BAF exhibits cell type specific activity. Overall, these data showed that B1 is likely a general regulator of viral intermediate gene expression.

The activity of G8R promoter was enhanced in L929-shBAF cells regardless of virus treatment. In L929-shBAF there was a 40-fold increase than in ts2-infected L929-shControl cells (Fig. 5.7C and D), while only modest increase was observed in other viruses. It is interesting to note that in ts2-infected L929-shControl cells, intermediate gene expression was more dramatically affected during ts2 infected than that of these viruses (WT, ts42, and ts24). Further, siRNA-mediated depletion of B1 from WT virus leads to a decrease in reporter activation, which can be rescued by the depletion of BAF (Fig. 5.9B). Together, these data strongly suggest that in the absence of B1, BAF

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is capable of impairing viral gene expression, and that the B1-BAF axis impacts VACV intermediate promoter activity.

In regard to the mechanism of BAF's inhibition of viral gene expression, data reported in chapter 3 showed that BAF's ability to capture DNA requires its DNA binding and dimerization properties. These properties allow BAF to bind and crossbridge/aggregate DNA (16-18), and also likely allow BAF to impair transcription in the absence of B1. To determine how BAF affects the transcription, ChIP assay (data not shown) and RT-qPCR were employed. ChIP assay data from our laboratory showed that multiple regions of the plasmid co-immunoprecipitate with BAF, including the promoter region of the reporter gene (data not shown). This showed that BAF directly interacts with the transfected DNA. Further, RT-qPCR data showed that luciferase-specific transcript accumulated in ts2-infected cells only when BAF was depleted. These results showed that BAF likely acts at the level of gene transcription in the absence of B1.

The activity of the BAF-B1 axis during transcription may be gene-specific. Gene expression of a reporter under a T7 promoter during infection with vTF7.3 (expresses the T7 polymerase) (Figure 5.11) is not affected by the BAF-B1 axis, while WT infection enhanced minP promoter activity and not CMV promoter. Whereas, the depletion of BAF has little if any impact on the activity of minP and CMV promoters, both responsive to RNA polymerase II and cellular transcription factors (Figure 5.12 A and B), the overexpression of BAF has an inhibitory effect of the reporter activity regardless of promoters (Figure 5.13 A and B). These

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results showed that overexpression of BAF likely inhibit promoter activity of minP and CMV. However, the role of BAF-B1 axis in regulating gene expression from a transfected plasmid needs further studies.

In summary, this study provides data showing that the BAF-B1 axis regulates viral intermediate gene expression at the transcription level. Based on these data, the model proposes the following scenario: BAF binds directly to foreign DNA in a sequence independent manner, but inhibits gene expression promoter-dependent manner and this in the absence of a defective B1.

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CHAPTER VI

B1 KINASE HAS A POTENTIAL ROLE DURING VACCINIA VIRUS MORPHOGENESIS IN U2OS CELLS.

The work described in this chapter is a preliminary study to assess the role of the BAF-B1 axis in post-replicative events in U2OS cells.

Our previous studies examined the antiviral actvities of the BAF-B1 axis in CV1, BSC40, 293, and L929 cell lines. In an effort to understand how this axis regulates other viral processes beyond viral DNA replication, U2OS cells (a human osteosarcoma cell line) were employed to assess the inhibitory effect of BAF on viral DNA replication and production. Our studies demonstrated that in U2OS cells, there is no difference in viral DNA replication between WT- and ts2-infections at 37ºC and 40ºC as determined by qPCR. However, the viral yield as determined by viral titration on BSC40 cells is about 10-fold lower in ts2-infected than in WT-infected cells at 40ºC, but equal at 37ºC. These data indicate that the presence of an active B1 may not be required for viral DNA replication in U2OS cells even at non-permissive temperatures (37ºC and 40ºC). However, the deficiency in viral yield in ts2 infection provides a new lead to the potential role of B1 in morphogenesis and/or viral release/spread. Thus, this study was initiated to further investigate the viral processes affected by the absence of an active B1, as well as the mechanism associated with it.

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6.1. Immunoblot analysis of BAF expression in U2OS cells.

To study the role of the BAF-B1 axis in U2OS cells, replication-incompetent lentiviral vectors were used to construct cells lines stably depleted of BAF or overexpressing 1XFlag-BAF from their genome. For the stable depletion of BAF, U2OS cells were transduced with replication-incompetent lentiviral vectors expressing either a BAF-specific or scrambled (control) shRNA, then selected with 20μg/mL puromycin. For the stable overexpression of BAF, U2OS cells were transduced with replication-incompetent lentiviral vectors expressing either 1xFlag-BAF or mcs (multi clonal site empty vector) (control), and then selected with 200μg/mL hygromycin.

Equivalent amounts of cell lysates extracted from U2OS-mcs, -1xFlag-BAF, -shBAF and -shScram were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 18% gel, transferred to PVDF, and incubated with 157+/+, a primary antibody against BAF, and a rabbit secondary antibody. Blots were developed with chemiluminescent reagents on a Bio-Rad Chemidoc XRS instrument. As shown in figure 6.1, the stable overexpression of 1xFlag-BAF is shown as a higher band and increase the total BAF within these cells. The stable depletion of BAF showed a decrease in the level of BAF detectable with the antibody.

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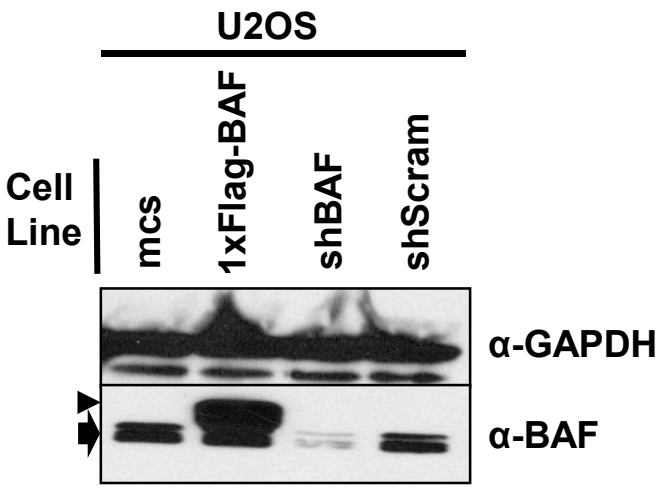

Figure 6.1. Immunoblot analysis of BAF expression. Equivalent lysates of U2OS-mcs, -1xFlag-BAF, -shBAF and -shScram were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 18% gel, transferred to PVDF, and incubated with a primary antibody against BAF, and a rabbit secondary antibody. Blots were developed with chemiluminescent reagents on a Bio-Rad Chemidoc XRS instrument. The migration of endogenous BAF is indicated with the arrow at the left, and that of 1xFlag-BAF by an arrowhead.

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6.2. Endogenous of BAF has little inhibitory effect on viral intermediate gene expression.

The observation that the BAF-B1 axis regulates viral intermediate gene expression in L929 cells prompted us to determine whether it is the same situation in U2OS cells. To this end, U2OS-mcs, -1xFlag-BAF, -shBAF or -shScram cells (shown in figure 6.1) were transfected with 10ng of pG8R-Luc for 7 hr at 37ºC; then infected with WT, ts2 or ts24 viruses (MOI = 3), and incubated at 40ºC overnight in the presence of 50 μM AraC. Cell lysate was collected at 16 hpi and assayed for luciferase activity.

As shown in Figure 6.2 left, in ts2-infected U2OS-mcs cells, there was only a 3-fold reduction in G8R promoter activity compared to WT-infected cells, and no effect was seen in ts24-infected cells, our control. This reduction of the intermediate gene expression is less pronounced than what that observed in L929 cells (see figure 5.4 in chapter 5). However, in ts2-U2OS-1xFlag-BAF infected cells, the activation of G8R promoter is 10-fold lower than that of WT-infected cells, suggesting the stable overexpression of BAF inhibits viral intermediate gene expression in the absence of an active B1. The stable overexpression of BAF inhibited the activity of G8R promoter in both WT and ts2-U2OS-1xFlag-BAF cells compared to that observed in WT- or ts2-infected U2OS-mcs cells. There were 8- and 25-fold decrease in luciferase activity respectively in WT- and ts2-U2OS-1xFlag-BAF compared to WT- and ts2-U2OS-mcs cells. However, the overexpression of BAF did not inhibit the G8R promoter activity in ts24-infected cells.

