THE PARVOVIRUS MINUTE VIRUS OF MICE MODULATES THE DNA DAMAGE RESPONSE TO FACILITATE VIRAL REPLICATION AND A PRE-MITOTIC CELL CYCLE BLOCK

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ABSTRACT

The DNA damage response (DDR) is a critical cellular network that affords cells the ability to repair DNA damage they have incurred from endogenous and exogenous sources. Recently, it has become appreciated that viruses, both DNA and RNA, can induce the DDR and have evolved the ability to interact with this ancient antiviral mechanism. Viruses can choose to inactivate or utilize this host response, which typically requires specific modulation for either case. Importantly, it has been shown that MVM *utilizes* this response to facilitate its replication in an ATM-dependent manner. MVM induces a DDR-dependent pre-mitotic, G2/M cell cycle block *via* activation of the checkpoint kinase, Chk2, and depletion of the RNA and protein of the key mitotic cyclin, cyclin B1. Unexpectedly, this cell cycle block was shown to be p21- and Chk1-independent.

MVM infection results in the recruitment and activation of numerous DDR proteins including Chk2, RPA32 and p53. Upon activation, *via* phosphorylation, p53, a critical tumor suppressor, is known to transactivate several hundred genes including the well-characterized CDK inhibitor, p21. However, previous work demonstrated a sustained, proteasome-dependent loss of p21 during infection, which was required for efficient replication. This depletion of p21 during infection was unexpected, given the activation of p53 during infection, and because p21 is a known, potent cell cycle inhibitor.

We investigated the loss of p21 during infection and found that siRNA knockdown of specific components of the $CRL4^{Cdt2}$ E3 ubiquitin ligase—Cul4A, DDB1 and

Cdt2—stabilized p21 during MVM infection. Importantly, siRNA knockdown of specific components of $CRL4^{Cdt2}$ reduced viral replication. DDB1 and Cdt2, the adapter protein and substrate recognition factor of the $CRL4^{Cdt2}$, respectively, were recruited to viral replication factories, termed autonomous parvovirus-associated replication (APAR) bodies, suggesting that MVM may be hijacking this important E3 ubiquitin ligase. The recruitment and utilization of this ligase is likely specific, as the APC/C^{CDC20} E3 ubiquitin ligase, which also targets p21 for proteasome-dependent degradation, was not recruited to APAR bodies nor did siRNA knockdown of specific components of this ligase stabilize p21 during infection. Taken together, these results suggest that MVM specifically utilizes the $CRL4^{Cdt2}$ E3 ubiquitin ligase to target p21 for degradation and that the activity of this E3 ubiquitin ligase is required for efficient MVM replication.

The requirements for the activity of $CRL4^{Cdt2}$ gave us a hint as to *why* MVM would target p21 for degradation. It has been shown that $CRL4^{Cdt2}$ activity requires interaction with PCNA, a DNA polymerase δ cofactor, and chromatin. Importantly, p21 is an inhibitor of PCNA activity. As PCNA and DNA polymerase δ are known to be required for parvoviral rolling hairpin replication, we hypothesized that MVM must target p21 for depletion to allow for PCNA activity, which is required for viral replication. p21 mutants that were unable to interact with either $CRL4^{Cdt2}$ or PCNA were stable during MVM infection, suggesting that p21 needed to interact with both the E3 ubiquitin ligase and PCNA for degradation during MVM infection. We next constructed p21 mutants that were resistant to ubiquitination, by mutating the seven lysine residues in p21 to arginine, and maintained or lost their ability to interact with PCNA. Importantly, a stable

p21 mutant that interacted with PCNA resulted in the significant depletion of MVM replication whereas a stable p21 mutant that no longer interacted with PCNA had no effect on replication. Taken together, this data suggests that MVM co-opts a cellular E3 ubiquitin ligase to target the CDK inhibitor p21 for degradation, which is required to allow the PCNA activity that MVM needs for efficient replication.

To induce a pre-mitotic G2/M cell cycle block, MVM depletes the key mitotic cyclin, cyclin B1, which is preceded by the loss of its encoding RNA. We next sought to determine *how* MVM programs the depletion of cyclin B1 RNA. Initial studies indicated a loss of cyclin B1 RNA between 18 and 24 hours post-infection in a NS2-independent manner. This loss of RNA and protein was seen in both human and murine cells lines, suggesting that programming the depletion of cyclin B1 is a critical hallmark of MVM infection. Interestingly, the viral mechanism which targets cyclin B1 could overcome the effects of an exogenous DNA damaging agent known to induce cyclin B1 levels. The stability of cyclin B1 RNA during MVM infection is comparable to doxorubicin-treated cells, while the production of nascent cyclin B1 RNA is substantially depleted.

Importantly, the chromatin landscape of the cyclin B1 promoter during MVM infection was consistent with an open conformation, yet significantly lower levels of RNA polymerase II (RNA pol II) were found to occupy the cyclin B1 gene. The NF-Y transcription factor and B-myb, a component of MuvB-B-Myb (MMB) complex, were both found to bind the cyclin B1 promoter during infection. However, the key G2/M transcription factor, FoxM1, was found to occupy the cyclin B1 promoter at significantly lower levels in MVM infected cells compared to mock- or doxorubicin-treated cells.

FoxM1, which requires hyperphosphorylation to activate its transcriptional activity, was found to have lower levels of phosphorylation during MVM infection, compared to mock- or doxorubicin-treated cells. Reconstitution of FoxM1 to the cyclin B1 promoter, *via* catalytically inactive Cas9 fused to the FoxM1 transactivation domain, upregulated cyclin B1 RNA and protein during MVM infection. Taken together, these results suggest that MVM prevents the activation and binding of the critical transcription factor FoxM1 to the cyclin B1 promoter, thereby reducing RNA pol II transcriptional activity and the production of nascent cyclin B1 RNA and its encoded protein, ultimately facilitating establishment of a pre-mitotic G2/M block.

An important RNA-binding protein, HuR, known to regulate two cyclins important for MVM infection, cyclin A and cyclin B1, was also investigated. We observed the predominantly nuclear HuR was heavily relocalized to the cytoplasm during MVM infection. Ectopic expression of NS1, but not NS2, resulted in the cytoplasmic relocalization of HuR in a Crm1-independent manner. Importantly, siRNA knockdown of HuR during MVM infection resulted in a further reduction of cyclin B1 protein, but not cyclin B1 RNA. This data suggested that HuR may promote the translation of specific RNAs during MVM infection. HuR was also shown to bind MVM RNA. While more work needs to be undertaken to fully understand the ramifications of this interaction, HuR is likely to be an important regulator of MVM RNA stability or translation.

Taken together, these observations suggest that MVM expertly modulates the DDR and other components of the cellular machinery to ensure an environment conducive to facilitation of viral replication. To establish this environment, MVM targets

several core cellular mechanisms including proteasome-mediated protein degradation, RNA transcription, RNA translation and subcellular localization of cellular proteins.

1 INTRODUCTION

1.1 PARVOVIRUSES

Parvoviruses, which derive their name from the Latin word for small, *parvus,* are genetically and physically minute, yet incredibly complex single-stranded DNA viruses that employ a multitude of mechanisms to ensure maximum usage of genetic coding capacity and optimal gene expression, to modulate cellular processes to facilitate establishment of an environment conducive to viral replication, and to co-opt cellular proteins to replicate its genome. The family *Parvoviridae* is divided into two subfamilies (illustrated in figure 1-1): the *Parvovirinae*, which infect vertebrates, and the *Densovirinae*, which infect invertebrates (46). The *Parvovirinae* subfamily contains eight genera (recently expanded from five): the heterotelomeric *Protoparvovirus*, *Amdoparvovirus*, *Bocaparvovirus* and *Aveparvovirus* and the homotelomeric *Dependoparvovirus*, *Erythroparvovirus*, *Tetraparvovirus* and *Copiparvovirus* (87).

To date, two parvoviruses have been utilized for human disease treatments. First, certain serotypes of adeno-associated virus (AAV), a member of the helper virus-dependent *Dependoviruses*, have been engineered for use as gene therapy vectors for a wide variety of target tissues and diseases (179, 375, 420). Productive AAV infection and replication requires co-infection with a larger DNA virus such as Herpes Simplex Virus – 1 (HSV-1) or Adenovirus (166). Interestingly, the requirement for helper virus co-infection can be abrogated by addition of certain genotoxic DNA damaging agents such as hydroxyurea, topoisomerase inhibitors or UV irradiation (437). AAV infection lacking either co-infection with a helper virus or presence of a DNA damaging agent results in

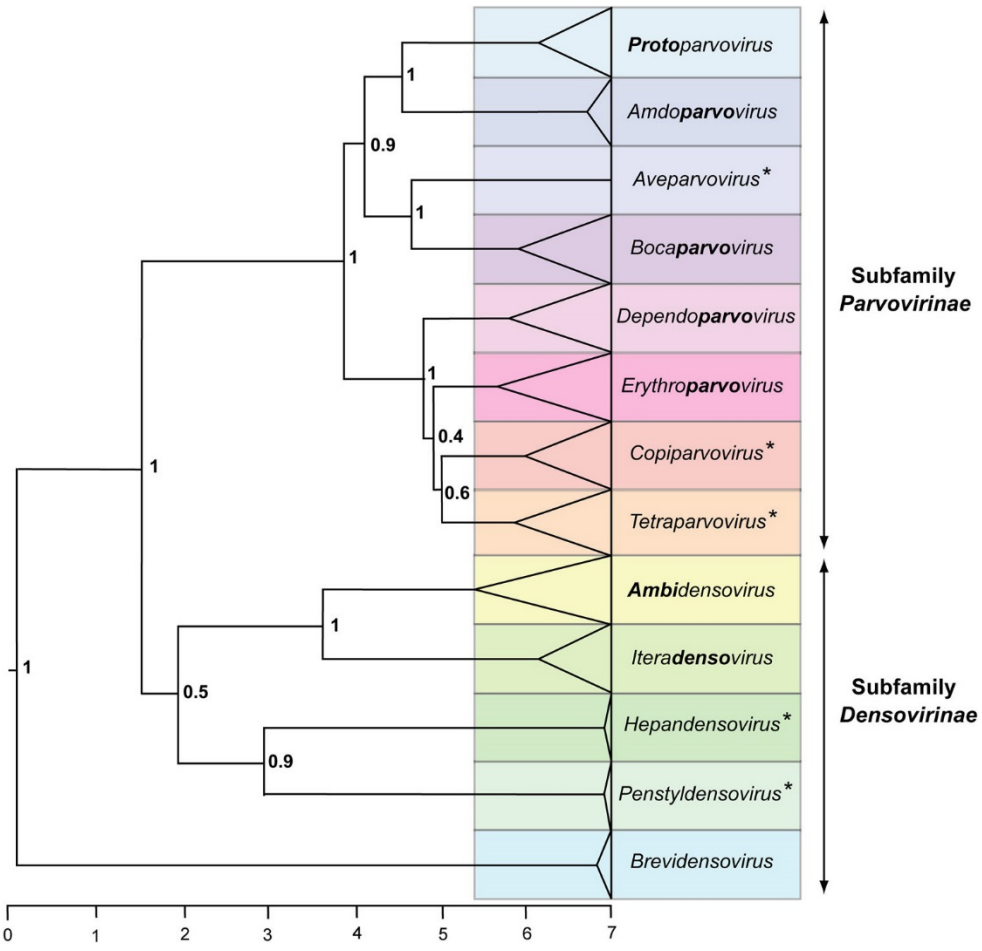
latency and integration, primarily into chromosome 19, which allows for long-term expression of a transgene delivered *via* AAV (122, 191). Second, H-1 parvovirus, a member of the autonomous *Protoparvoviruses*, capable of completing their viral life cycle independent of co-infection with other viruses, was recently utilized in clinical trials as an oncolytic therapy (87, 277). Minute Virus of Mice (MVM), the prototype virus of the *Protoparvovirus* genera, will be discussed in more detail later.

Parvoviruses package their genome in incredibly rugged, non-enveloped virions 18-28 nm in diameter with T=1 symmetry (88). Viral particles are constructed from the 2-4 virion protein species, depending upon the virus, termed VP1-4 (88). The ssDNA genome of Parvoviruses is flanked by imperfect palindromic hairpin termini that serve as origins of replication (102). These palindromic termini can be homotelomeric, identical at either end and forming part of the inverted terminal repeats (ITRs), or heterotelomeric, possessing disparate ends, with both types serving as replications of origin for the rolling hairpin synthesis scheme employed by parvoviruses to replicate their genomes. In addition to its origin serving role, there is evidence that suggests that the hairpin's telomeric structure influences the ratio of ssDNA packaging polarity (percent positive: negative strands in virion) (90, 98).

To replicate its genome and generate ssDNA for packaging, an incredibly complex, uni-directional DNA polymerase δ–dependent rolling hairpin replication mechanism, first proposed in 1976, is utilized (407). Briefly, the incoming MVM genome is extended into a duplex, covalently closed monomeric form, which is then used to

Figure 1-1: Phylogenetic tree of the genera within the Family *Parvoviridae*.

Phylogenetic analysis was performed based on amino acid homology of the conserved helicase domain, containing the AAA+ ATPase residues, of the main replicative non-structural protein, NS1. Asterisks indicate newly named genera. Adapted from (87).