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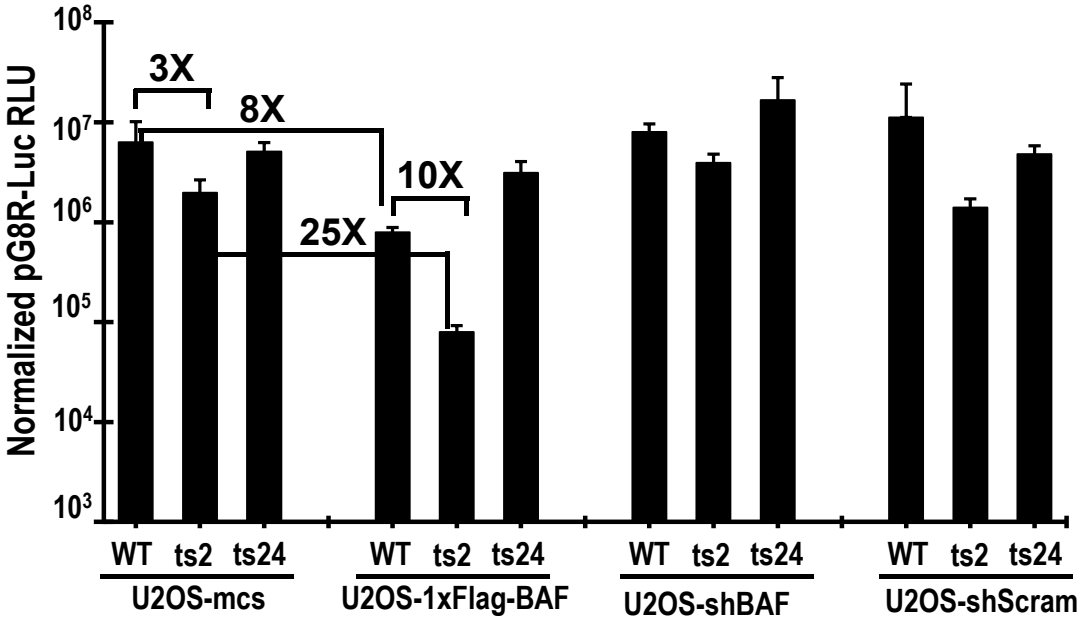

Figure 6.2. Endogenous BAF has little inhibitory effect on viral intermediate gene expression. U2OS-mcs, -1xFlag-BAF, -shBAF or -shScram cells were transfected with 10ng of pG8R-Luc for 7 hr at 37ºC; then infected with WT, ts2 or ts24 viruses (MOI = 3), and incubated at 40ºC overnight in the presence of 50 μM AraC. Lysates were collected in 300 μl of 1X Reporter Lysis buffer at 16 hr after infection and assayed for luciferase activity. RLU shown is normalized to total protein measured by BCA assay measured by BCA assay. Data were obtained from triplicate experiments, and the error bars represent standard deviation.

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The stable depletion of BAF modestly enhanced the activity of the G8R promoter regardless of viruses employed (see figure 6.2, right). This is expected considering the inhibitory effect of BAF. This result is in line with what was observed in L929 cells (see figure 5.7 in chapter 5).

The observation that there was only 3-fold reduction in the G8R promoter activity between WT and ts2-infected U2OS-mcs at 40ºC suggests that either 1) the endogenous BAF within U2OS cells may not be functional as observed in other cell lines such as L929 or 2) the residual activity of B1 from ts2 virus was efficient to perform its normal function during intermediate gene expression or 3) other cellular factors in U2OS cells may complement the role of B1 to allow viral intermediate gene expression. These results showed that the role of an active B1 is modest during intermediate gene expression in U2OS cells compared to L929 cells (105). Considering the little inhibitory effects of the endogenous BAF on viral intermediate gene expression, it is likely that the endogenous BAF is not able to fully exert its antiviral activity in U2OS cells.

6.3 Viral DNA replication is inhibited in cells overexpressing BAF.

The observation that the overexpression of BAF inhibits viral intermediate gene expression prompted us to assess if it also affects viral DNA replication and production. The goal is to assess whether overexpression of BAF can restore its inhibitory effect on DNA replication as seen in CV1 cells (11). To this end, U2OS-1xFlag-BAF or –mcs cells were infected with WT or ts2 at MOI= 3 at 40ºC. At 8 or 24 hpi, cell lysates were collected in 100 µl of 1XPBS and 50% were used for

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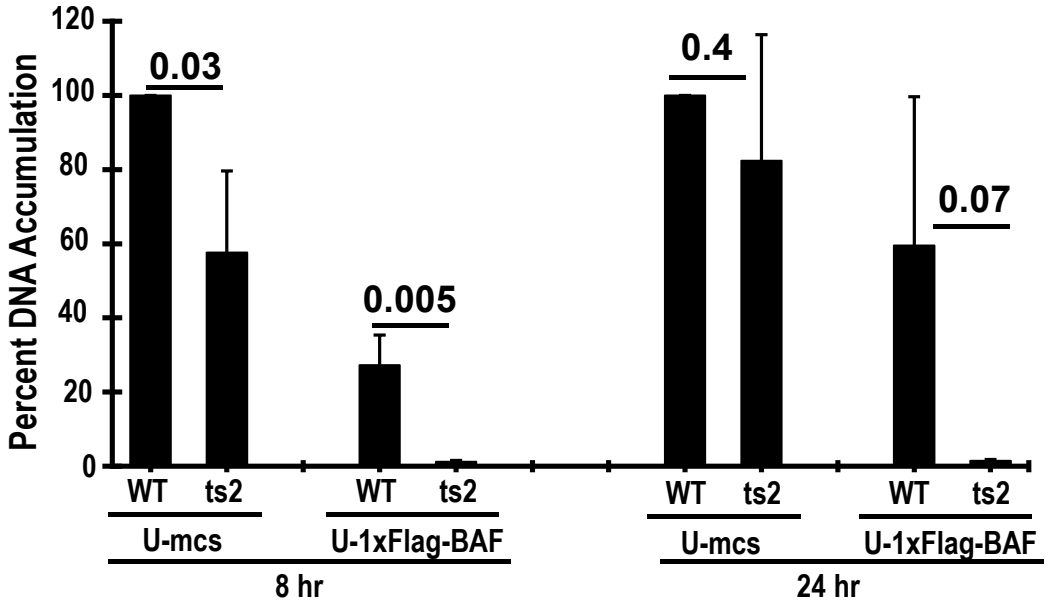

Figure 6.3. Viral DNA replication is inhibited in cells overexpressing 1xFlag-BAF. DNA replication assay: U2OS-mcs or -1xFlag-BAF cells infected with WT or ts2 at MOI= 3 for 8 hr or 24 hr at 40ºC. Lysates were collected in 100 µl of 1XPBS, DNA extracted and quantified for viral DNA accumulation by qPCR at both 8 hr and 24 hr. Data were obtained from triplicate experiments, and the error bars represent standard deviation.

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viral DNA replication assay, and the remaining 50% for viral titration on BSC40 cells. The 8 hpi incubation time was selected to detect any early difference in viral DNA replication. After viral DNA extraction, qPCR was employed to quantify the viral DNA accumulation at both 8 and 24hrs. As shown in figure 6.3, in U2OS-mcs cells at 8 hpi, DNA accumulation in WT-infected cells is statistically significant and higher than that in ts2-infected cells. At this early point, ts2 virus showed a delay in viral DNA replication with 1.7-fold less DNA accumulation in ts2- than in WT-infected U2OS-mcs cells. However, at 24hpi this difference become 1.2-fold and is not statistically significant. This result suggests that the absence of an active B1 has a minimal effect on viral DNA replication, but is less pronounced at later times after infection.