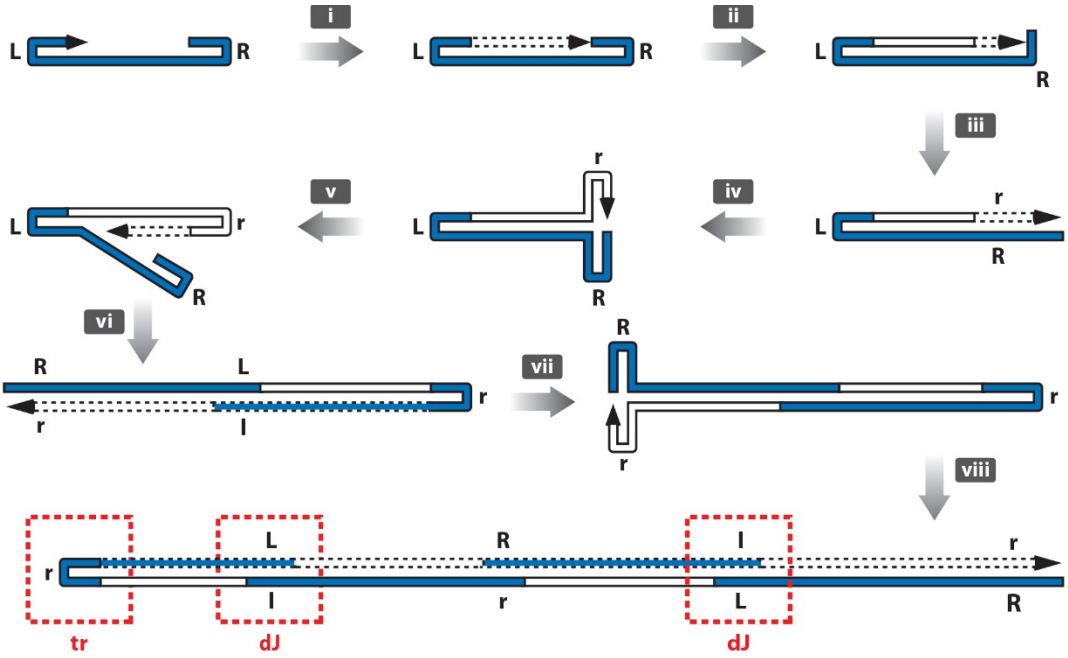

Protoparvovirus
Amdoparvovirus
Aveparvovirus*
Bocaparvovirus
Dependoparvovirus
Erythroparvovirus
Copiparvovirus*
Tetraparvovirus*
Ambidensovirus
Iteradensovirus
Hepandensovirus*
Penstyldensovirus*
Brevidensovirus
Subfamily Parvovirinae
Subfamily Densovirinae
1
0.9
1
1
1
0.4
0.6
1
1
0.5
0.9
0 1 2 3 4 5 6 7

generate the viral non-structural protein, NS1 (89, 93, 101, 102, 257). Replication beyond this initial step requires further NS1 expression and subsequent phosphorylation of NS1 to bind the origin and generate a nick in the covalently closed right-end hairpin (24). This step also requires the cellular host protein from the high mobility group 1/2 family (99). After nicking, NS1 remains covalently attached to the 5' end of the nicked DNA and recruits RPA, which appears to be a rate-limiting step in the processivity of the single-strand displacement mechanism (75). Next, a complex series of melting and re-annealing steps, which require the activity of NS1, replicate the right-end hairpin, and utilize either terminal or junction resolution, for right-end or left-end hairpin resolution, respectively, to generate extended form, duplex dimer and then tetramer concatameric molecules (89). Unit length genomes are then excised from the concatemers *via* site and strand-specific nicks (102).

To ensure maximum utilization of their limited coding capacity parvoviruses rely on varying combinations of the usage of 1-3 promoters, alternative splicing and alternative polyadenylation (90, 101, 102). These transcripts are translated into a variety of non-structural or structural proteins that differ between the genera, illustrating the diversity among the *Parvoviridae* family members. For example, the autonomous parvoviruses at a minimum generate the non-structural protein NS1, but members of the *Bocaparvovirus* genera also generate NP1, a viral protein required for RNA processing (132, 402), and Amdoparvoviruses encode three non-structural proteins (187). Although the proteins generated by parvoviruses are not identical, in general, the basic

Figure 1-2: Rolling Hairpin Replication

Illustration of the mechanism of rolling hairpin replication utilized by parvoviruses for replication. The process is characterized by at least eight separate steps. Blue DNA represents incoming, template viral genome and white DNA represents the nascent, replicating DNA. The dotted lines and arrows signify the directionality of the replication fork. The red dotted boxes designate the areas that generate the hairpins including the turnaround form (tr) that forms the right-end palindrome and the dimer junction (dJ) that forms the left-end palindrome. Adapted from (102).


i
ii
iii
iv
v
vi
vii
viii
L
R
r
l
tr
dJ
dJ

necessities are well conserved (i.e., NS1 nicking and helicase functions, VP1 PLA2 activity, VP2 and VP3 forming the capsid).

1.2 MINUTE VIRUS OF MICE

Minute virus of mice (MVM) is the prototype virus of the *Protoparvovirus* genera and is the most widely studied virus of that genera. MVM exists as two strains: the lymphotropic strain, MVMi, which infects murine lymphocytes and can cause runting syndrome as a result of mild immune deficiency, and the prototype, fibrotropic strain, MVMp, which infects murine fibroblasts, primarily affects neonates and can cause intestinal hemorrhaging (47, 199, 220, 373). Wildtype MVMi cannot infect murine fibroblasts whereas MVMp cannot infect murine lymphocytes (282). Although the major viral determinant of host range for MVMi or MVMp is the capsid, the non-structural protein, NS2, also plays a role in supporting host-specific replication (106, 282). A single nucleotide change in the MVMi large-intron 3' splice site allows for the accumulation R2 transcripts, and thus the non-structural protein NS2, and is sufficient to allow replication of MVMi in fibroblasts (69).

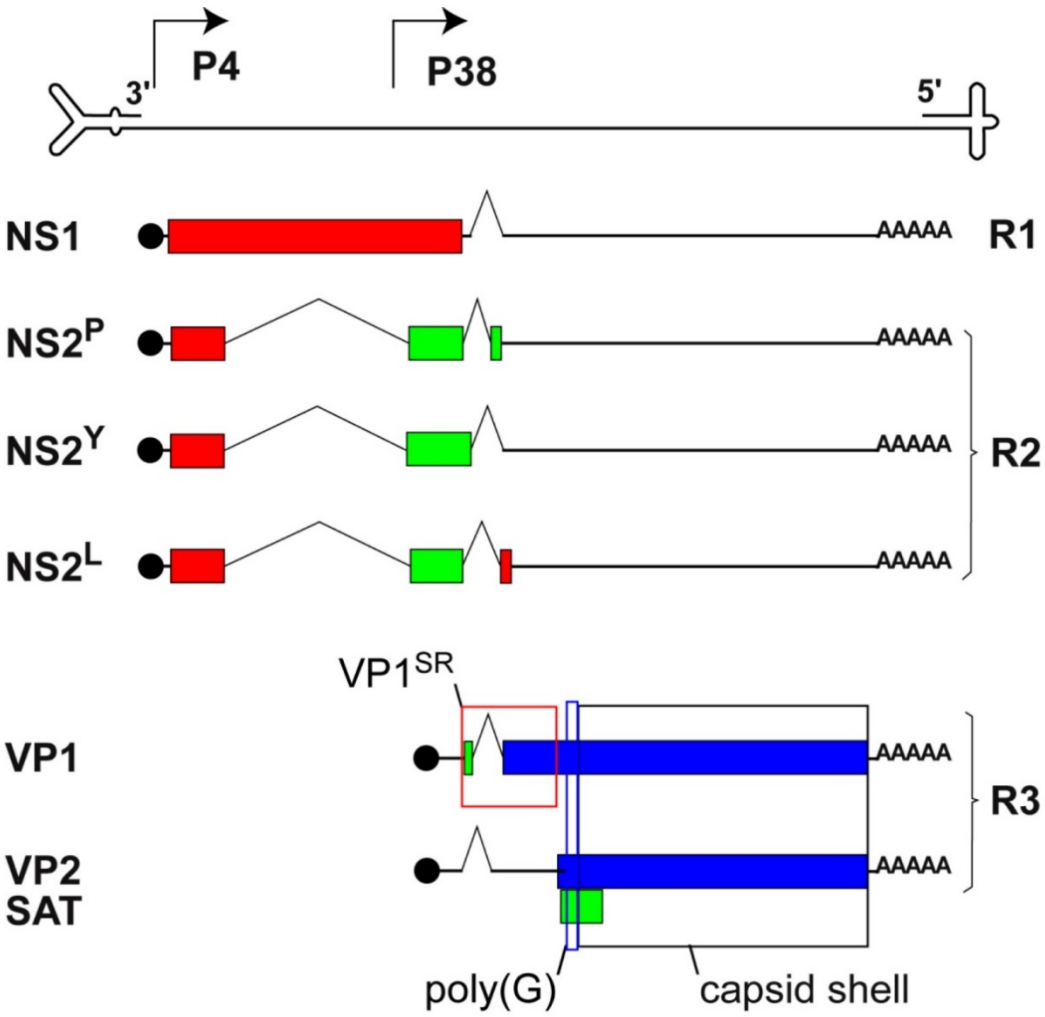
GENETIC ORGANIZATION OF MVM

The MVM genome is approximately 5,000 nucleotides in length and is flanked by disparate palindromic telomere hairpins, which serve as origins for replication. As with all parvoviruses, MVM utilizes several strategies to maximize its coding potential. The genetic map of MVM, illustrated in Figure 1-3, outlines many of these features. RNA transcription initiates at two promoters, designated P4 and P38, which generate two major pre-mRNAS ultimately generating, *via* intron splicing, three transcripts: R1 (4.8 kb in size), R2 (3.3 kb in size) and R3 (3.0 kb in size) (341). MVM mRNAs are very stable (>6

hours) and can make up to 25 percent of total RNA at late times during infection (341, 380). All three transcripts polyadenylate at a single polyadenylation site, although there are at least four canonical polyadenylation signals in the MVMp genome at the right-hand end (22, 79). RNA transcription during infection is temporal in nature with the P4 promoter being activated first, in early S phase, once the cells have passed an early restriction point, which requires an E2F motif in the proximal region of P4 (113, 114). Once activated, the P4 promoter generates the R1 and R2 transcripts. R1 is the unspliced transcript whereas splicing of the large intron results in R2 transcripts, encoding NS1 and NS2, respectively (94). Further splicing of the small intron in R2 transcripts generates three isoforms of the NS2 protein (78, 380). An intronic splicing enhancer (ISE), which binds the cellular RNA-processing proteins TIA-1 and TIAR, determines the rate of splicing of the large intron ultimately governing the relative ratios of R1:R2 transcripts (70, 450). There is approximately twice as much R2 as R1 in a cell during MVM infection (380). Sufficient expression of NS1 is required for NS1 to bind and transactivate the p38 promoter, which generates the R3 transcript, ultimately encoding for the capsid proteins VP1-3 (262, 264). The R3 pre-mRNA is alternatively spliced at the small intron to generate two mRNAs, which encode VP1 or VP2 (206, 232). During the late stages of infection, R3 transcripts accumulate in greater amounts than the combined P4 transcripts due to an increased frequency of initiation of the p38 promoter (380).

Figure 1-3: Transcriptional Map of MVM

The MVM genome, indicated at the top, possesses two disparate hairpins and two viral promoters, P4 and P38. MVM generates three classes of mRNA transcripts from two pre-mRNAs. Initiating at p4 and splicing only the small intron generates the mRNA (R1) encoding the main replicative protein, NS1. Splicing of the large intron (R2) generates NS2, which is further processed by splicing of the small intron into three isoforms. NS1 transactivation of the p38 promoter allows the mRNAs (R3) encoding for the capsid proteins, VP1 and VP2, to be generated. VP3 is generated by post-translational cleavage of VP2. SAT, a small alternatively translated protein, is also generated from R3 transcripts. SAT's role in MVM infection is not fully understood. Adapted from (102).


P4
P38
3'
5'
NS1
AAAAA
R1
NS2P
AAAAA
NS2Y
AAAAA
R2
NS2L
AAAAA
VP1SR
VP1
AAAAA
R3
VP2
SAT
AAAAA
poly(G)
capsid shell

NS1

NS1 is an 83 kDa non-structural protein of MVM and is absolutely essential for viral replication in both murine and human cells (322, 323). NS1 is a stable protein with a half-life greater than 6 hours, which results in a massive accumulation of the protein during infection (320). NS1 predominantly resides and accumulates within the nucleus and exhibits extensive phosphorylation, which is required for a variety of functions (100, 377). A triple lysine motif in residues 214-216 is critical for nuclear localization of NS1 during infection (327).

It has been proposed that temporal phosphorylation of NS1 regulates the progression of many events in MVM replication (324). NS1 can be phosphorylated *in vitro* by members of the kinase families casein kinase II (CKII), protein kinase A (PKA) and protein kinase C (PKC) (322, 323). However, only PKC family members PKCλ and PKCη have been shown to modulate distinct NS1 functions *in vivo* as a result of phosphorylation (233, 324). Specifically, PKCλ phosphorylates NS1 at Ser-283, Thr-403, Thr-435, Ser-473, Thr-585 and Ser-588 *in vivo* (84, 85, 107). To initiate viral replication, NS1, *via* its DNA binding domain, binds an (ACCA)2 motif in the palindromic hairpin origins (91, 301, 432). NS1 can bind non-MVM DNA if it contains (ACCA)2-3 motifs (301), yet it is unclear if NS1 does indeed bind these motifs in cellular DNA nor if nicking can occur (330). It is interesting to speculate that NS1 binding to cellular (ACCA)2-3 motifs and nicking cellular DNA could be a primary or ongoing source of DNA damage during infection. NS1 also blocks cellular DNA replication, which could stall replication forks and cause collisions between the replication and transcription machinery resulting in cellular

DNA damage (176, 177, 330). PKC phosphorylation of NS1 at Thr-435 and Ser-473 allows for NS1 to unwind the viral origin of replication and nick the DNA (119, 321, 324). NS1 requires oligomerization for activation of its helicase function, utilizing its NTP-binding domain to achieve formation of higher order oligomer, yet phosphorylation by PKC remains critical for helicase activity (115, 320, 327, 348). Importantly, a cellular protein belonging to the KDWK family, parvovirus initiation factor (PIF), must be recruited and form a ternary complex with NS1 and the origin (OriL) for nicking to occur (72, 74). The cellular protein high mobility group-1 (HMG-1) is required for initiation of replication at the right-hand end of the genome (92, 99).

Another critical function of NS1 is transactivation of the p38 promoter, allowing for the transcription of the R3 transcripts and generation of the structural capsid proteins (262, 264). NS1 binds (ACCA)2-3 sequences, in an ATPase-dependent manner, upstream of the p38 promoter in the trans-activation region (TAR), which is critical for efficient transactivation of the p38 promoter (73, 262). Also located upstream of the p38 promoter, GC and TATA boxes are critical for binding of general transcription factor SP1 and recruitment of transcriptional machinery, respectively (11, 151). Importantly, NS1 interacts with both SP1 and general transcriptional machinery TBP and TFIIA (228, 263). NS1 is a critical player in an efficient MVM infection, relying on its multiple domains for oligomerization, DNA unwinding, DNA nicking and promoter transactivation.

Interestingly, NS1 is also capable of transactivating the cellular *c-erbA-1* gene, which encodes the thyroid hormone (T3) receptor (THR) α, in FREJ4 cells (415). Although

the physiological significance of this finding is still not completely understood, it has been speculated that this upregulation causes cytopathic effects (CPE) (414). Ectopic expression of NS1 is also capable of inducing CPE *via* a not completely understood mechanism(s) including altering phosphorylation of cellular proteins, possibly beta-tubulin and other cytoskeletal proteins, likely by modulating CKII activity (17, 325, 326). As with other functions of NS1, phosphorylation plays a key role in this activity with residues Thr-585 and Ser-588 appearing to be required for NS1 induced CPE (107). Possibly contributing further to CPE and cellular death, ectopic NS1 expression results in a transient S phase arrest, dependent upon p53 accumulation, which transitions into a permanent G2 arrest, dependent upon p53 and p21 accumulation (10, 331). Although the cause of cellular death following NS1-induced CPE and G2 arrest is not understood, NS1 expression induces p53-independent apoptosis in transformed rat fibroblasts (291).

NS2

NS2 is a 25 kDa non-structural protein with a required, yet not fully understood role during replication. However, this role should not be understated as NS2 expression is critical for replication of MVMp in murine cells (56, 69). NS2-null viruses are capable of establishing very early-stage APAR bodies (Class I), suggesting that early steps in initiation of infection are not adversely affected (367). However, by 6 hours post-infection the block to APAR body progression is apparent, confirming previous reports that NS2 expression is critical to productive infection (69, 309, 367). NS2 also plays a role in ensuring efficient translation of viral mRNA (310). Intriguingly, NS2 is not

required for MVM infection in human NB324K cells, suggesting that NS2 may play a role in abrogating the function of a host restriction factor in murine but not human cells (309). For reasons not yet fully elucidated, a Bocaparvovirus ancillary protein, NP1 can partially complement specific defects resulting from NS2 depletion during MVM infection (287).

NS2 is generated from R2 transcripts, owing to splicing of the large intron, and shares an 84 amino acid N-terminal region with NS1 (320). This shared region is required for interaction of both NS1 and NS2 with survival motor neuron (SMN), a nuclear protein involved in the assembly of snRNPs, essential components of spliceosomal machinery (445, 446). The physiological relevance of these interactions is not yet known. NS2 contains a nuclear export signal (NES), which allows it to shuttle from the nucleus to the cytoplasm, presumably transporting cargo important to facilitate viral replication (124). Consistent with this shuttling property, NS2 was shown to very strongly interact with the host cell protein exportin 1, also known as chromosomal maintenance 1 (Crm1) (40). Although the full role of NS2 during MVM infection has yet to be elucidated, NS2 does seem to be required for nuclear egress of progeny virions (124, 288). It has also been proposed that in an MVMi murine *in vivo* infection, NS2 sequesters Crm1 in the nucleus of the infected cell, which increases the general fitness of the virus in a natural host (261). NS2 has also been shown to interact with 14-3-3 protein family members, possibly the epsilon, beta or zeta isoforms (45). While we do not yet understand how this interaction affects MVM infection, we can speculate from known 14-3-3 functions that cell cycle regulation, DNA damage response modulation

and cell death induction, all known critical hallmarks of parvovirus infection, may be involved (286).

The small intron located in the middle of the genome, as with all other MVM transcripts, is spliced from the P4-generated pre-mRNA along with the large intron to form the NS2-encoding R2 transcript (152, 168, 169, 353, 449). Alternative splicing of the small intron generates three NS1 isoforms that vary at their C-termini (78, 95). These isoforms, NS2-P (major form), NS2-Y and NS2-L (rare form), are expressed in a 5:1:<1 ratio (366). While the isoforms are expressed in different molar amounts, there are no known differences in their localization, phosphorylation status or stability (95). Altering the expression levels of NS2-P or NS2-Y can depress NS1 accumulation during infection (366). Overall, the NS2-P isoform appears to be the most critical to infection while NS2-Y modulates a NS2 dosage effect (366).

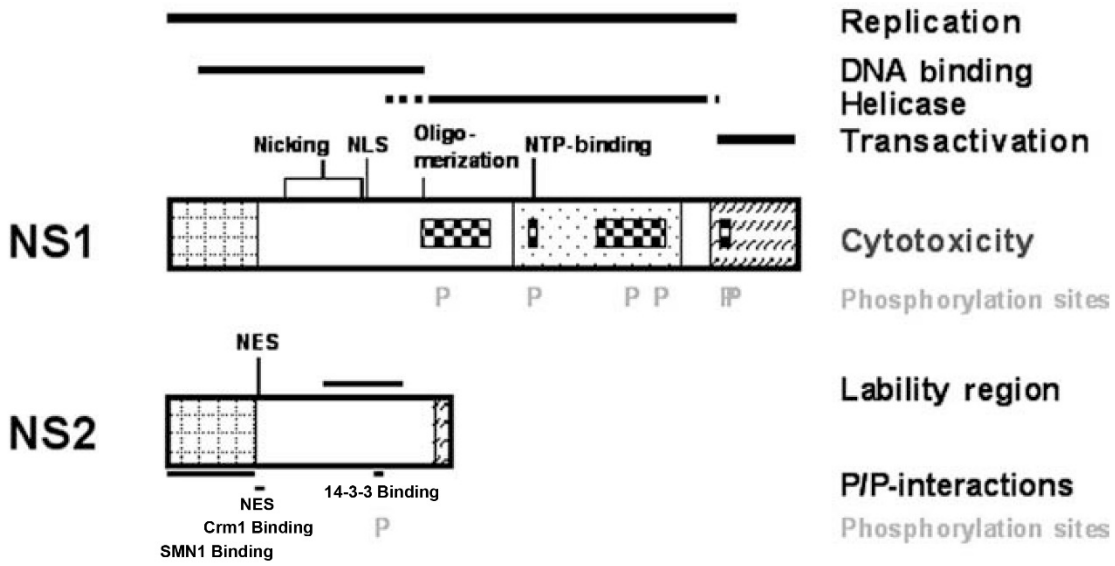
Like NS1, NS2 exists in phosphorylated and unphosphorylated forms and can be phosphorylated at multiple residues by multiple cellular kinases (320). Phosphorylation is required for NS2 interaction with 14-3-3 proteins while the unphosphorylated form interacts with Crm1 (124). While NS1 is an extremely stable protein, NS2 contains a different lability region, which confers a relatively short half-life of approximately 90 minutes (289, 380). Surprisingly, degradation of NS2 occurs in a proteasome-dependent, ubiquitin-independent manner (289).

Figure 1-4: Functional domains of MVM NS1 and NS2

Map of the domains of the non-structural proteins of MVM, NS1 (672 amino acids) and NS2 (188 amino acids). The black lines above each protein schematic indicate the placement of specific domains within NS1 and NS2. The cross-hatched boxes at the N-terminus of each indicate the 84 amino acids shared between NS1 and NS2.

NS1 contains replication, DNA binding, helicase and transactivation domains. The nicking, NLS, oligomerization and NTP (ATPase) –binding domains are also indicated directly above each schematic. Each domain is elaborated on within the text. The six residues in NS1 known to be phosphorylated are indicated with a light grey P (Ser-283, Thr-403, Thr-435, Ser-473, Thr-585 and Ser-588). The checkered boxes indicate domains implicated in causing cytopathic effects. The dotted box represents the helicase domain. The hatched box indicates the region required for p38 transactivation.

NS2 contains a lability domain, indicated above the schematic, which confers a much shorter (90 minute) half-life to NS2 than the very stable NS1. NS2 contains a nuclear export signal (NES). NS2 is phosphorylated at Thr-149, which is indicated with the light gray P. NS2 also has several regions, indicated below the schematic, that are important for protein-protein interactions with SMN, 14-3-3 proteins and Crm1. The hatched box at the C-terminus of NS2 indicates the alternatively spliced region which generates the three NS2 isoforms, NS2-Y, NS2-P and NS2-L. Adapted from (320).


Replication
DNA binding
Helicase
Transactivation
Nicking
NLS
Oligo-merization
NTP-binding
NS1
Cytotoxicity
P
P
P P
PP
Phosphorylation sites
NES
NS2
Lability region
P/P-interactions
NES
14-3-3 Binding
Crm1 Binding
SMN1 Binding
P
Phosphorylation sites

CAPSID PROTEINS

MVM capsids are incredibly stable, T=1 icosahedral viral particles assembled from 60 copies of the three structural viral proteins, VP1 (83 kDa), VP2 (64 kDa) and VP3 (61 kDa) (88). VP1 and VP2 are generated from alternative splicing of the R3 transcript produced by the NS1 transactivated p38 promoter (232). VP3 is generated from the post-translational cleavage of the N-terminus of VP2 (130, 406). VP2 and VP3 compose the majority of protein species utilized to form the functional capsid. However, VP2 is capable of binding ssDNA and forming full capsid particles when expressed alone, though these particles are not infectious (431). Although controversial, there is some evidence that cell cycle phase may affect assembly of parvovirus nuclear capsids (153).

Comprising a minor component of the fully formed capsid, VP1 is critical for infectivity of the particle (259, 411). The N-terminus of VP1 is externalized when the capsid is within the endosome due to pH changes (259, 275, 365). The N-terminus of VP1 contains a nuclear localization signal (NLS), presumably to allow it to locate and enter the nucleus, and a catalytic phospholipase A2 (PLA2) domain, which is critical for escaping the endosome after cellular entry and trafficking (130, 131, 447).

MVM REPLICATION AND LIFE CYCLE

Like all viruses, MVM's journey through its lifecycle first requires entry into a host cell. MVM capsids bind sialic acid to gain entry and infection can be prevented by treatment of cells with neuraminidase (97). The precise sialic acid bearing receptor bound by MVM is unknown, but glycan array analysis revealed that MVMp prefers to

bind oligosaccharides with α2-3 linkages, specifically 3'SiaLN-LN-LN and 3'SiaLN-LN motifs (312, 416). After binding, MVM is taken up in cells by receptor-mediated endocytosis, likely mediated by clathrin and/or calveolin, which can be prevented by treatment with bafilomycin A1 (145, 336, 364). Once the endosome traffics further into the cell towards the nucleus, the lower pH within the endosome causes MVM's capsid to rearrange and expose VP1's PLA2 domain (131, 447). The exact mechanism of escape from the endosome is not yet clear, however experiments utilizing alpha-sarcin or dextrans with canine parvovirus (CPV) suggest that the endosome is not completely disrupted by VP1 PLA2 activity (336, 401). Once the capsid enters the cytoplasm the ensuing events are less clear. It is thought that endocytic exit occurs in a perinuclear region, but whether active microtubule-associated dynein transport or passive movement aids in perinuclear localization and nuclear import is controversial (271, 400, 401, 417). It is also possible that the vimentin intermediate filament network is involved in endosomal trafficking and escape by MVM (133).

Once at the nuclear periphery, the mechanism of entry is not yet fully elucidated and remains controversial. Microinjection experiments suggest that presence of the MVM capsid at the nuclear envelope causes distinct breaks in the nuclear membrane, possibly by host cell caspases, which could be used to gain access (81, 82). More detailed studies suggest that the MVM capsid attaches directly to the nuclear pore complex *via* nucleoporins and requires the activity of PKCα, CDK1/2 and caspase-3 to facilitate nuclear entry (347). Once inside the cell, the MVM genome is released from

the capsid *via* an unknown process, which does not require full capsid disassembly (363).

After reaching the nucleus and ejecting its DNA from the capsid, the virus must wait for the cell to complete and re-enter the cell cycle, until entry into S phase (433). Once past an early S phase restriction point and after Cyclin A activation, MVM extends its ssDNA genome into the duplex form that serves as a template for replication and transcription and generates NS1, which is required for all subsequent steps in replication as described earlier (31, 102). This microenvironment of NS1 and viral DNA replication eventually becomes a viral replication factory, termed an autonomous parvovirus-associated replication (APAR) body (32). APAR bodies develop through stages defined as 0 to IV, progressing from a cloud of nuclear puncta to intensely stained, large nuclear foci, which progressively take over the entire nucleus (367). As the infection progresses, an ATM-dependent DNA damage response (DDR) is activated as evidenced by increasing levels of the DNA damage marker γ-H2AX and tumor suppressor p53, and the phosphorylation and activation of ATM, Chk2, p53 and RPA32, among others (7). Interestingly, all of these proteins, as well as many other cellular proteins including DDR, cellular replication and cell cycle proteins, and an E3-ubiquitin ligase, are recruited to APAR bodies (7-10, 32). This data suggests that MVM is hijacking or co-opting many different types of cellular proteins for its own use, to prevent cellular usage *via* sequestration or both.

1.3 DNA DAMAGE AND REPAIR

It is of critical importance that every organism safeguards its genome from unwanted modifications, as this is the information passed onto daughter cells and utilized for RNA transcription. Cells face daily insults from endogenous and exogenous sources of DNA damage (See Figure 1-5) (175). Endogenous sources typically generate DNA damage at a higher rate than exogenous sources, estimated at up to 100,000 endogenous DNA damage events per cell per day (185). Endogenous DNA damage can arise *via* spontaneous reactions, which occur due to dNTP misincorporation during DNA replication or interconversion, loss, or modification of DNA bases *via* deamination, depurination, or alkylation, respectively (27, 76, 252). Reactive oxygen species (ROS), generated as a natural byproduct of metabolism, can oxidize DNA bases and create DNA breaks (76, 185). APOBEC proteins, which act as cytidine deaminases, provide cells with a method to resist retroviral infection and mobile retroelements by introducing C-to-U base substitutions (167, 374). Cells can also harness the DDR to generate the crucial diversity of B and T cell immune repertoires *via* VDJ recombination (111). As would be expected, dysregulation of APOBEC family proteins or VDJ recombination can result in genomic instability and cancer.

Exogenous DNA damage occurs as a result of environmental exposure to physical or chemical genotoxic agents. Ionizing radiation (IR) and ultraviolet light (UV) are physical sources of DNA damage (175). IR can be introduced in the form of cosmic radiation or X-rays, inducing oxidation of DNA bases and resulting in single-stranded and double-stranded DNA breaks (SSBs and DSBs, respectively) (76). UV, primarily from

sunlight, can generate cyclobutane pyrimidine dimers and (6-4) photoproducts (76, 175, 185). Exposure to certain chemicals, especially those which crosslink or intercalate DNA, topoisomerase inhibitors, illegal recreational drugs (26, 383), pharmaceuticals (i.e. chemotherapy agents) or radioactivity can also result in DNA damage (175). Perhaps the most easily preventable exposure to an exogenous DNA damaging agent, other than excessive sunlight, is nicotine containing tobacco, which has been shown in many systems to induce oxidative damage in the form of 8-hydroxydeoxyguanosine (20).

Over the past 15 years, it has become increasingly evident and well-studied that certain viruses induce a DDR, which they must negotiate to facilitate their replication (251, 265, 428). It is also interesting to speculate, as many have, that the DDR may have served as an ancient antiviral detection and suppression mechanism. The viruses that interact with the DDR tend to be DNA viruses, but there are some cases of RNA viruses, even those that remain entirely cytoplasmic, interacting with or inducing a DDR as well (368). There are multiple sources capable of generating DNA damage during viral infection including viral gene products, viral replication and presence of unusual, foreign DNA structures (251, 368, 428). Some viruses may also either cultivate an environment or generate a protein product capable of causing direct lesions to cellular chromatin.