The stable overexpression of 1xFlag-BAF blocked viral DNA replication in both WT- and ts2-infected cells at both 8 and 24 hpi. As shown in figure 6.3, at 8 hpi, viral DNA accumulation is inhibited 5-fold in WT-U2OS-1xFlag-BAF cells compared to WT-U2OS-mcs cells, while it was inhibited more than 50-fold in ts2-U2OS-1xFlag-BAF cells compared to ts2-U2OS-mcs cells. In U2OS-1xFlag-BAF cells at 8hpi, there is 22-fold more viral DNA replication in WT- than in ts2-infected cells. However, there is a 2-fold increase in DNA replication in WT-U2OS-1xFlag-BAF at 24 hpi compared to that at 8 hpi. There is no significant change in DNA accumulation in ts2-infected cells at 24 hpi compared to 8hpi. There is 40-fold difference in DNA accumulation between WT and ts2 at 24 hpi in U2OS-1xFlag-BAF.

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These results showed that the endogenous BAF in U2OS cells is not effective to function as an antiviral factor against poxvirus, but BAF's overexpression has a potent inhibitory effect on viral DNA replication and viral yield in the absence of an active B1.

6.4 Stable depletion of BAF has little effect on viral DNA replication

As reported in 6.4, the overexpression of BAF has an inhibitory effector on viral DNA replication, however, the extent to which the endogenous BAF is implicated in these events is not known, as well as the role of the BAF-B1 axis. To assess the degree to which the endogenous BAF functions as an inhibitory of poxvirus infection, stable depletion of BAF from U2OS cells was employed to assess viral DNA replication and production. To test this possibility, U2OS-shBAF and -shScram cells were infected with WT or ts2 at MOI= 3 for 8hr or 24hr at 40ºC. Cell lysates were collected and half used for viral DNA assay and the other half for viral titration assay.

As shown in figure 6.4, in U2OS-shScram infected cells, there is no difference in DNA accumulation in WT and ts2 infection at 8hpi versus 24hpi. However, stable depletion of BAF increased viral DNA replication by about 3-fold in WT infection, and a minor increase in ts2 infection compared to similar infections in U2OS-shScram at 8 hpi. At 24 hpi, depletion of BAF had a minor effect on viral DNA replication during both WT and ts2 infections compared to these infections in U2OS-shScram.

The depletion of BAF in U2OS cells has a minor rescuing effect on viral DNA replication in both WT and ts2 infections compared to what is observed in cells

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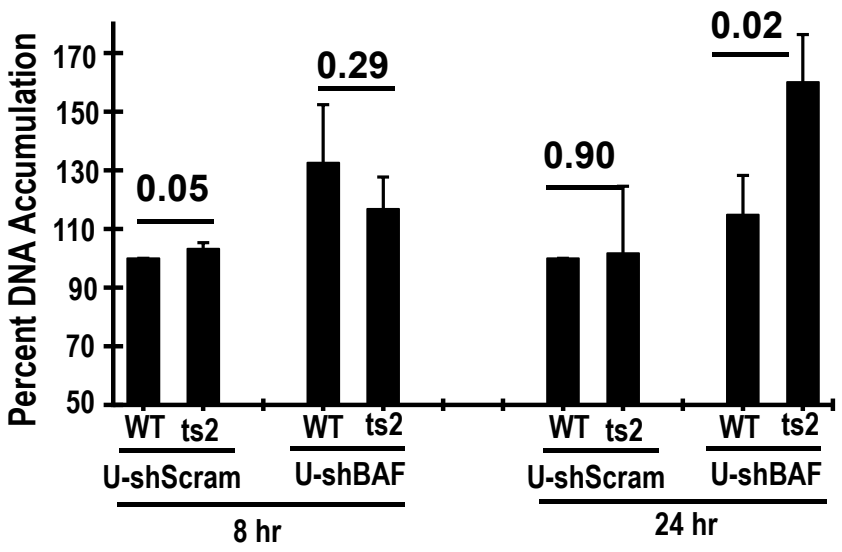

Figure 6.4. Stable depletion of BAF enhances both DNA replication. DNA replication assay: U2OS-shBAF or -shScram cells infected with WT or ts2 at MOI= 3 for 8 or 24hr at 40ºC. Lysates were collected in 100 µl of 1XPBS, DNA extracted and quantified for viral DNA accumulation by qPCR at both 8 and 24 hr. Data were obtained from triplicate experiments, and the error bars present standard deviation.

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used in this and published reports (11). While these results highlight the minor inhibitory role of the endogenous BAF on VACV DNA replication, they prompted us to investigate whether the endougenous BAF is present at ts2 DNA replication sites.

6.5. BAF is present at ts2 DNA replication sites.

The ineffective activity of the endogenous BAF on viral DNA replication compared to 1XFlag-BAF prompted me to determine whether BAF can be present at the ts2-viral DNA replication sites. Previously, it was shown that the inhibitory effect of BAF on VACV DNA replication is associated with its presence at DNA replication sites (11). To assess BAF's cellular distribution, U2OS cells were infected with WT or ts2 virus at MOI=3 at 40ºC. At 9hpi, cells were processed for immunofluorescence imaging. Rabbit α-157+/+ (against endogenous BAF) and rabbit α-I3 (against the viral ssDNA-binding protein) primary antibodies were used, followed by Alexa Fluor 488 secondary antibody and DAPI. The anti-BAF α-157+/+ was recently designed and tested for the detection of endogenous BAF.

In WT-infected U2OS cells, BAF is mostly diffuse in the cytoplasm (figure 6.5, panel B) but absent from WT DNA replication sites, which are shown in DAPI (figure 6.5, panels A and G), and also detected with the I3 antibody (figure 6.5, panel H). This diffuse distribution of BAF in WT-infected cells is in accordance with previous report (11). However, in ts2-U2OS infected cells, BAF is present at cytoplasmic ts2-DNA replication sites (figure 6.5, panels D to F look to arrowheads), also in accordance with a previous report (11).

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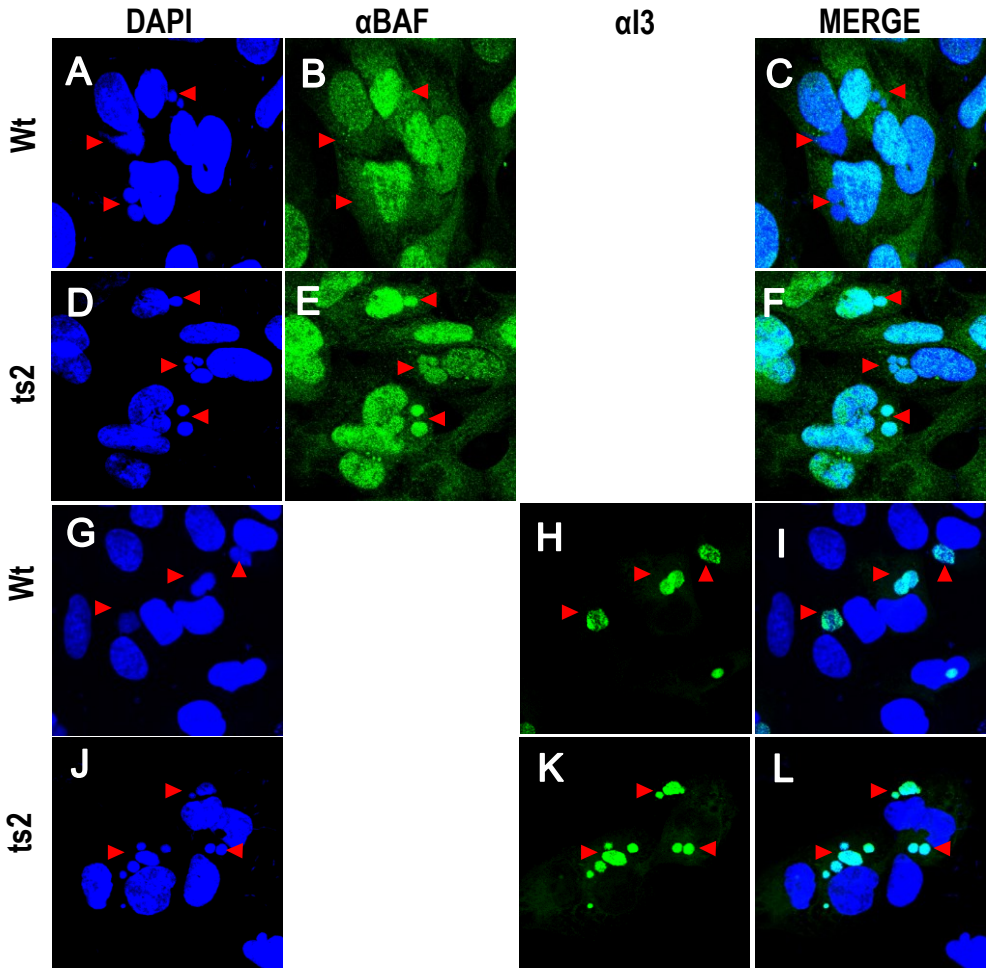