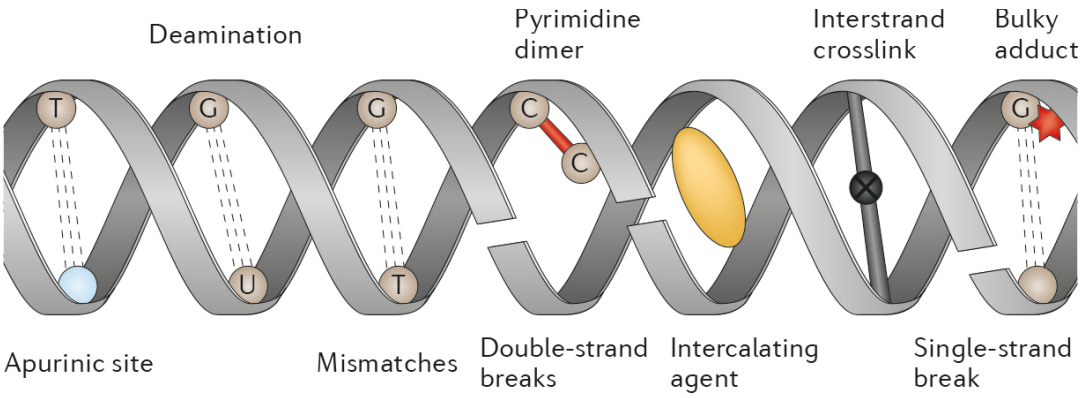
THE DNA DAMAGE RESPONSE PATHWAY

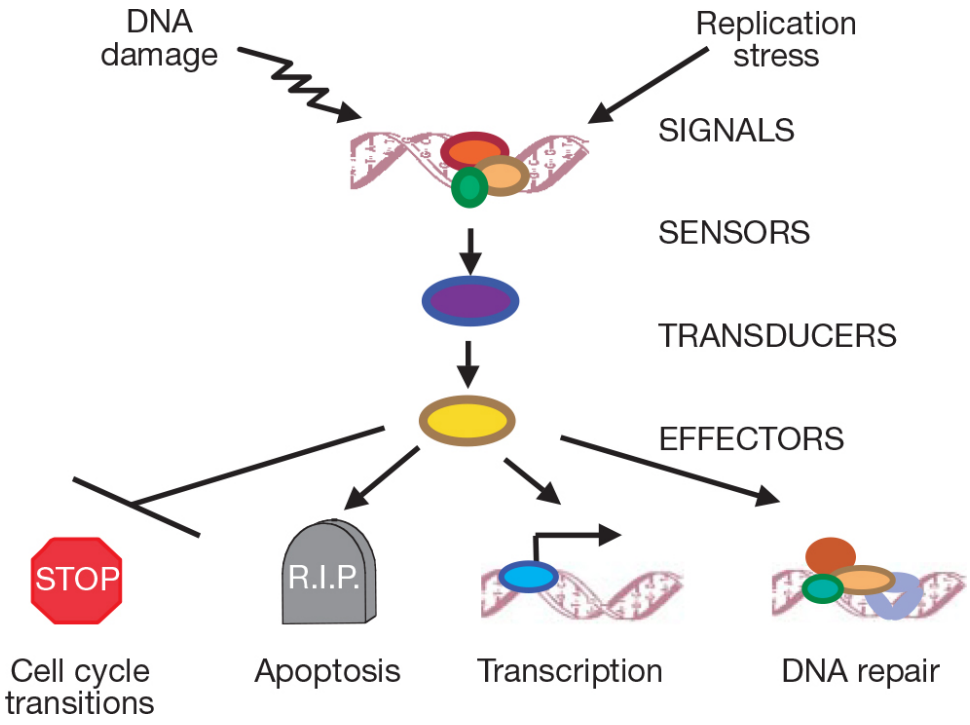
Maintenance of cellular chromatin is critical for cellular survival. Failure to repair mutations can directly impede a cell's ability to replicate its genome or transcribe RNA

Figure 1-5: General overview of DNA damage, DDR signaling and potential outcomes

Top: Different sources of DNA damage are indicated on the DNA helix. Endogenous sources of DNA damage include spontaneous base loss (apurinic or apyrimidinic sites), deamination, replication errors (mismatches) and damage from free radical species. Exogenous sources of DNA damage include ionizing radiation (double-stranded breaks) and ultraviolet radiation (pyrimidine dimers). Chemical sources of DNA damage such as platinum based compounds (Cisplatin) can cause bulky adducts or interstrand and intrastrand crosslinks ultimately resulting in single-stranded breaks. Intercalating agents such as Doxorubicin are known to cause frameshifts and double-stranded breaks. Adapted from (175).

Bottom: A general outline of the DNA damage response is represented in this schematic. The schematic illustrates that the DDR is a cascade, which requires many proteins to generate its effects, with the ultimate goal being DNA repair and re-entry into the cell cycle. Direct DNA damage or replication stress, such as stalled forks, serve as signals for a cell to induce the DDR. Sensor proteins, such as the MRN complex and Ku proteins, sense DNA damage and signal to transducers. Transducers, such as ATM or ATR, signal to downstream effector proteins and also participate in directing and repairing DNA. Effector proteins, such as Chk1 or Chk2, can be kinases, phosphatases, DNA binding, scaffolding or transactivating proteins, E3 ubiquitin ligases or other protein types, or exert direct effects themselves on the DDR. The DDR ultimately culminates in halting cell cycle transitions, apoptosis, transcriptional regulation and/or DNA repair. Adapted from (451).


Deamination
Pyrimidine dimer
Interstrand crosslink
Bulky adduct
T
G
G
C
C
G
U
T
Apurinic site
Mismatches
Double-strand breaks
Intercalating agent
Single-strand break


DNA damage
Replication stress
SIGNALS
SENSORS
TRANSDUCERS
EFFECTORS
STOP
R.I.P.
Cell cycle transitions
Apoptosis
Transcription
DNA repair

(451). Mutations in critical genes, either oncogenes or tumor suppressors, such as Ras or p53 (350, 362), respectively, or genes required for cellular survival, can abrogate a cell's ability to repair further insults, prevent halting of the cell cycle so damage can be repaired or can themselves lead to induction of DNA damage. These situations can result in cells becoming cancerous, and ultimately forming tumors, or developing heritable mutations, thus disrupting the faithful transmission of the original genetic information (198, 451). Unrepaired DNA can also result in cellular death as a result of indefinite cell cycle arrest, ultimately leading to senescence, or an apoptotic death, which can occur *via* multiple mechanisms that are not all clearly understood (342). For neurological cells, which inherently possess a limited capacity for regeneration, the DDR is critical for lifelong maintenance and persistence (29).

To combat and repair the aforementioned damage, and potential consequences thereof, a complex, multifaceted set of mechanisms, collectively termed the DNA damage response (DDR), has evolved to ensure preservation of a eukaryote's genetic code. Cells have specific systems in place poised to repair DNA bases and single-stranded and double-stranded DNA breaks *via* error-prone and non-error-prone methods. The DDR pathways include: direct repair (by replicative and repair polymerases), base excision repair, nucleotide excision repair, mismatch repair, single-stranded break repair, double-stranded break repair, and interstrand crosslink repair. In this chapter, we will focus on double-stranded break repair as this is both the most well-studied form of DNA damage repair and the form most applicable to virally-induced DNA damage.

Importantly, eukaryotic cells have integrated cellular replication, cell cycle control and DNA repair pathways allowing for a cellular circuitry which promotes crosstalk between each (76, 451). This allows a cell to sense DNA damage, halt the cell cycle prior to DNA replication in S phase or anaphase / telekinesis in mitosis, thereby minimizing the risk of passing on mutations, and then repair the damage, thus attempting to preserve genomic fidelity at multiple steps within the process.

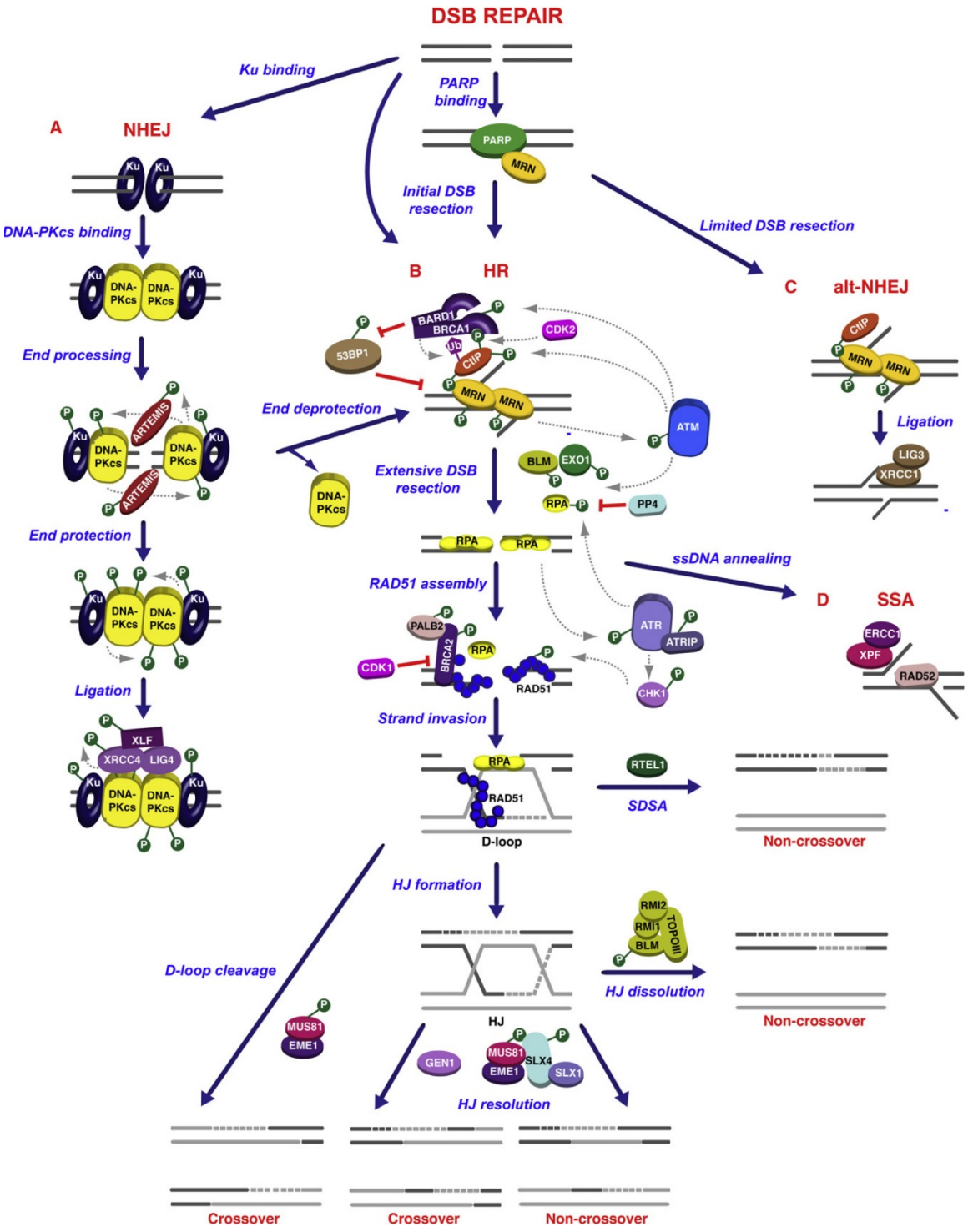
The DDR is a multilayered signal-transduction pathway (see Figures 1-5 and 1-6) that, in very simplified terms, utilizes sensor proteins to first detect the DNA damage signal. This leads to the localization and activation of many mediator and transducing proteins that in turn modulate effector proteins. These effectors then help the cell decide its fate: cell cycle halting and restarting, transcriptional up- or down-regulation of critical RNAs, DNA repair or apoptosis. In addition, other proteins such as E3 ubiquitin ligases, kinases, phosphatases, chromatin remodelers, RNA processing proteins, sumoylation and desumoylation proteins, and many others help the cell fine-tune this response (76, 198). The end goal of the DDR pathway is always to either repair the damage and re-enter the cell cycle or determine that the damage is too extensive and terminate the cell.

DOUBLE-STRANDED BREAK REPAIR

Double-stranded DNA breaks (DSBs), although not as frequent as other types of damage, are the most severe and life-threatening types of DNA damage (76, 198). This severity lies within how difficult DSBs are to repair, as they lack a complementary DNA

Figure 1-6: Schematic of double-stranded break repair and repair of single-stranded DNA after processed DSB

Methods of DSB repair and the DSB DDR cascade are shown in this schematic. Error-prone NHEJ and error-free HR repair methods are specifically detailed within the text. HR strand invasion and the resulting non-crossover and crossover resolved Holliday junctions are shown, with grayscale indicating parent and sister chromatids used in repair. Adapted from (76).


DSB REPAIR
Ku binding
PARP binding
A
NHEJ
PARP
MRN
Initial DSB resection
Limited DSB resection
DNA-PKcs binding
B
HR
C
alt-NHEJ
End processing
End deprotection
Ligation
End protection
Extensive DSB resection
ssDNA annealing
RAD51 assembly
D
SSA
Ligation
Strand invasion
RTEL1
SDSA
Non-crossover
D-loop
HJ formation
D-loop cleavage
HJ dissolution
Non-crossover
HJ
HJ resolution
Crossover
Crossover
Non-crossover

strand to serve as a template for accurate DNA repair. At least four sensor proteins can detect DBSs: PARP, Ku70/Ku80, MRN and, with additional factors, the ssDNA binding protein RPA (76). PARP is an early DSB sensor protein that facilitates the activation of ATM by mediating the recruitment of the MRN complex, although this may be somewhat controversial as many posit that the MRN complex acts completely independent of other proteins (50, 164). PARP can also promote alt-NHEJ and HR repair by competing with KU70/80 for binding of DNA resected ends. Ku70/80 sense and bind DSBs within seconds of formation and stabilize DNA ends to prevent end resection (76, 279). The MRN complex is composed of three proteins, Mre11, Rad50 and NBS1, which interact to form a heterohexameric DNA binding complex, and can sense DNA damage, recruit DDR proteins and promote ATM activation *via* NBS1 activity (237, 403). Mre11 can form stable dimers that possess endo- and exonuclease functions, which contribute to NHEJ and HR repair (237). RPA is a heterotrimer that binds ssDNA and promotes the recruitment of ATR (76).

DSBs can be repaired by at least four independent pathways: homologous recombination (HR), non-homologous end joining (NHEJ), alternative-NHEJ (alt-NHEJ) and single-strand annealing (SSA) (197). The driving force behind choosing a repair pathway lies in how much DNA processing must be done to accomplish repair. The two most commonly utilized pathways are the HR and NHEJ pathways. HR repair occurs during S or G2 phase of the cell cycle and has a higher fidelity of repair. NHEJ repair occurs during G1 phase and exhibits more error-prone repair. Apart from cell cycle phase, a key player in choosing between HR and NHEJ is 53BP1, a protein first identified

as a binding partner with p53 (425). 53BP1 promotes NHEJ by protecting damaged DNA from end resection, as well as helping to promote ATM signaling through interactions with Rad50, a member of the DNA damage sensing MRN complex (335). Alt-NHEJ requires extensive deletions at repair sites and uses microhomology regions to direct repair (244). The SSA pathway requires larger homologous repeats for repair and is more error-prone (36). HR and NHEJ, the most common DSB repair pathways, will be described briefly. It is important to note that these pathways are capable of crosstalk, and collaborate with and compete with one another, ensuring maximum likelihood of optimum DNA damage repair.

NHEJ

DSBs are bound at the ends by Ku70 and Ku80, which forms a heterodimer, within seconds of the formation (204). Binding of Ku70/80 helps protect the DNA from end resection and also tethers the DSB ends (60). DNA-PKcs (catalytic subunit) is then recruited by the Ku heterodimer and binds to the DSB, which provides additional support to prevent end resection and stabilize the DNA ends. After DNA binding, DNA-PK autophosphorylates at Thr-2609, which requires a phosphatidylinositol 3-kinase-related kinase (PIKK) interaction motif in the C-terminal region of Ku80, which has homology to the C-termini of ATRIP and NBS1 (128). Following autophosphorylation, DNA-PKcs destabilizes its interactions with the DNA ends, allowing ARTEMIS to cleave single-stranded overhangs (244). Next, the XRCC4-ligase IV complex and the stimulatory

factor XLF are recruited, separately, by the Ku heterodimer and together promote the religation of the broken ends (316, 355, 440).

HR

Homologous recombination repair, regarded by many as “error-free” (278), is the least error-prone method of repairing DSBs, accounting for approximately ten percent of total DSB repairs (76, 165). HR repair is restricted to S or G2 cell cycle phases, as sister chromatids are available for use as repair templates during this window (335). HR can initiate and complete repair through the MRN-ATM and/or RPA-ATR pathways (76, 165). Specifically, the MRN-ATM pathway is activated and localized to DSBs and the RPA-ATR pathway is activated and localized to DSB end-resected ssDNA sections in close proximity to the original DSB (201). The hallmark of HR, and the source of its error-free repair, is the invasion of one homologous strand into the other to use as a template for repair (201).

To initiate HR, the MRN complex binds to DSBs, then recruits and activates ATM in a process that is not yet fully elucidated (246, 247). There is some controversy over whether MRN can act without the help of other proteins or if there is an earlier role for PARP1 in recruiting the MRN complex (50, 164). MRN unwinding of the DNA ends is critical to activation of ATM, as is the binding of NBS1 to ATM *via* a C-terminal motif (76, 278). ATM then undergoes dimer dissociation and autophosphorylation at Ser-1981, to become fully activated (23, 384). There are at least five phosphorylation and one acetylation sites on ATM, although the significance of every site is not yet known (227).

Once activated, ATM phosphorylates Chk2, p53, NBS1, BRCA1 and H2AX, to activate γ-H2AX, along with many other proteins (53, 212, 281). Next, BRCA1, CtIP and the MRN complex interact to form the end resecting BRCA-1C complex whose activity is closely regulated by ATM. End resection is a key element of HR-dependent repair and can be undertaken by BRCA-1C, Mre11 and Exo1 (188). 53BP1 is a negative regulator of HR end resection and can push a DSB towards NHEJ repair (52). Following end resection there should be free, open 3' ssDNA ends, which bind the heterotrimer RPA, which serves to stabilize these regions. ATR is then activated and promotes HR-dependent repair by inhibiting CDK activity, thus stimulating the BRCA1-PALB2 interaction as a result of switching phosphorylation from Ser-64, a CDK site, to Ser-59, an ATR site, on PALB2 (51). As a result, the BRCA2-PALB2-Rad51 complex associates with BRCA-1 on the resected DSB (51, 76). The Rad51-associated ssDNA nucleoprotein filament then searches for homologous DNA and, when found, initiates Rad51-dependent strand invasion, a critical hallmark of HR (76, 188). Following strand invasion and formation of D-loop structures the 3' invading strand can be extended by DNA polymerases (synthesis-dependent strand annealing) or ligated, forming Holliday junctions (76). In all cases, these Holliday junctions are resolved in a crossover or non-crossover manner, either through dissolution by the BLM/TOPOIII complex or cleavage by the endonucleases GEN1, MUS81/EME1 or SLX1/SLX4 (76). These crossover events are highly regulated as they can lead to genomic rearrangements, which would introduce potentially severe genomic instability into an otherwise error-free process (76).

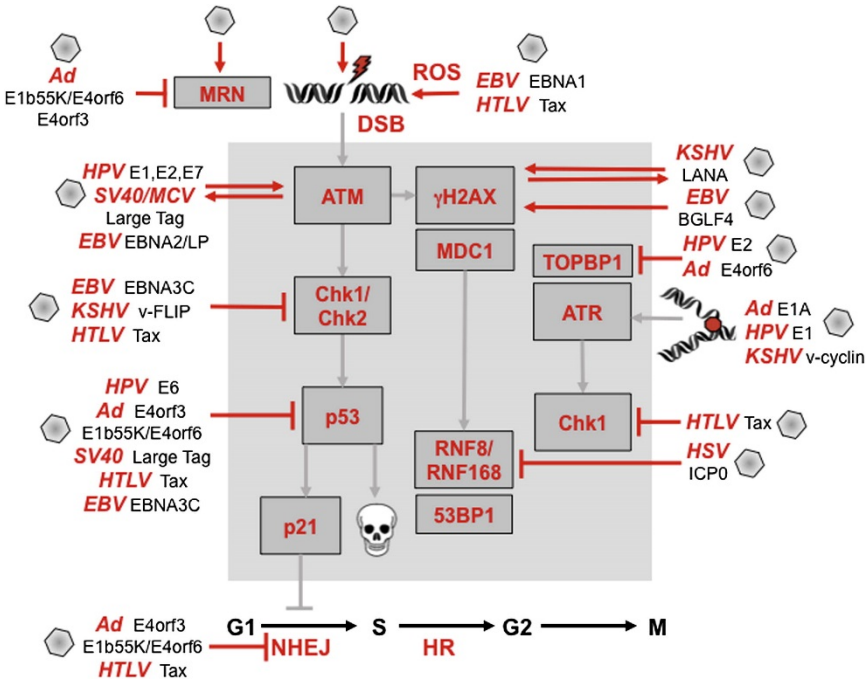
INTERACTION OF VIRUSES WITH THE DDR

As previously discussed, it has recently become appreciated that viruses, almost exclusively DNA viruses, have evolved the ability to interact with, modulate and utilize the DDR to promote their life cycle (428, 430) (See figure 1-7). In some cases the DDR is absolutely required for viral replication, as with SV40 that requires both ATM and ATR activity to resolve concatameric replication intermediates prior to packaging (396, 397). Other viruses must expertly modulate the DDR to ensure an environment conducive for viral replication, as with Adenovirus, which must temporally inactivate a MRN-ATM DDR *via* viral proteins E1B-55K and E4orf3 while allowing a MRN-independent ATM DDR required for replication (382). Adenovirus utilizes another protein, E4orf4, to inhibit both ATM and ATR activity, which likely inhibits cellular DNA damage repair in addition to aiding viral replication (44). Given the requirement for viruses to negotiate this intricate response, it seems likely that the DDR is an innate antiviral mechanism (265).

The method by which a virus activates the DDR is both controversial, at times, and varied depending upon the virus in question. For certain viruses, such as Herpes simplex virus 1 and 2 (HSV-1, HSV-2), Epstein-Barr virus (EBV), polyomavirus, SV40, adenovirus, human cytomegalovirus (HCMV), human papillomavirus (HPV), AAV and MVM, viral replication is the key to activating a DDR (7, 428). Viral activation of the DDR, as evidenced by ATM or ATR activation *via* autophosphorylation, can be detected during early points of infection in these viruses. Viruses that activate a DDR as a result of replication commonly recruit specific DDR proteins to their viral replication factories (428).

Figure 1-7: – Summary of viral interactions with the DDR

Viral infections and viral proteins that modulate and interact with the DDR are indicated outside the large gray box. Activation of the DDR by viruses or viral gene products are shown at the top of the schematic. Proteins that activate and are acted upon by DDR proteins are indicated by two-way arrows. Viral proteins that inhibit DDR proteins are indicated by a red T. Viral proteins that affect the cell cycle are indicated at the bottom of the schematic. Adapted from (430).


Ad
E1b55K/E4orf6
E4orf3
MRN
ROS
EBV EBNA1
HTLV Tax
DSB
HPV E1,E2,E7
SV40/MCV
Large Tag
EBV EBNA2/LP
ATM
γH2AX
KSHV
LANA
EBV
BGLF4
MDC1
TOPBP1
HPV E2
Ad E4orf6
EBV EBNA3C
KSHV v-FLIP
HTLV Tax
Chk1/
Chk2
ATR
Ad E1A
HPV E1
KSHV v-cyclin
HPV E6
Ad E4orf3
E1b55K/E4orf6
SV40 Large Tag
HTLV Tax
EBV EBNA3C
p53
Chk1
HTLV Tax
HSV
ICP0
RNF8/
RNF168
53BP1
p21
Ad E4orf3
E1b55K/E4orf6
HTLV Tax
G1
NHEJ
S
HR
G2
M

What is not always immediately clear is whether the recruitment of DDR proteins is a cellular attempt at reducing or a viral attempt at enhancing viral replication.

Viruses can also activate the DDR by expression of viral proteins. SV40 large T-antigen expression alone is sufficient to induce a DDR in a Bub1-interaction dependent manner (174). HPV E7 protein is sufficient for activation of Chk2 and associates with a complex that contains activated ATM (295). AAV2-Rep protein is capable of producing cellular nicks, thus creating novel sites of DNA damage and activating ATM (35). However, although these viral proteins are capable of producing these effects when expressed ectopically, it is not always clear if the activity of these proteins is the direct activator of the DDR during infection or if multiple sources culminate during viral infection to induce the response.

Finally, some viruses induce a DDR *via* introduction of foreign nucleic acid structures found within their genomes. Because not all viruses induce the DDR in this manner, one could speculate that only certain viral genomes exhibit the specific secondary structures or contain cis- or trans-acting sequences that can directly interact with or activate the DDR machinery. HSV-1, a linear dsDNA virus, is capable of inducing a DDR simply by depositing its genome into the host nucleus, resulting in rapid recruitment of MDC1 and γ-H2AX to viral DNA (250, 265). Peter Beard's lab showed that UV-inactivated AAV2 was able to induce an ATR-dependent DDR, which requires the sequence of the p5 promoter (207). Interestingly, UV-inactivated MVM is unable to induce an ATR-dependent DDR despite both being parvoviruses and both introducing a ssDNA genome into the host cell's nucleus, which would be predicted as sufficient to

activate the ATR pathway (8, 313, 439, 453). One possibility is that MVM prevents the activation of ATR signaling by NS1 binding to nicked, free 5' ends, as HSV-1 does by recruiting the viral helicase/primase complex (293), while AAV2 simply lacks the ability to inhibit ATR signaling (8).

Although it has been hypothesized that the DDR serves as an innate antiviral response, unsurprisingly, some viruses have evolved to utilize, and in some cases require, the DDR. Ablating DDR signaling with RNAi or specific chemical inhibitors reduced viral replication of HSV-1, polyomavirus, SV40, EBV, HCMV and MVM (7, 251, 265). However, many viruses that *need* a DDR to efficiently replicate still inactivate or modulate specific aspects of the virally-induced DDR. Viruses utilize several strategies to modulate or completely shut off the DDR: mislocalization of DDR proteins, sequestration of DDR proteins, reprogramming the activity of cellular kinases, phosphatases and histone acetylases, altering the sumoylation of cellular proteins and programming the degradation of cellular proteins by redirecting cellular E3 ubiquitin ligases (251, 428). This is not an exhaustive list and there are likely more strategies that have not yet been fully elucidated. A few key examples of these viral abilities will be elaborated upon in this chapter.

HSV-1 has been shown, *via* shRNA knockdown experiments, to recruit and require ATR pathway proteins including ATRIP, RPA70, TopBP1, Claspin and CINP (292). Yet despite recruiting and requiring these proteins for viral replication, paradoxically, and as described above, HSV-1 utilizes it primase/helicase proteins (UL8/UL5/UL52) to specifically disable cellular ATR signaling (293). HSV-1 is also capable of programming

the targeted depletion of DNA-PK *via* its ICP0 protein, which can mediate E3 ubiquitin ligase activity (391). ICP0 can also target PML, a nuclear protein implicated in defense against viral infection, for sumo-dependent and -independent proteasomal degradation (104, 202).

HCMV, the most genetically complex viral pathogen of humans, activates an ATM-dependent DDR, which may be necessary for viral infection like other herpesviruses (123, 436). Although HCMV needs certain aspects of this DDR for replication, HCMV mislocalizes activated ATM and Chk2 to the cytoplasm preventing activation of downstream targets, such as CDC25A (147). It is likely that this modulation ultimately results in HCMV promoting nucleotide excision repair of its genome while preventing repair of the host genome (328). 53BP1, a key protein for promoting classical NHEJ, is lost during infection but the mechanism is not yet clear (266).

Adenovirus is a master of utilizing several of the aforementioned strategies to facilitate effective viral replication. Adenovirus early protein E4orf3 can mislocalize PML, Mre11, Rad50 and Nbs1, which inhibits the antiviral protein PML and prevents DDR initiation and signaling from the MRN complex, respectively (18, 125, 126, 251). Adenovirus can also use its E1B55K and E4orf6 proteins to degrade the three components of the MRN complex, to further ensure abrogation of MRN-dependent DDR signaling, the Bloom helicase, which is required for end resection prior to DNA damage repair, and p53, the critical tumor suppressor and important regulator of the DDR (332, 351, 352, 399). E1A, which is also expressed early in adenovirus infection, can bind several histone acetyltransferases, including P/CAF, TRRAP/GCN5 and p300/CBP, which

allows for the virus to reprogram cellular transcription and redirect the machinery to viral promoters (239, 361). Adenovirus is also capable of altering the post-translational modifications of cellular proteins by binding protein phosphatase 2A (PP2A), thus inducing apoptosis and altering junB transcription (222, 387), and promoting the sumoylation of p53 and SP100A, allowing the virus to inactivate p53/SP100A tumor suppressive and antiviral activity (34, 307).

The DDR is typically thought of as a response to DNA viruses, as these viruses inhabit and replicate within the host cell's nucleus. However, it is becoming clear that RNA viruses, including those that replicate exclusively in the cytoplasm, can activate and inhibit the DDR (368). Human immunodeficiency virus – 1 (HIV-1) utilizes its VPR protein to induce a G2/M cell cycle arrest and is thought to activate DDR pathways to accomplish this (368). VPR can induce DSBs, directly bind cellular chromatin to activate ATR, and activate ATM, which has been shown to enhance HR repair (235, 311, 404). It has been speculated that these activities of VPR enhance HIV-1 integration.

Hepatitis C virus (HCV) is known to elevate levels of nitric oxide (NO) and reactive oxygen species (ROS), which cause DNA damage and are thought to contribute to the genetic abnormalities seen in HCV infected cells (368). HCV infected cells have reduced abilities to repair UV-induced DNA damage and to utilize NER and BER repair pathways (181, 333, 412). NS3/4A, HCV's protease, interacts with ATM, causing it to relocalize to the cytoplasm, and inhibits repair of IR-induced damage (234). HCV NS2 inhibits DDR signaling by mislocalizing p53 to the cytoplasm (37). The HCV core protein interacts with Nbs1 and interferes with MRN localization at DBSs, thus preventing ATM activation

(273). Many of the DDR modulations that HCV induces target and mislocalize proteins in the ATM pathway, so it is not surprising that ATM and its downstream kinase, Chk2, have been implicated in HCV RNA replication (19). Taken together, these examples demonstrate how critical the DDR is to the lifecycle of numerous, diverse viruses.