Figure 6.5. BAF colocalizes with ts2 DNA replication sites. U2OS cells were infected with WT (A to C and H to I) or ts2 (D to F and J to L) at MOI= 3 at 40ºC. At 9 hpi, cells were fixed and permeabilized with 0.5% saponin. For immunofluorescence imaging, rabbit α-157+/+ (against endogenous BAF, A to F) and rabbit α-I3 (G to L) primary antibodies were used, flowed by Alexa Fluor 488 secondary antibody (A to L), and DAPI. Representative images shown were taken using a confocal microscopy at 60X magnification. Arrowhead shows viral DNA replicates sites in DAPI (A, D, G and J), BAF locations (B and E) and viral replications using I3 antibody (H and K).

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In both WT- (figure 6.5, panels G to I) and ts2-infected cells (figure 6.5, panels J to L), viral replication sites are formed as detected by the I3 specific antibody. This showed that both WT and ts2 viruses were equally replicating their DNA. Although, BAF is able to sense and relocalize to viral DNA replication sites. These results provide strong evidences that the endogenous BAF in U2OS cells is not sufficiently active to inhibit viral DNA replication, contrary to what was previously shown in other cells (11). However, the degree and how the defective B1 expressed from ts2 contributes to viral DNA replication in the presence of the endogenous BAF is not known. But previously, it was reported that in ts2-infected U2OS cells, BAF is more phosphorylated than in uninfected cells (11). Thus, it is possible that the residual activity of ts2-B1 inactivates BAF, and excludes the possible involvement of BAF with the events leading to defect in viral production.

6.6. The viral B1 kinase is involved with viral spread

Although both WT and ts2 viruses perform DNA replication with the same efficiency, viral yield is consistent about 20-fold higher in WT-infected than in ts2-infected U2OS-mcs or -shScram cells (Figures 6.3 and 6.4). This is an indication that B1 is mediating at least one post-replicative event. Our hypothesis is that the defect in B1 kinase affects late gene expression therefore viral morphogenesis or release/spread. Our rational is based on the fact that since both WT and ts2 viruses replicate with the same efficiency, the difference seen in viral yield is due to defects in late gene expression and/or during morphogenesis. During viral intermediate gene transcription assay, there was a minor 3-fold decrease in intermediate gene expression in ts2-infected U2OS cells compared to WT-

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infected U2OS cells (figure 6.2). This result showed a modest role of B1 in intermediate gene expression in U2OS cells.

To investigate a potential defect in viral spread, two recombinant viruses expressing red fluorescent protein (RFP) under an early/late promoter, WT-RFP and ts2-RFP, were used. These viruses efficiently spread in cell monolayer, thus providing a mean to assess viral spread. The pattern of expression of RFP is used as a marker of viral growth and spread. Specifically, the expression of RFP is considered as a sign of successful viral growth. In addition, the pattern of cells expressing RFP is used as a marker of viral spread. Specifically, clustered cells expressing RFP and that are organized in a plaque are considered to be a sign of viral spread. To this end, U2OS cells were infected with 100, 1000 or 10,000 pfu (plaque forming unit) of WT-RFP or ts2-RFP and incubated for 24 or 48 hr at 40ºC. Cells were fixed and stained with DAPI, images shown were taken using a confocal microscopy at 10X magnification.

As shown in Figure 6.6 (panels A, E and I) at 24 hpi, in WT-RFP-infected cells, large RFP-expressing plaques are visible, and there is a direct increase in plaque number of cells proportional to the amount of pfu used. However, in ts2-RFP-infected cells, only isolated RFP-expressing cells were observed (Figure 6.6 panels F and J). Clearly, at 24 hpi the number of RFP-expressing cells organized in a plaque is much higher in WT-RFP than in ts2-RFP-infected U2OS cells.

At 48 hpi, regardless of the number of input pfu, all WT-RFP-infected samples have RFP-expressing cells forming large plaques (Figure 6.6C, G and

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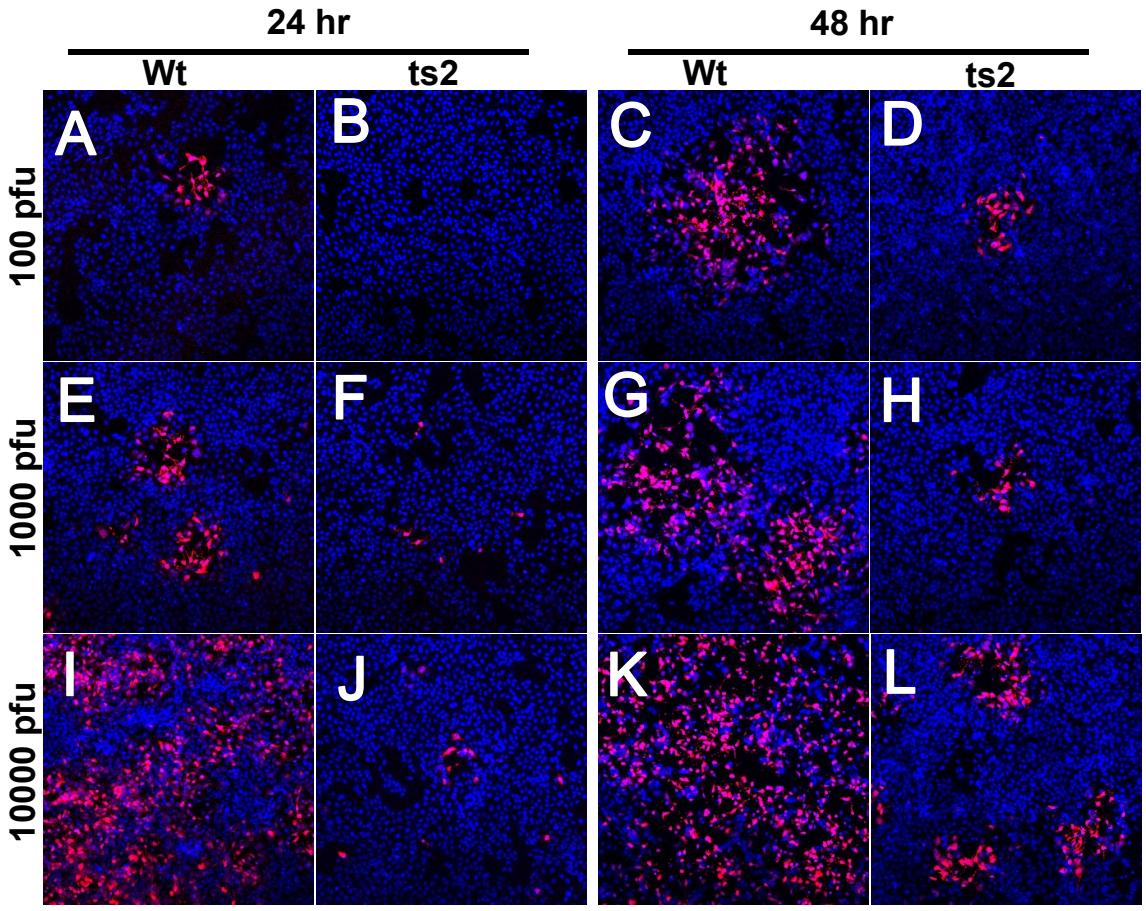

Figure 6.6. The viral B1 kinase is involved with viral spread. U2OS cells were infected with 100, 1000 or 10000 pfu of WT-RFP (A, E, I, C, G and K) or ts2-RFP (B, F, J, D, H, and L) at 40ºC. At 24 or 48 hpi, cells were fixed and stained with DAPI. Representative images shown were taken using a confocal microscopy at 10X magnification.

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K). However, in ts2-RFP infected cells, RFP-expressing cells emerge and form small plaques, but with less frequency than in WT-RFP-infected cells (Figure 6.6D, H and L). The RFP-expressing cells in ts2-RFP infection at 48 hpi showed some similarity to those in WT-RFP infection at 24hpi (Figure 6.6 A vs. D, E vs. E). The existence of single RFP-expressing cells at 24hpi then a clustered of RFP-expressing cells in ts2 infection at 48hpi showed that the absence of an active B1 may lead to a delay in viral morphogenesis, exit and spread. These results showed that the absence of an active B1 is likely causing a delay in viral growth and spread.

6.7. Multi-step viral growth indicates a defect in viral spread in ts2 infection.

Based on data showing the potential implication of B1 in viral growth and spread (Figure 6.6), I sought to determine the effect of B1 on viral spread using a one- and multi-step viral growth assays. In one-step growth, all cells are synchronously infected with more than one pfu, leading to a single cycle of infection and viral burst. However, during a multi-step growth, only one cell in 10 or more is infected, therefore many cycles of infection occur. My rationale is that since viral yield depends absolutely on the ability of the virus to grow, and to be released to infect adjacent cells, a multi-step growth infection provides a mean to quantify any defect in viral formation and spread in viral yield. Considering the functional relationship between BAF and B1, the potential impact of BAF on viral growth and spread is of interest is also addressed.

To this end, U2OS-mcs, 1xFlag-BAF, -shScram or –shBAF cells were infected with WT or ts2 at MOI= 0.01 for 48 hr or at MOI= 3, or 5 for 24 hr at

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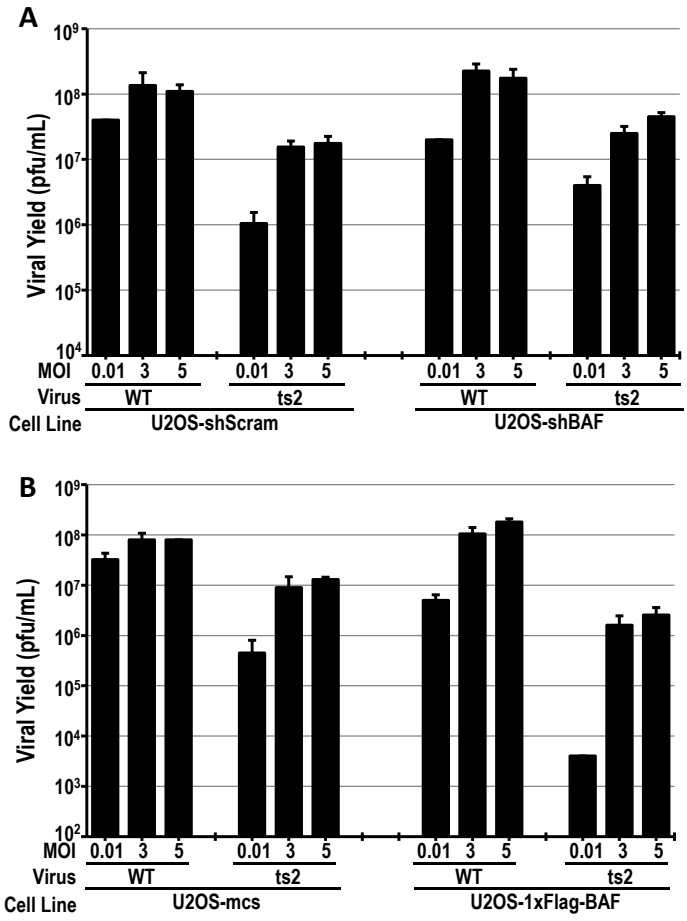

Figure 6.7. Multi-step viral growth indicates a defect in viral spread in ts2 infection. U2OS-mcs, 1XBAF, -shScram or -shBAF cells infected with WT or ts2 at MOI= 0.01 for 48 hr or MOI= 3, or 5 for 24 hr at 40ºC. Lysates were collected, and viral production was quantified by a plaque assay on monolayers of BSC40 cells at 32ºC. Data were obtained from duplicate experiments, and the error bars present standard deviation.

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40ºC. Cell lysates was collected, and viral production was quantified by a plaque assay on monolayers of BSC40 cells at 32ºC.

As shown in figure 6.7A and B, in WT-infected cells, regardless of MOIs and cell types, the viral yield range from 10^7 to 10^8 pfu/ml suggesting viral production and spread are not affected. This is somewhat expected because WT virus expresses an active B1. However, in ts2-infected U2OS-mcs and -shScram cells at MOI=3 and 5, viral yield is in average 10-fold lower than that of WT infections (Figure 6.7A and B). At MOI=3 and 5, in U2OS-shScram cells viral yield in ts2 infection is approximately 10-fold lower than in WT infection (Figure 6.7A). In BAF-depleted cells, the viral yield in both WT and ts2 infections increases, but viral yield in ts2 infection is still 4-folf lower than in WT infection. Depletion of BAF increases viral yield 6-fold higher in ts2-infected U2OS-shBAF than in ts2-infected U2OS-shScram cells, while only a minor increase was observed in WT-infected U2OS-shBAF compared to WT-U2OS-shScram

Stable overexpression of 1xFlag-BAF inhibited viral yield about 100-fold in ts2 infection compared to WT infection at both MOI of 3 and 5. In ts2-infected U2OS-mcs, the viral yield was about 10-fold higher than in ts2-infected U2OS-1xFlag-BAF cells.These results suggest that the endogenous BAF has a modest inhibitory activity on viral yield during ts2 infection.

In the contrary to the one-step growth data (MOI=3 and 5), during a multi-step viral growth (MOI=0.01), the viral yield in ts2-U2OS-shScram cells is about 40-fold lower than that in WT-U2OS-shScram cells (Figure 6.7A). Depletion of BAF slightly increased viral yield is 2-fold higher in ts2-infected U2OS-shBAF

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compared to ts2-infected U2OS-shScram cells, and 5-fold lower than in WT-infected U2OS- shBAF (Figure 6.7A).

In ts2-infected U2OS-mcs cells (MOI=0.01), the viral yield is about 40-fold lower than in WT-U2OS-mcs infected cells (Figure 6.7B). In U2OS-1xFlag-BAF, the viral yield during ts2 infection is 1250-fold lower to that in WT-infected U2OS-1xFlag-BAF cells. Overexpression of BAF inhibited viral yield more than 70-fold in ts2-infected U2OS-1xFlag-BAF than in ts2-infected U2OS-mcs cells at MOI=0.01. These results showed that the overexpression of BAF has a tremendous effect on ts2 virus's viral yield. This may be due mainly to BAF's inhibitory effect on viral DNA replication as shown by BAF's overexpression data in Figure 6.3A. However, there is 110-fold less virus produced in ts2-infected U2OS-1xFlag-BAF cells than in ts2-U2OS-mcs infected cells, which may be due essentially to the overexpression of 1xFlag-BAF.

Using a multi-step viral growth in U2OS-shBAF and -1xFlag-BAF showed that the defect in viral yield during ts2 infection is likely due abnormalities during viral morphogenesis. Based also on data reported on figure 6.3A, the defect on viral yield is likely independent of BAF. Further, because almost all proteins mediating morphogenesis are expressed during late gene expression, it is possible that defects in viral yield are the result of B1's effects on late gene expression.