1.4 PARVOVIRUSES AND THE DNA DAMAGE RESPONSE

Due to their small genome, and thus limited genetic arsenal, parvoviruses must rely on and steal from their host cell for many of their basic lifecycle requirements including entry into S phase, transcription *via* RNA polymerase II and utilization of replicative machinery. Dependoparvoviruses, such as AAV, further rely on the additional presence and actions of a helper virus. HSV-1, HPV and Adenovirus, each of which AAV is able to utilize as a helper virus, all activate and modulate the DDR during their replicative lifecycles (148, 392, 429). Parvoviruses have evolved to provide themselves with a hospitable cellular environment, in part, due to their ability to negotiate and modulate the DNA damage response, which sits at the crux between cell cycle regulation and replication control. Prior to the first report characterizing the DDR's involvement in autonomous parvoviral replication (7), there were hints that perhaps the DDR was important for parvoviruses. One publication suggested that NS1's ability to induce G2/M arrest involved p53 (331), a critical mediator of the DDR, and another suggested that parvovirus replication was inversely correlated to a cell's ability to repair X-ray induced DNA damage (408).

It is not currently known if the palindromic hairpins—which a cell might sense as an unusual, foreign DNA structure—parvoviral proteins, either structural or non-structural, or parvoviral replication serve as the initial or ongoing source of DNA damage during viral infection. One can also speculate that NS1 is binding to (ACCA)2-3 elements within the genome and nicking the cellular chromatin, which could prompt the initiation of a DDR that the virus could then exploit for establishing or spreading an infection

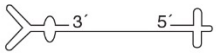
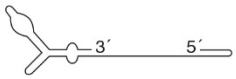
within a cell (221, 344). What is clear is that the DDR invoked by parvoviruses is critical to establish and facilitate a robust infection (7, 117, 267). Unsurprisingly, the different genera of the *Parvovirinae* subfamily have developed several strategies to modulate and negotiate this complex cellular response (101, 270) (See figure 1-8).

PROTOPARVOVIRUS

MVM, the prototype virus of the genus *Protoparvovirus*, induces an ATM-dependent DNA damage response (7, 367). Hallmarks of a canonical ATM-dependent DDR are observed during MVM infection including phosphorylation of the DNA damage marker, γ-H2AX at Ser-139, autophosphorylation of ATM at Ser-1981 and phosphorylation of p53 at Ser-18 (7, 367). Importantly, ATM activity, as determined by inhibition utilizing a specific ATM inhibitor, was critical for MVM replication and for establishing the G2/M, pre-mitotic block observed during infection (7). Further study implicated Chk2, a serine/threonine checkpoint kinase downstream of ATM, as an important signaling component for the G2/M block (9). Mre11, which acts in concert with Nbs1 and Rad50 to form the DNA damage sensing MRN complex and signals to ATM, is depleted by a proteasome-dependent mechanism during infection, which suggests that although MVM requires ATM activity, it still modulates specific aspects of the virally-induced DDR (7). A similar effect is seen during MVM infection where p53 is upregulated and activated; however protein levels of p21, a critical target of p53 transactivation, remain unexpectedly low during infection, further suggesting that MVM specifically targets key facets of the DDR for modulation (10). Surprisingly, ATR is not

Figure 1-8: Parvovirinae and the DDR

Represented viruses from each Parvovirinae genera are included. The virus utilized for the studies is indicated next to each genera. The structure of the viruses listed is indicated within the table. The known phosphatidylinositol 3-related-kinase related (PIKK) mediators of each viral DDR are shown, as is the consequence of DDR activation. Adapted from (270).

Genus	Virus	Genome structure	Mediator of the DDR	Consequence of the DDR
Dependovirus	AAV2		ATR (UV-AAV2)	ATR triggers G2/M arrest and apoptosis in UV-AAV2-infected cells
			DNA-PKcs and ATM (AAV2/adenovirus)	MRN complex limits AAV2 replication
			DNA-PKcs (AAV2/HSV-1)	Unknown
Erythrovirus	B19V		ATR and DNA-PKcs	Facilitates B19V replication; dispensable for B19V infection-induced G2/M arrest
Parvovirus	MVM		ATM	Facilitates MVM replication; partially mediates G2/M arrest
	H-1PV		Unknown	Unknown
Bocavirus	MVC		ATM	Facilitates MVC replication and triggers p53-dependent apoptosis; partially mediates G2/M arrest
Amdovirus	AMDV		Unknown	Unknown

activated during infection (8), as one might predict due to the presence of RPA-coated ssDNA during infection (313, 453). This is likely due to lack of binding of Rad9 to MVM chromatin (112). DNA-PK also does not appear to be activated or required for efficient replication, in spite of Ku70/80 and DNA-PK CS recruitment to APAR bodies in the presence of single-stranded MVM DNA (7). The lack of ATR and DNA-PK activity, coupled with the aforementioned Mre11 and p21 modulation, suggests that MVM is able to precisely activate or deactivate specific features of the host DDR machinery.

Much less is known about the interaction between the DDR and parvovirus H-1 (H-1PV), a protoparvovirus that has been used for oncolytic therapy as previously discussed. However, it is clear that expression of H-1PV NS1 alone is sufficient to elevate γ-H2AX levels, which suggests that NS1 is causing DNA damage *per se* or NS1 is stimulating a DDR *via* a cell cycle block or another unknown function (186). One report suggests that NS1 is inducing reactive oxygen species (ROS), which have been shown to activate the DDR (30, 186). Although it is still not clear how H-1PV NS1 is triggering ROS production, which DDR signaling pathways are involved or if the DDR is required to facilitate H-1PV replication, given the oncolytic nature of H-1PV, one would predict that the virus would need to interact with and modulate both the cellular DDR and cell cycle machinery to fulfill its oncolytic nature (186, 270).

BOCAPARVOVIRUS

Bocaparvoviruses, specifically Minute Virus of Canines (MVC), appear to have some key similarities to the Protoparvovirus DDR. Both activate an ATM-dependent DDR

and both observe a loss of Mre11 during later stages of infection (267). As with MVM, MVC infection requires ATM activation to phosphorylate γ-H2AX, p53 is activated *via* phosphorylation and DNA-PK signaling seems to play no known role (267). However, in contrast to MVM, MVC does activate ATR, as evidenced by phosphorylation of ATR, and this activation is required for RPA32 phosphorylation (267). Perhaps a key to the differences in DDR signaling between the two viruses lies in the additional non-structural protein, NP1, that MVC expresses (402).

Human bocavirus -1 (HBoV1) is an emerging human pathogenic virus, which also belongs to the *Bocaparvovirus* genera. In contrast to MVC, HBoV1 activates all three phosphatidylinositol 3-related-kinase kinases (PIKKs): ATM, ATR and DNA-PK (117). Utilizing specific inhibitors individually demonstrated that HBoV1 does require the activity of each PIKK for efficient replication, though the contributions of each PIKK are not yet known (117). Surprisingly, induction of the DDR appears to allow HBoV1 to replicate in *non-dividing* human airway epithelium cells, which is distinct from all other parvoviruses that require infection of a dividing cell (117). HBoV1 can also replicate in dividing HEK293 cells and still activates all three PIKKs, which suggests that their concurrent activation may simply be a hallmark of HBoV1 replication (116). HBov1 is also the only parvovirus that has been demonstrated to utilize DNA repair polymerases η and κ, opening the possibility that DNA polymerase δ is not the sole DNA polymerase involved in replication or repair of parvoviral genomes (117).

ERYTHROPARVOVIRUS

Human parvovirus B19 (B19V) is an Erythroparvovirus whose only known natural host cell is the human erythroid progenitor cell (EPCs) (444). B19V is the etiological agent of 'fifth disease', a mild disease commonly found in young children (427). It is important to note that B19V can cause more severe symptoms in pregnant women during the second trimester, inducing hydrops fetalis (238), a serious fetal condition of accumulation of abnormal levels of fluid in two or more fetal compartments, and chronic pure red cell aplasia in immunocompromised patients (302), an anemia which affects the precursors of all red blood cells.

Similar to HBov1 and MVC, B19V activates all three PIKKs during infection of EPCs and recruits each cellular PIKK to viral replication centers (269). It is not clear if these viruses require separate activities from each PIKK to replicate or process its genome, or if there are additional specific cellular effects induced by activating certain PIKKs. As with many parvoviruses, B19V recruits several additional key DDR proteins to its APAR bodies including Chk1, Chk2 and KU70/80, which are involved in ATR, ATM and DNA-PK activity, respectively (269, 270). However, unlike MVM, B19V infection activates ATR and Chk1, which facilitates B19V replication, as evidenced when inhibiting ATR during infection (269). ATM, although activated during B19V infection, is not specifically required for replication as inhibition of ATM could be tolerated without affecting viral replication (269). This striking difference in the requirement for ATM and ATR could be due to dissimilarity in the hairpins within each genome or perhaps roles fulfilled by ancillary non-structural proteins. What is consistent between all of the autonomous

parvoviruses known to induce a DDR is the necessity of DDR signaling to arrest cells in a pre-mitotic late S (4N DNA content) or G2/M phase (9, 62, 117, 267, 268, 270, 299).

DEPENDOPARVOVIRUS

Adeno-associated virus (AAV) was first isolated in 1965 as a contaminant in Adenovirus isolates (375). Since then, it has been well established that AAV requires a helper virus, typically in the form of a larger DNA virus (HSV-1, HPV or Adenovirus), for efficient replication (375), and that these helper viruses typically invoke, interact with and modulate the DDR (148, 392, 429). Failure to infect in the presence of a helper virus induces latency and results in eventual integration, almost exclusively, into chromosome 19 (189, 190, 192, 226, 376). In contrast to the previously discussed parvoviruses, AAV have identical terminal repeats (ITRs), which form “T” shaped hairpins flanking their genome (270, 375). It is unclear if the hairpins of any parvoviruses play a role in inducing or providing an ongoing stimulus for the DDR.

It is important to note that virtually all of the research undertaken concerning AAV and the DDR has been done in the most well-studied AAV, AAV2. However, there could be key differences among the different naturally occurring serotypes, of which there are at least 15 representative members (21, 434). If differences in DDR pathways among the multiple AAV do indeed exist, they could be due to variances in the viral genomes or viral replication, yet it is just as likely, given the different tropisms of the AAVs, that infection in different cell types invokes different DDR pathways.

The AAV genome within an infected cell, much like the autonomous parvoviruses, will be present as a single-stranded DNA molecule coated with RPA (75, 315). This type of molecular structure has been well documented as a signal that activates ATR signaling *via* binding of RPA, ATRIP, the 9-1-1 complex and other required proteins (313, 439, 453). Peter Beard's lab found that introducing UV-inactivated AAV2, which is no longer replication competent, was sufficient to induce an ATR-dependent DDR (207). Introduction of UV-inactivated AAV2, *via* Chk1 activation, was also sufficient to cause a G2 cell cycle arrest (207). It was determined that cis-acting sequences in AAV2, specifically within the p5 promoter, were required for invoking a DDR (137). The same p5 sequence is also required for integration of both wild-type AAV and genetically engineered recombinant AAV (339, 340), raising the possibility that AAV integration induces and requires a localized DDR. Interestingly, recombinant AAV2, which was engineered to express green fluorescent protein (GFP) instead of the viral genome, flanked by AAV2 ITRs, was unable to induce a DDR (137).

In addition to AAV2 p5 inducing a DDR, AAV2 replication proteins Rep68 and Rep78 can both induce an ATM-dependent DDR (35, 381). It has been proposed that the nicking activity of the Rep proteins may induce nicks in cellular chromatin, which is sufficient to activate ATM (35, 270). In contrast to the ATR DDR induced by p5, which results in a G2 block, the ATM DDR activated by Rep78 causes an S phase cell cycle block (35). It is possible that during an infection the Rep proteins do cause a transient S phase block that transitions into a pre-mitotic G2 block. However, coinfection studies suggest that the majority of DDR signaling results from viral replication, which may also

influence the resulting cell cycle block (381). It is also important to note that the co-infecting virus will also play an important role in modulating the ensuing DDR and potentially impact the cell cycle block (83, 290, 418).

AAV has been used for many years as a gene therapy vehicle to target a wide variety of cell and tissue types and genetic diseases (21, 375). Interestingly, it has been demonstrated that introducing unusually structured DNA *via* AAV vectors, such as the AAV2 genome itself, induces a DDR in p53 signaling deficient cells and results in their apoptotic death (354). One could speculate that AAV vectors, designed to express specific unusually structured DNA, could also be utilized as cancer therapeutic reagents, similar to H-1PV's use as an oncolytic therapy.

1.5 CELL CYCLE CONTROL

Dividing cells utilize the cell cycle, a highly controlled and precise process, to replicate their DNA and divide into two daughter cells (298). The cell cycle is divided into four distinct phases: G1, S, G2 and M phase (See figure 1-9). The gap phases, G1 and G2, occur prior to S phase, where cells replicate their DNA, and prior to mitosis, where the cells prepare to enter mitosis and divide, respectively. These gap phases provide cells with extra time for cell growth, which typically takes longer than the duplication and segregation of chromosomes (298). Gap phases also serve as key regulatory transition periods, allowing for the progression of cell cycle to be controlled by many signals (38). Maintaining this control requires cells to expertly regulate cell cycle proteins *via* protein modifications, localization, interactions and, at the correct times, destruction.

Cell cycle progression is controlled by the activity of cyclin-dependent kinases (CDKs), which are serine and threonine kinases whose activity depends on the association with their activating subunits, cyclins (38) (See figure 1-10). The activity of cyclin-CDK complexes is regulated through phosphorylation and ubiquitination, which controls protein-protein interactions, protein localization and expression levels (298). CDK inhibitor proteins, from the INK4a(p15/16/17), Cip1(p21) and Kip1(p27) families, provide further control of CDK activity by inhibiting CDKs or cyclin-CDK complexes when required (319). In contrast to yeast, which possesses a single CDK, Cdc28 (CDK1 equivalent), animal cells express multiple cyclins and CDKs, which is hypothesized to allow for precision and flexibility in controlling cell cycle progression (38). There are at least 20 members of the mammalian cyclin and CDK families, but only a few key cyclin-

CDK complexes directly regulate the progression of the cell cycle (193). In the simplest explanation of cell cycle progression: early G1 events are regulated by D-type cyclins and CDK4 or 6, Cyclin E-CDK2 regulates entry into S phase, completion of S phase is regulated by Cyclin A-CDK1/2 and Cyclin B-CDK1 regulates entry into mitosis (184). Recently, it is becoming more appreciated that cyclins and CDKs also fulfill non-canonical roles including regulating transcription, DNA damage repair, cell death, cell differentiation, immune responses and metabolism (193). However, redundancies are likely built into this incredibly complex and vital system as mice individually lacking CDKs -2, -4 or -6 or Cyclins -A2, -B2, -E1, or -E2 are viable, albeit with certain defects (184).