6.8. Discussion

The finding that B1 is involved in viral intermediate gene expression, in addition to its role in viral DNA replication, suggests the possible involvement of

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B1 in other post-replicative events such as late gene expression. Indeed, the ts2 infection of U2OS cells did not lead to a decrease in viral DNA replication compared to a WT infection at 40ºC, a non-permissive temperature (Figure 6.3A and 6.4A, U2OS-mcs and U2OS-shScram cells). This is not in accordance with what was previously shown in CV1, BSC40, 293 and L929 cells (11). However, the level of expression of BAF does not vary between U2OS, CV1, BSC40 and L929 (see figure 5.4), and sequence analysis of the BAF ORF of U2OS cells does not reveal any difference with BAF's sequences from other cells (Ma and Wiebe, unpublished observations).

There is no difference in viral DNA replication between WT and ts2 infection in U2OS cells, but the viral yield is always about 10-fold lower in ts2 than WT infections (Figure 6.3B and 6.4B), suggesting that a postreplicative event may require an active B1. The presence of BAF at ts2 viral DNA replication sites (Figure 6.5E) and the slight increase in DNA replication and viral yield in BAF-depleted cells (Figure 6.4) showed that the endogenous BAF may not be sufficiently functional to exert its inhibitory activity on viral DNA replication. However, both viral DNA replication and yield are inhibited when BAF was stably overexpressed (Figure 6.3), suggesting that the defective B1 from ts2 virus has a residual activity capable of inactivating the endogenous BAF to allow DNA replication. These data suggest a modest role of BAF during VACV life cycle, and B1 may play a role in another post-DNA replication event.

A previous report showed that in ts2-infected U2OS cells, BAF is more phosphorylated than in uninfected cells (11). This suggests that B1 expressed

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from ts2 virus possess some residual activity; but it is unclear whether BAF at the ts2-DNA replication is still active or not. If the defective B1 was efficient to inactivate BAF, this annuls the potential implication of BAF with post-replicative events, and provides an explanation of the minor role of BAF in viral intermediate gene expression seen in U2OS cells compared to other cells (see figure 5.4)

Defects in viral DNA replication results in abnormal virus productions (181, 238), however our data showed that defects in viral production in U2OS cells is not associated with viral DNA replication (Figure 6.3A and 6.4A, U2OS-mcs and U2OS-shScram cells). This is likely due to defects associated with morphogenesis. Based on the results of a one- and multi-step viral growth assays, ts2 virus showed a defect in viral growth and spread (Figure 6.7 and 6.6) compared to WT virus. The multi-step viral growth data would indicate that the failure of ts2 virus to produce progeny viruses that can successfully infect neighboring cells, or viral morphogenesis is altered or the newly formed virions are not infectious. Further, the immunofluorescence data showed defects in both viral growth and spread in the absence of an active B1, supporting the potential role of B1 in morphogenesis and viral spread.

In summary, this study has provided evidence to support the role of B1 during viral morphogenesis and viral spread. However, the implication of late gene expression is not exclude. This study also reveals that BAF is not fully associated with B1's role in morphogenesis. It is unclear whether B1 affects one step or the whole morphogenesis process, or whether B1 affects the release of newly formed virions and their ability to infect other cells. It is also unknown if B1

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associates with and/or targets other viral proteins to regulate viral morphogenesis. Further studies are needed to fully understand how B1 regulate viral morphogenesis.

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CHAPTER VII.

DISCUSSION &SUMMARY

7.1. BAF as a DNA sensing factor

Poxviruses, such as vaccinia virus, encode numerous homologs of cellular proteins as part of their strategy to evade or inactivate the host immune system (156, 230-233). Virus-host interactions occurring during vaccinia infection provide insights into how critical aspects of the host immune system can be targeted by viral proteins within the cytoplasm. Based on its role during viral infection, the viral B1 kinase, an homologue to the cellular VRK proteins should be counted among vaccinia's immune evasion strategies (11).

The viral B1 is essential for poxvirus DNA replication as it phosphorylates BAF, which consequently stimulates viral DNA replication (11). The ability of BAF to relocalize to DNA both during a ts2 virus infection (11) or transfection is similar ro other cellular mechanisms that sense pathogen-derived DNA. The DNA-sensing system, composed of Toll-like receptors and cytosolic DNA-sensors, is critical for the activation of the innate immune responses (239). With this logic, the potent antiviral activity of BAF contributes to cellular innate mechanisms designed to counter VACV infection and cytosolic dsDNA.

Detection of pathogen-derived DNA within the cytoplasm may require specific molecular features intrinsic to these DNA-sensing proteins. For example, DNA-sensing proteins should directly bind to the target DNA or indirectly through

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an interacting partner. Our data provide the first evidence that intrinsic features of BAF, namely DNA-binding and dimerization, are essential for BAF's relocalization to cytoplasmic dsDNA. Further, our data highlight the direct interaction between BAF and DNA, suggesting that BAF mediates complex formation. BAF is not an IFN-stimulated protein, and there is no evidence of BAF triggering IFN production (Wiebe, unpublished observations), thus further studies are needed to fully understand the mechanisms by which BAF regulates vaccinia virus.

Our studies also demonstrated certain cellular proteins, such as DNA repair proteins are present at BAF-DNA complexes. It is well documented that DNA repair proteins, including recently Ku86, are avoided or manipulated by viruses because of their antiviral potential (227, 240). Although some of these proteins relocalize to cytosolic DNA independently of BAF, BAF is likely a stabilizing factor as BAF-DNA nucleoprotein complexes are resistant to up 700 mM salt concentration (6, 12), suggesting that BAF stabilizes theses complexes and serves as a scaffold for other proteins to be recruited and perform their biological functions. Based on our data and other published reports, our current model of BAF's functions is shown in figure 7.1. The following scenario is proposed: within the nucleus, the interactions of BAF with LEM-domain proteins and with chromatin are regulated by the cellular VRK1's phosphorylation of BAF (12). The shuttling of BAF between the nucleus and cytoplasm occurs, and the cytoplasmic pool of BAF may be dephosphorylated by a phosphatase, possibly

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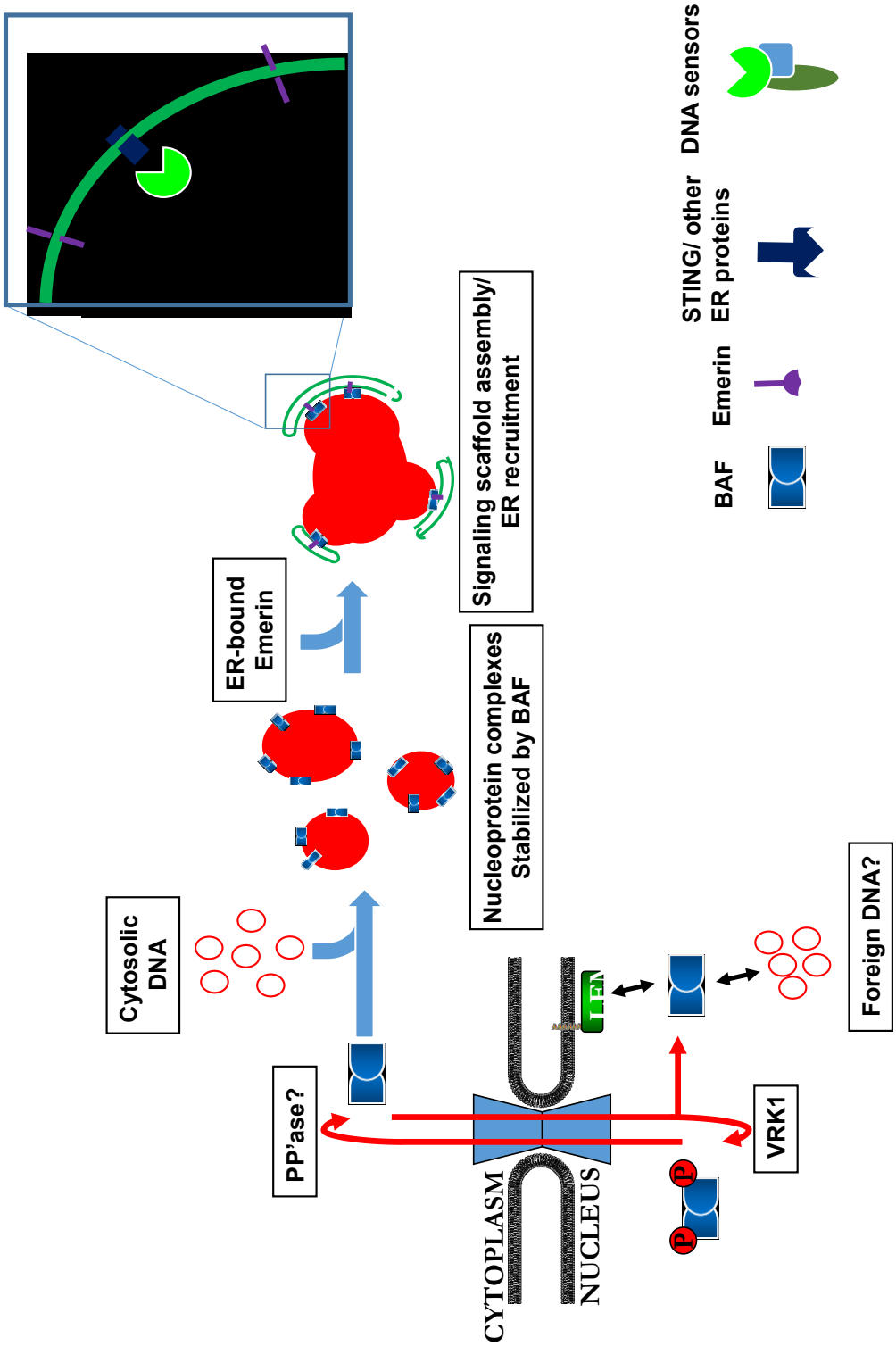

Figure 7.1. Proposed Model of BAF's activity in the presence of dsDNA in the cytoplasm (adopted and modified from Dr. Wiebe).

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the protein phosphatase 4 (241). Cytoplasmic BAF senses the presence of a cytoplasmic dsDNA (for vaccinia virus DNA in the absence of B1), and binds to it to form high order nucleoprotein complexes. These complexes are stabilized by BAF, and other cellular proteins are also recruited in both a BAF-dependent and independent manner. The proximity of these complexes to the ER may suggest that they are platforms for the recruitment other proteins with that have cell signaling properties.

7.2. BAF as a transcriptional regulation of foreign DNA

The cytoplasmic life cycle of vaccinia virus is regulated by poxvirus-encoded proteins, and a highly regulated gene expression that occurs in three sequential stages (107). Although the expression of both intermediate and late genes requires DNA replication, the latter is also dependent on the early B1 kinase (70). In addition to its required role in viral DNA replication, B1 also regulates intermediate gene expression by an unknown mechanism (105). With the discovery that BAF is the substrate of B1, the molecular mechanism employed by B1 to enable viral DNA replication has been elucidated (11). B1 kinase phosphorylates Thr2/Thr3/Ser4 at the N' terminus of BAF to inactivate and abrogate BAF's DNA-binding properties (11, 12). In addition the role of B1 in the regulation of viral DNA replication and intermediate gene expression (11, 105), our data showed for the first time that BAF is an inhibitor of viral intermediate gene transcription in the absence of an active B1.

Previous reports showing that DNA-bridging proteins are regulators of gene expression by affecting promoter's accessibility by the transcriptional

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machinery and associated factors (242-244). As a DNA-binding/bridging protein, we hypothesized tha BAF regulates viral gene expression. For example, two bacterial DNA-bridging proteins, H-NS and *LacI*, repress transcription by bridging foreign DNA and blocking promoter accessibility (244). In eukaryotes, nuclear proteins, such as histones and high mobility group B (HMGB), regulate the functional state of chromatin by affecting the structure of DNA (245-247).

Like the bacterial DNA-bridging proteins, BAF inhibits gene expression from transfected plasmid. ChIP assay data from our laboratory also showed that BAF directly interacts with several sections of a transfected plasmid, supporting previous report on BAF's ability to compact dsDNA by forming intramolecular loops and cross-bridging leading to the formation of high-order nucleoprotein (17). Based on our studies and other independent studies, our proposed model of how BAF regulates gene expression is through compaction of DNA, which structurally interferes with binding of transcription factors to their target sequences and promoters; consequently transcriptional is inhibited.

The role of the BAF-B1 axis in regulating intermediate gene transcription is consistent in several cell lines and regardless of intermediate promoters tested, an indication that the early inactivation of BAF during viral infection rescues both DNA replication and intermediate gene expression. Stable depletion of BAF in the absence of an active B1 rescues viral intermediate gene transcription, but not to the level of WT infection, suggesting additional viral or cellular factors contribute to this process independent of BAF, but B1-dependent.

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The existence of another factor contributing to B1's role in intermediate gene expression is an indication that B1 has other cellular and viral substrates, which may include the substrates of VRKs (248-250). On the contrary, most substrates of VRKs are nuclear proteins (196, 207, 209), therefore they may have less involvement in vaccinia productive infection.

One potential candidate that may contribute to the viral intermediate transcription in B1-dependent manner is the viral protein H5 (251). H5, a phosphoprotein that is also targeted by F10, another poxvirus kinase with roles in morphogenesis (85, 182, 251). Virion-associated, early and constitutively expressed during vaccinia virus life cycle, H5 is conserved in all orthopoxviruses. It binds DNA and RNA (139), and is also a late transcription factor or VLTF-4 (139). Using a yeast two-hybrid system, Black et al. (1998) showed that G2R (initiation factor) and A18R (termination factor) interact with H5R to form a complex that regulates postreplicative transcription (142). Further, B1, A20 (DNA replication protein), A2 and G8 (both initiation factors) and A49R (unknown function) also interact also with H5 (252, 253).

Through these interactions, H5 is considered to be a multifunctional protein with roles in DNA replication, transcription of late gene and morphogenesis (139, 144, 253, 254). Based on the interaction between H5 and B1 (253), H5 is a likely candidate to elucidate B1-dependent, but BAF-independent mechanism of regulation of intermediate viral transcription. Because H5 binds DNA and RNA (254), our assumption is that the phosphorylation of H5 by B1 (251) may regulate its role during intermediate gene expression, and

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possible its interactions with G8R and A2L. Our model also favors a mechanism independent of the BAF-B1 axis. Since H5 is also part of a transcription complex because it interacts A18R and G2R (142), it is probable that when phosphorylated by B1, H5 mediates transcription initiation and elongation. Therefore, depletion of H5 can provide preliminary evidences of H5-B1's role in intermediate gene expression.

7.3. B1 as a potential regulator of morphogenesis

The viral kinase B1 is produced prior to the onset of viral DNA replication, and is required for viral DNA replication (180, 184). Since viral DNA replication is itself necessary for post-replicative events, the regulation of these events by B1 may remain hidden. With its role in intermediate gene expression, B1 is likely to directly or indirectly regulate viral late gene expression, and subsequently viral morphogenesis. Our data demonstrated that B1, independently of its role in DNA replication, is also needed for viral late gene expression and/or morphogenesis.

In U2OS human osteosarcoma cells, viral DNA replication is less dependent on B1, the opposite of what was shown in CV1, BSC40 and L929 cells (11, 193). The absence of an active B1 as well as the presence of BAF at the ts2 DNA replication sites did not block viral DNA replication, but in WT-infected cells BAF is diffuse in accordance with previous report (11). Based on our data, the residual activity from the defective B1 gene may be sufficient to allow viral DNA replication regardless of BAF's presence. The level of expression of BAF does not vary between U2OS, CV1, BSC40 and L929 (see figure 5.4),

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and sequence analysis of the BAF ORF of U2OS cells did reveal any difference with BAF's sequences from other cells (Ma and Wiebe, unpublished observations). However, it is possible that cell type-specific factors may contribute to the viral phenotype observed, or a cellular pathway regulating BAF or active innate responses may be dysfunctional.