Cell cycle checkpoints are fail-safes built into the cell cycle that allow the cell to halt cell cycle progression until it can ensure that an earlier process, such as DNA replication or DNA repair, was satisfactorily completed (319). The eukaryotic cell cycle possess three checkpoints at the borders of the G1/S and G2/M phases, and at the metaphase/anaphase boundary during mitosis (319). There is an additional "G1" checkpoint in mammalian cells, termed "Start" in yeast, which is the early restriction point cells use to determine if they can initiate division in the environment, both external and internal, they currently reside in. Proper regulation of this restriction point is critical as passage marks an irreversible transition in which the cell is now committed to completing the cell cycle (319). Cell cycle checkpoints are activated by different cellular stresses including DNA damage, unreplicated DNA and chromosomes not being attached to mitotic spindles (319). These checkpoints are activated and deactivated by a multitude of proteins, many of which are key DDR proteins including ATM, ATR, Chk1

Figure 1-9: The eukaryotic cell cycle

The eukaryotic cell cycle is divided into four discrete phases: G1, S, G2 and M. The black R represents a critical, early cell cycle restriction point that must be passed to continue into the cell cycle. Key cyclin proteins are illustrated next to the cell cycle phases they regulate. Adapted from (297).

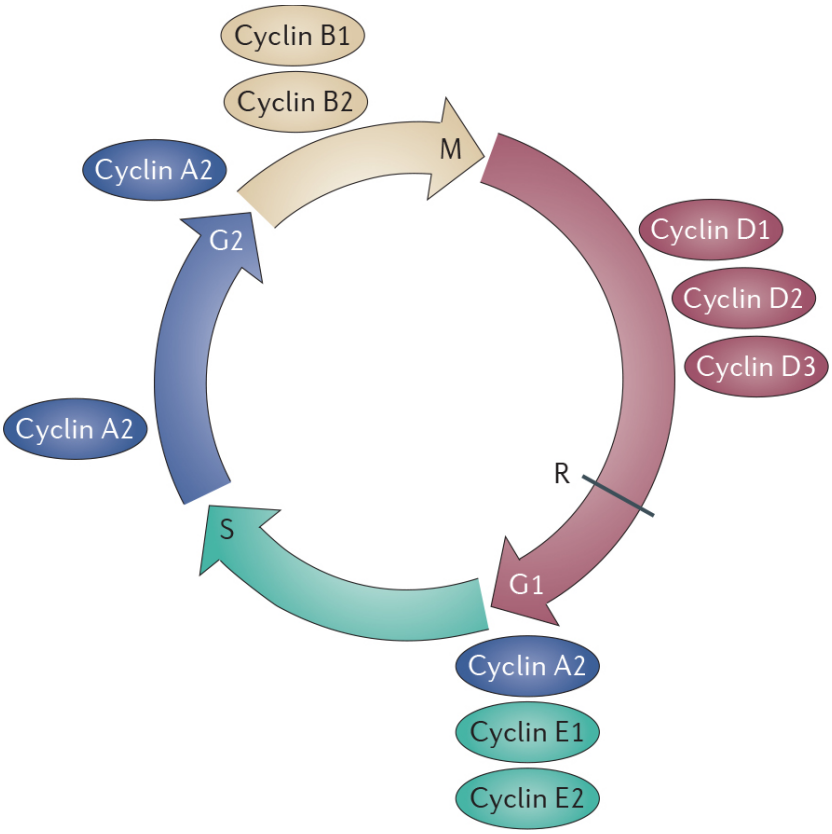
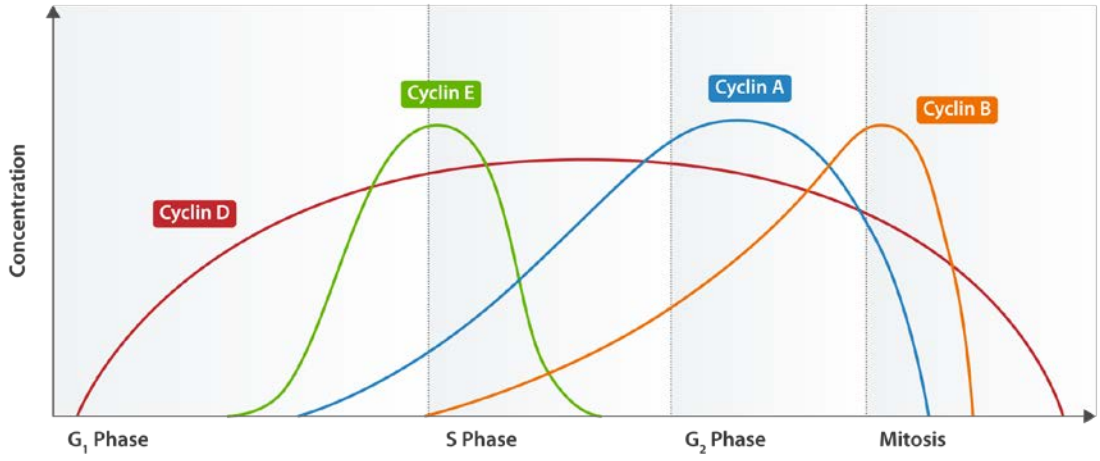

Cyclin B1
Cyclin B2
M
Cyclin A2
Cyclin D1
Cyclin D2
Cyclin D3
G2
Cyclin A2
R
S
G1
Cyclin A2
Cyclin E1
Cyclin E2

Figure 1-10: Key cyclin types which regulate the cell cycle

Schematic illustrating the relative expression levels of key cyclin types, which regulate entry and transit through the phases of the cell cycle. Cyclin expression is not regulated in an “on/off” manner, rather they are expressed at low levels until the requirement for their availability is approaching. They are then upregulated and bind to the appropriate cyclin-dependent kinase (Cdk). Progression through the cell cycle occurs once the correct cyclin-Cdk complex is formed, modified and localized and minimum threshold levels activity are achieved.

Figure borrowed from Wikipedia Commons. Retrieved 1/29/2017.

https://upload.wikimedia.org/wikipedia/commons/thumb/c/ce/Cyclin_Expression.svg/2000px-Cyclin_Expression.svg.png


Concentration
Cyclin D
Cyclin E
Cyclin A
Cyclin B
G1 Phase
S Phase
G2 Phase
Mitosis

and Chk2 (211). The cell cycle, checkpoint and DNA repair pathways are integrated into a network that facilitates appropriate cellular responses to halt the cell cycle, repair DNA damage or complete the required process and then re-enter and complete the cell cycle. Failure within this network can result in genomic instability, aberrant cellular proliferation and tumorigenesis. This chapter will focus on two key regulators within this network which MVM targets during infection: the CDK inhibitor, p21 and the mitotic cyclin, Cyclin B.

THE CDK INHIBITOR, p21

p21 is a small 165 amino acid protein, also known as $p21^{WAF1/Cip1}$, which belongs to the Cip/Kip family of CDK inhibitors (1, 39). The Cip/Kip family also includes p27 and p57. p21 is a well-characterized target of the tumor suppressor p53 and is generated *via* p53 transactivation of the CDKN1A gene during a DDR (1). p21 has many roles including cell cycle arrest, DNA repair, cellular differentiation, cellular proliferation and apoptosis (57). Given the differing roles that p21 is capable of fulfilling, p21 is thought to act as both a tumor suppressor and an oncogene.

Many of the effects p21 produces are a result of its interactions with other proteins. p21 accomplishes many of these effects through the use of its two main domains, a carboxy-terminal PCNA binding domain and an amino-terminal CDK-cyclin inhibitory domain (64). It also has less well-characterized domains, Cy1 and Cy2 domains located in the N-terminal and C-terminal halves of p21, respectively (66). p21 utilizes these motifs to disrupt interactions between CDKs and substrates that bind to CDK-

cyclin complexes through similar motifs including p107, p130, retinoblastoma family proteins and CDC25 (372, 386, 452).

p21 AND CELL CYCLE ARREST

p21's chief role, typically in response to p53 transactivation, is to arrest cells during G1 or G2 phase when cells face DNA damage or loss of replication fork integrity. The ability of p21 to arrest cells in G1 relies on its ability to bind and inhibit the activity of Cyclin E and Cyclin A-CDK2 (48). By interfering with CDK2 activity, p21 prevents the phosphorylation of Retinoblastoma (Rb), which in turn prevents the activation of E2F dependent genes, and prevents the formation of pre-replication complexes and origin firing (42). p21 also indirectly affects origin firing by competing with the histone methyltransferase, Set8, and the licensing factor, Cdt1, for binding to PCNA, a required cofactor for polymerase-δ DNA replication (2, 219).

In addition to its role in inducing a G1 block, p21 is also involved in mediating and sustaining a G2 arrest. p21 can bind to Cyclin B-CDK1 complexes and mediate nuclear retention of the complex, thus preventing its activation by CDC25 and CDK-activating kinase (CAK) and its recruitment to the centrosome (61). One report suggests that p21 also mediates the degradation of Cyclin B, likely *via* the APC/C^{CDC20} E3 ubiquitin ligase, which would prevent the activity of its binding partner, CDK1 (154). p21 has also been shown to be necessary and sufficient for mediating p53-dependent gene repression of genes regulating mitotic entry including CDC25C, CDC2 (encoding CDK1), CHEK1 (encoding Chk1) and CCNB1 (encoding Cyclin B1) (1, 258).

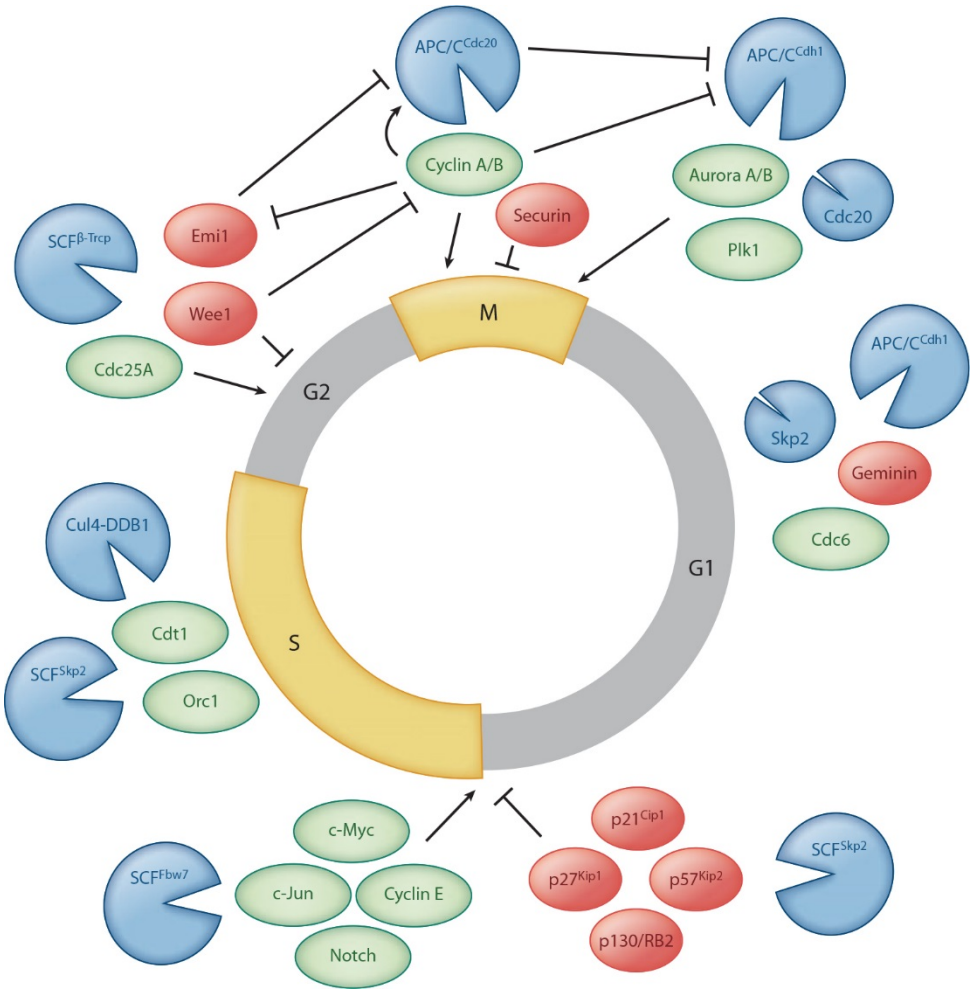
Because p21 is such a crucial and potent inhibitor of CDK and PCNA activity, which inhibits cell cycle progression and DNA replication, respectively, cells require the ability to repress p21 activity. Although p21 can be repressed at the transcriptional level, post-transcriptional control of p21 affords fast, flexible inhibition. p21 is a relatively unstable protein with a half-life of 20 to 60 minutes, but newly synthesized p21 is protected from proteasomal degradation by the activity of FKBPL, which recruits HSP90 to p21 (200). Thus, cells need a way to quickly and reliably target p21 for degradation to derepress cell cycle arrest and restart replication forks.

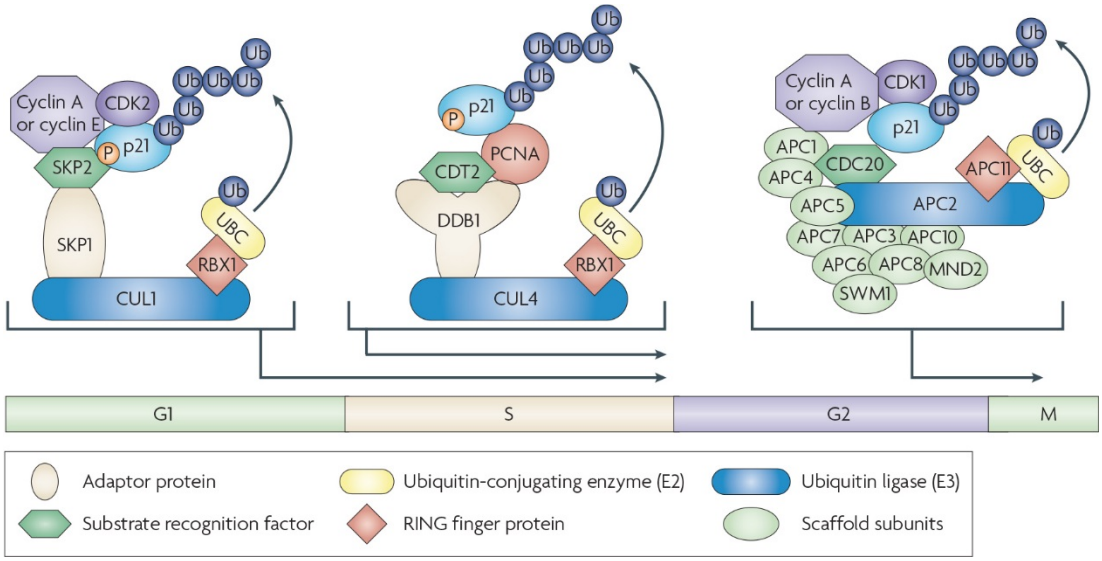
Proteasomal degradation is mediated by interaction of the protein targeted for degradation with an E3 ubiquitin ligase. There are three types of E3 ubiquitin ligases: RING, HECT and RBRs (300). The process of conjugating ubiquitin to a protein targeted for degradation has been reviewed extensively (224). In the simplest of terms, an E3 ubiquitin ligase targets a protein for degradation and conjugates poly-ubiquitin to, typically, specific lysine residues within that protein, resulting in subsequent trafficking to and degradation within the 26S proteasome. This chapter will focus on the Cullin-RING family given their importance in regulating cell cycle and DNA replication (See figure 1-11, top). Cullin-RING ubiquitin ligases (CRLs) are a superfamily of multisubunit complexes composed of one of seven Cullin proteins, which act as a scaffold, an adapter protein, such as Skp1 or DDB1, which bind substrate recognition factors that recognize and recruit the protein targeted for degradation, and a catalytic core (RING protein), HRT1, RBX1 or ROC1, which conjugates poly-ubiquitin to the protein targeted for degradation (338).