While no defect in viral DNA replication was observed in the absence of an active B1, viral yield was drastically lower in ts2 infection compared to WT infection suggesting a defect in specific step between intermediate gene and morphogenesis. Because of the modest role of B1 in viral intermediate gene expression in U2OS cells, our data showed for the first time that a defective B1 kinase is likely associated with defects in late gene expression and morphogenesis. This novel function of B1 in late gene expression and morphogenesis is independent of its role in DNA replication, but the mechanism through which B1 regulates these events is not known.

Based on the results presented here, our model of the BAF-B1 axis during poxvirus infection is shown in figure 7.2. Early during infection, poxviruses express B1, which then inactivates BAF by phosphorylating residues at its N' terminus (11, 12). However, in the absence of an active B1, BAF blocks DNA replication and subsequently inhibits viral lifecycle (11). Our data showed that independently of its inhibitory effect on viral DNA replication, BAF also inhibits viral intermediate gene expression. Independently of the BAF-B1 axis, but viral intermediate gene is stimulated in a B1-dependent pathway.

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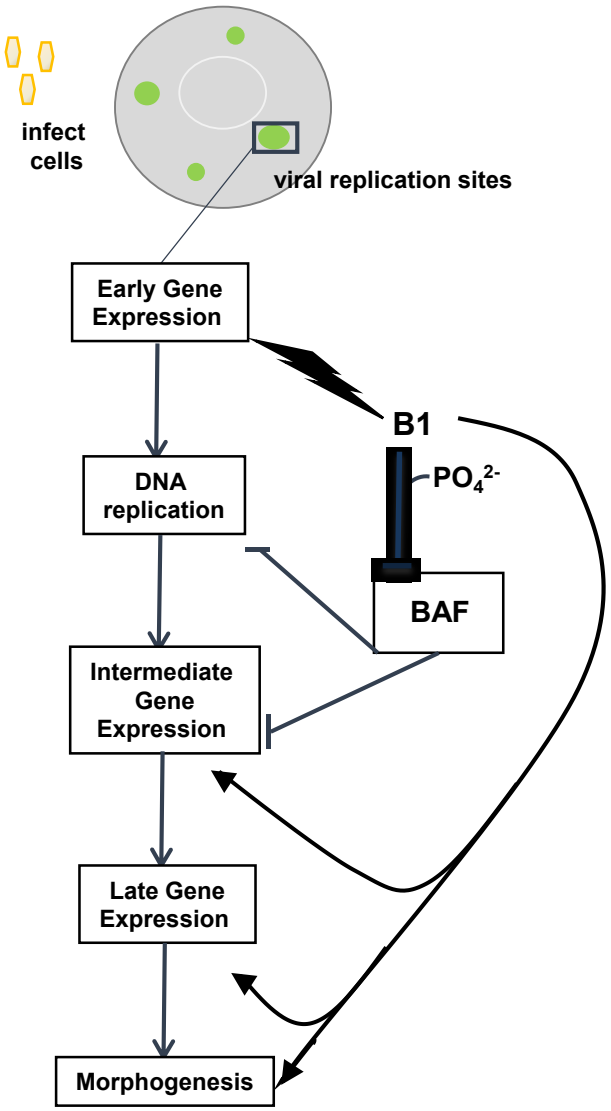

Figure 7.2. Proposed Model of how BAF-B1 axis regulates vaccinia virus lifecycle (adopted and modified from Dr. Wiebe).

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Further, our data showed that B1 regulates late gene expression and morphogenesis in BAF-independent manner. Therefore our data support a model in which B1 has multiples roles during productive infection.

Vaccinia morphogenesis is a multi-step process mediated by many proteins expressed during late gene expression (85, 143). It is unclear from our data which step (membrane formation, encapsidation of viral DNA, formation of IV, MV or the exit of WV) is malfunctioning. In addition, considering the number of proteins specifically involved in each step, it is not clear which protein is associated with the defect in the formation of new virions in relation to B1. Based on previous reports showing that viral phosphoproteins are essential players in virion morphogenesis (144, 255, 256), the following viral proteins can be suitable candidates to elucidate the potential B1-dependent defects leading to defects in late gene expression/morphogenesis.

The viral protein H5, is a credible candidate. Because of its role in viral DNA replication and late gene transcription (254) and being a substrate of B1 (253), it is a likely candidate to affect virion formation due to its role in late gene expression. Indeed, targeted mutagenesis study showed that H5 is involved in virus assembly (144). However, the late protein F10 is unlikely associated with the role of H5 in late gene expression, suggesting B1 may regulates it.

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Another viral protein is the late phosphoprotein F17, which is essential for the assembly of the mature virion. While a substrate of F10, no impact on virion morphogenesis was observed when F10 phosphorylation sites within the F17ORF were mutated (211). Another substrate of F10 is A14 which is, like F17, associated with virion morphogenesis, however mutational studies showed that the phosphorylation of the residue Ser85 of A14 is not essential for its role in morphogenesis (256). While F17 and A14 are not reported as substrates of B1, they may harbor other phosphorylation sites targeted by B1. Additional potential phosphoproteins that be targets of B1 include A17 (257), A11 (258), and L4 (259). Because most of the viral proteins involved with virion morphogenesis are participating as a multiprotein complex (85), it is possible that B1 regulates the assembly of these complexes.

7.4. The potential implications of the BAF-B1 axis on other cellular pathways

Based on our data showing the impacts of the BAF-B1 axis on poxvirus infection, I speculate that the BAF-B1 axis may inhibit or enhance cellular processes and pathways associated with BAF as well as those regulated by the cellular VRKs. The role of BAF during mitosis and its nuclear interacting partners, such as the core histone H3 and the linker histone H1.1 (20), may be disrupted during a poxviral infection as BAF is constantly phosphorylated by B1. In addition, the repression of Crx-mediated transcription by BAF (58) may also be abrogated as a consequence of the BAF-B1 interaction. In addition of BAF, B1 has other cellular substrates such as the ribosomal Sa and S2, p53 and JIP1

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(Banham et al., 1993; G. Beaud, Sharif, Topa-Massé, & Leader, 1994; C. R. Santos, Vega, Blanco, Barcia, & Lazo, 2004; C. R. Santos, Blanco, Sevilla, & Lazo, 2006; C. R. Santos et al., 2006)(188, 204, 249, 250, 260). Through its interaction with JIP-1, B1 regulates the MAPK and JNK signaling (204), which is already regulated by VRKs (Sandra Blanco, Marta Sanz-García, Claudio R. Santos, Pedro A. Lazo, 2008; Sevilla et al., 2004; F. M. Vega, Sevilla, & Lazo, 2004)(207, 209, 261). This supports the potential of B1 to regulate other cellular signaling pathways to enhance viral infection

Several cellular proteins act as effectors to initiate or enhance an intracellular signaling pathway to regulate cellular metabolism or respond to external stimuli, such as an infection (156, 157). Based on its anti-poxviral role, BAF may be associated with a specific cellular signaling pathway as an effector or as an intermediate protein (for example during a cross-talk between pathways). For instance, cellular proteins involved with DNA repair and damage responses (Ku86 and RPA32) were found at BAF-DNA complexes in the presence of a cytosolic dsDNA. This may suggest a possible direct implication of BAF with the cellular DNA repair/damage pathway. This suggestion is supported by the fact that during VACV infection, both Ku86 and RPA32 as well as BAF are not find at viral DNA replication sites. While VACV may employ other mechanism to target the cellular DNA repair/damage pathway, it is likely that by inactivating BAF, poxviruses affect also cellular pathways using BAF as an effector or intermediate protein.

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In summary this study has contributed to our understanding of how the interplay between BAF and B1 affects VACV lifecycle. The following finding are novel functions of BAF and B1 during VACV productive infection, and prior to this study they were not known or published:

1. DNA-binding and dimerization are essential for BAF's ability to detect cytoplasmic DNA and then form nucleoprotein complexes.
2. Several cellular proteins are also present at these BAF-dsDNA. BAF is required for the formation of these complexes; but certain cellular proteins interact with these complexes in a BAF-independent fashion.
3. In the absence of an active B1, BAF recruits emerin to DNA replication sites.
4. BAF is a transcriptional inhibitor in the absence of an active B1.
5. The absence of an active B1 is associated with defective virion formation, in part due to B1's role in regulating late gene expression.

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