Figure 1-11: Regulation of cell cycle proteins by ubiquitin ligases

Top: Multiple ubiquitin ligases target cell cycle regulators for ubiquitin-mediated degradation. Key ubiquitin ligases include the APC/C, Cul4 and SCF complexes. Blue circles represent E3 ubiquitin ligases. Green ovals represent activators of cell cycle progression while red ovals represent inhibitors. The placement of E3 ubiquitin ligases in proximity to specific cell cycle phases indicates the phase in which they are active. Adapted from (409).

Bottom: Three ligases are known to target p21 for degradation during normal cell cycle progression: SCF^{Skp2}, $CRL4^{CDT2}$ and APC/C^{CDC20}. Placement of each E3 ubiquitin ligase represents the cell cycle phase in which they are active. The blue ovals represent the scaffold of the ubiquitin ligase and the gray oval represents the adapter protein. The green hexagon represents the substrate recognition factor of each E3 ubiquitin ligase that specifically recognizes the protein to be targeted for degradation. The $CRL4^{CDT2}$ E3 ubiquitin ligase requires interaction with chromatin and proliferating cell nuclear antigen (PCNA) for activity. Adapted from (1).


APC/C^Cdc20
APC/C^Cdh1
Cyclin A/B
Aurora A/B
Cdc20
Plk1
Securin
SCF^β-Trcp
Emi1
Wee1
Cdc25A
M
G2
G1
S
APC/C^Cdh1
Skp2
Geminin
Cdc6
Cul4-DDB1
Cdt1
SCF^Skp2
Orc1
c-Myc
SCF^Fbw7
c-Jun
Cyclin E
Notch
p21^Cip1
p27^Kip1
p57^Kip2
p130/RB2
SCF^Skp2


Cyclin A
or cyclin E
CDK2
Ub
P
p21
SKP2
SKP1
CUL1
RBX1
UBC
PCNA
CDT2
DDB1
CUL4
Cyclin A
or cyclin B
CDK1
APC1
CDC20
APC4
APC11
APC5
APC2
APC7
APC3
APC10
APC6
APC8
MND2
SWM1
G1
S
G2
M
Adaptor protein
Ubiquitin-conjugating enzyme (E2)
Ubiquitin ligase (E3)
Substrate recognition factor
RING finger protein
Scaffold subunits

Multiple CRLs are utilized to regulate cell cycle progression by targeting many key cell cycle proteins. Here, we will focus specifically on the targeted depletion of p21. There are three E3 ligases known to target p21 for degradation: SCF^{SKP2}, $CRL4^{CDT2}$ and APC/C^{CDC20} (1) (See figure 1-11, bottom). SCF^{SKP2} targets p21 during the G1/S transition and S phase, and promotes the ubiquitylation and degradation of p21, which has been phosphorylated by CDK2 on Ser-130 (41, 423). This activity de-represses CDK2 and allows cell cycle progression to resume. $CRL4^{CDT2}$ targets p21 for degradation during S phase and also during an active DDR (1). The activity of this ligase requires the interaction of the E3 ligase complex and p21 with PCNA and chromatin (3, 317). There are key residues within p21 which are critical for the activity of this ligase, the PIP box domain (PCNA-interacting protein), required for PCNA interaction, and the "+4" arginine four residues downstream of the PIP box responsible for recruitment of $CRL4^{CDT2}$ to chromatin (171). Destruction of p21 permits PCNA activity to resume, which is required for polymerase-δ DNA replication, which allows cellular DNA replication to recommence following DNA damage repair or resolution of other cellular stress (240). The final E3 ligase known to target p21 for proteolysis is APC/C^{CDC20} (anaphase-promoting complex – cell division cycle 20). This E3 ligase recognizes Cyclin A-CDK1 or Cyclin B-CDK1 bound forms of p21. The degradation of these bound forms of p21 allows for CDK1 activity which is critical for entry and progression into mitosis (15).

VIRAL REGULATION OF p21

Given its central role in cell cycle arrest and cellular proliferation, it is unsurprising that many viruses, especially tumor viruses, have found ways to exploit p21 for their own purposes. HPV E6 protein can downregulate p21 activity independently of p53, which promotes apoptosis in the infected cell (135). During an HPV coinfection, AAV2 downregulates p21, which results in an increase in Cyclin E-CDK2 activity, preventing further progression through S phase (13). HCV can utilize its core protein to inhibit p21 by targeting multiple pathways, including repressing the p21 promoter through inhibition of the TGF-β pathway and at a post-translational level by targeting it for proteasomal degradation and caspase cleavage (249, 329, 443). This results in alleviating CDK2 inhibition and promotes, at least in part, HCV-mediated tumorigenesis. Finally, the oncogenic human gammaherpesvirus Kaposi's sarcoma-associated virus (KSHV) employs a virally encoded microRNA, miR-K1, to repress p21 at the post-transcriptional level, which contributes to cellular transformation and tumorigenesis (156, 296).

THE MITOTIC CYCLIN, CYCLIN B

Maturation-promoting factor (MPF) was discovered in 1971 to be the driving force mediating mitotic entry (280). It was later shown that MPF was a complex of cyclin B1 and CDK1 (149). There are two B-type cyclins, Cyclin B1 and Cyclin B2, which are generated from different genes, CCNB1 and CCNB2, respectively. The two B-type cyclins differ in their cellular localization and function. When activated, Cyclin B1 is localized to the nucleus at mitotic spindles and is involved in chromosomal alignment, microtubule

attachment and disassembly of the nuclear lamina (67). Cyclin B2 is localized in the cytoplasm, primarily bound to the golgi apparatus, and is specifically involved in disassembly of the golgi apparatus, which allows an approximately equal number of vesicles to go to each daughter cell after telekinesis (121). Interestingly, swapping the N-terminal region responsible for subcellular localization could direct Cyclin B1 to the cytoplasm and confer to it the ability to disassemble the golgi apparatus (121). Redirecting Cyclin B2 in a similar manner does not result in microtubule reorganization or nuclear lamina breakdown, suggesting that while Cyclin B1 can substitute for Cyclin B2 function, the reverse is untrue. Furthermore, mice can tolerate deletion of Cyclin B2, which suggests that Cyclin B1 can indeed compensate for the loss of Cyclin B2 (43). In this chapter, the term Cyclin B will refer exclusively to Cyclin B1.

Cyclin B has been described as the quintessential mitotic cyclin. The critical regulatory step for the activation of the Cyclin B-CDK1 complex is the level of Cyclin B protein available to bind CDK1 and form a complex (253). As a result, Cyclin B expression is controlled at multiple levels including subcellular localization, protein degradation and transcriptional regulation. Cyclin B shuttles between the nucleus and the cytoplasm as many cell cycle regulators do (253). The nuclear export of Cyclin B outweighs the nuclear import during much of the cell cycle resulting in a predominantly cytoplasmic localization (163, 438). During late G2 and early M phase, phosphorylation of the cytoplasmic retention sequence (CRS) enhances Cyclin B nuclear import up to 40-fold (162, 297). It has been suggested that as Cyclin B accumulates in the nucleus and the Cyclin B-CDK1 complex becomes activated, Cyclin B-CDK1 phosphorylates nuclear pore

complexes, which accelerates Cyclin B nuclear import at a higher rate than phosphorylation of the CRS alone can achieve (150).

Once sufficient levels of Cyclin B have been generated, the formation of the Cyclin B-CDK1 complex is not sufficient to trigger entry into mitosis. CDK1 must be phosphorylated in its T loop on Thr-161 by CAK, a heterodimer of Cyclin H and CDK7, to be active (55). CDK1 has additional residues which regulate its activity, Thr-14 and Tyr-15, which, contrary to archetypal activation *via* phosphorylation, keep the complex inactive when phosphorylated (253). Phosphorylation of Thr-14 and Tyr-15 is balanced by the activities of the Wee1 and Myt1 kinases and the CDC25 phosphatases. Interestingly, it has been suggested that the phosphorylation events of Thr-161 and Thr-14 are coupled, providing further regulation to prevent the premature activation of the Cyclin B-CDK1 complex (103).

Once the activity of the Cyclin B-CDK1 complex reaches a threshold level required for activity, cells enter mitosis, segregate their chromosomes and disintegrate their nuclear lamina. The Cyclin B-CDK1 complex is maintained in the nucleus during these mitotic events until the spindle checkpoint proteins—Mad1, Mad2, Bub1, BubR1 and Mps1—release the cell from the spindle assembly checkpoint (SAC) (413). Following release from the SAC, the APC/C^{CDC20} E3 ubiquitin ligase, with the APC10 co-activator bound, targets Cyclin B for proteasomal degradation thus reducing the activity of the Cyclin B-CDK1 complex below threshold levels (196, 413).

Transcriptional regulation of Cyclin B is the final layer of regulation that will be discussed in this chapter. The Cyclin B promoter is complexly regulated and contains

binding sites for multiple transcription factors including NF-Y (CCAAT-boxes), SP1 (GC-boxes), Myc (E-boxes), Myb-MuvB (cell cycle genes homology region (CHR)), and p53 (p53-responsive element) (59, 80, 139, 195, 229, 254, 285, 306, 442). NF-Y is bound as a heterotrimer at the Cyclin B promoter and synergizes with the p300 cofactor to activate transcription (426). p53 represses Cyclin B transcription, usually in response to DNA damage, in a NF-Y- and p21-independent, SP1-dependent manner by binding as a dimer to head-to-tail p53 responsive elements in the promoter (195, 229, 254).

It has been suggested that the CHR provides the largest contribution to the transcriptional activation of Cyclin B (426). Many cell cycle genes, especially G2/M associated genes, are regulated by CHR sites, including Cyclin A, Cyclin B1 and B2, CDK1, CDC25 and Polo-like kinase-1 (PLK1) (305). The promoters of CHR regulated genes can be divided into two classes: those with a cell-cycle dependent element (CDE), generally located four nucleotides upstream of the CHR (Class I), and those without a CDE (Class II), such as Cyclin B1. Transcriptional activation *via* CHR/CDE sites still requires binding of the NF-Y transcription factor (59, 305, 426), perhaps acting as a pioneer factor (136).

Recently, it has been discovered that CHR promoter elements do indeed play a key role in regulating cell cycle-dependent gene regulation, and this is accomplished by binding of the DREAM and MMB complexes (306). The DREAM complex is a multisubunit complex formed by: DP (p130-E2F4 Dimerization Partner), RB-like pocket proteins p130 and p107, the repressive E2F4 and MuvB (multi-vulval class B) (369). MuvB is a complex of five proteins: LIN9, LIN37, LIN52, LIN54 and RBBP4 (369, 370). The DREAM complex has been shown to repress most, if not all, cell cycle gene expression

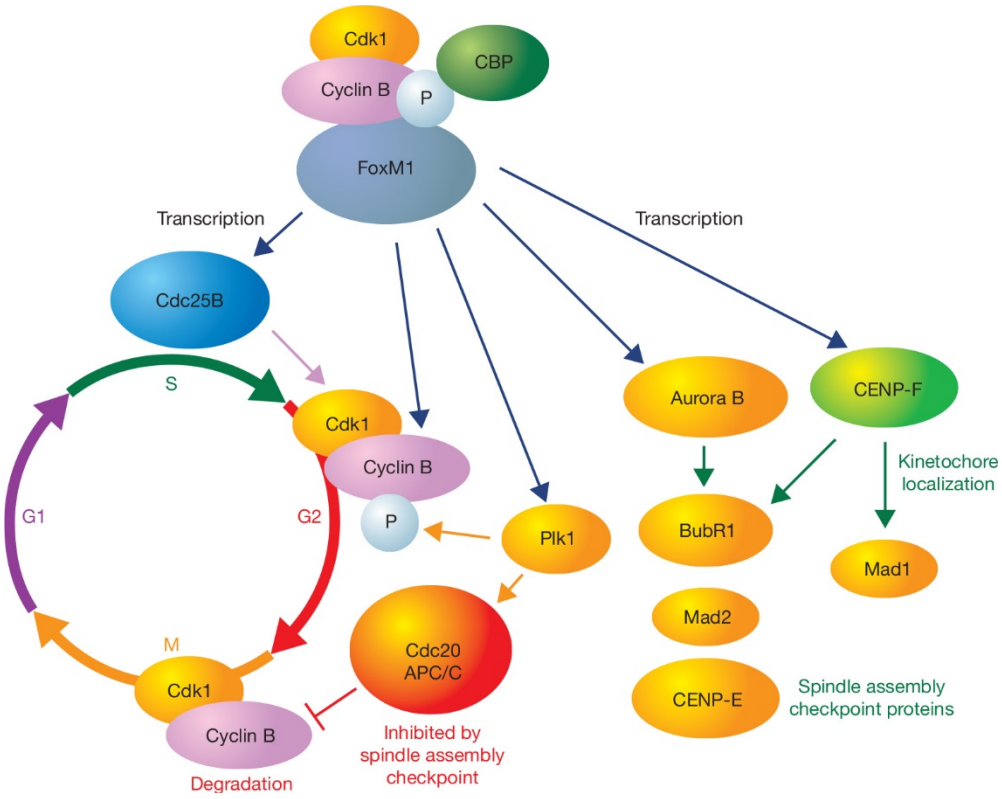
during quiescence (G0) (369). The phosphorylation of Ser-28 in LIN52, a component of MuvB, by dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) is required for p130 binding to the MuvB core and DREAM complex assembly (255). As cells transit through the cell cycle (G1 to S), the repressive elements of the DREAM complex—DP, p130, p107 and E2F4—dissociate from the complex. MuvB, which is still bound to the CHR element, then successively recruits B-MYB (forming the MMB complex) and the forkhead transcription factor, FoxM1 in late S phase and G2/M phase (306, 370) (See figure 1-12 bottom).

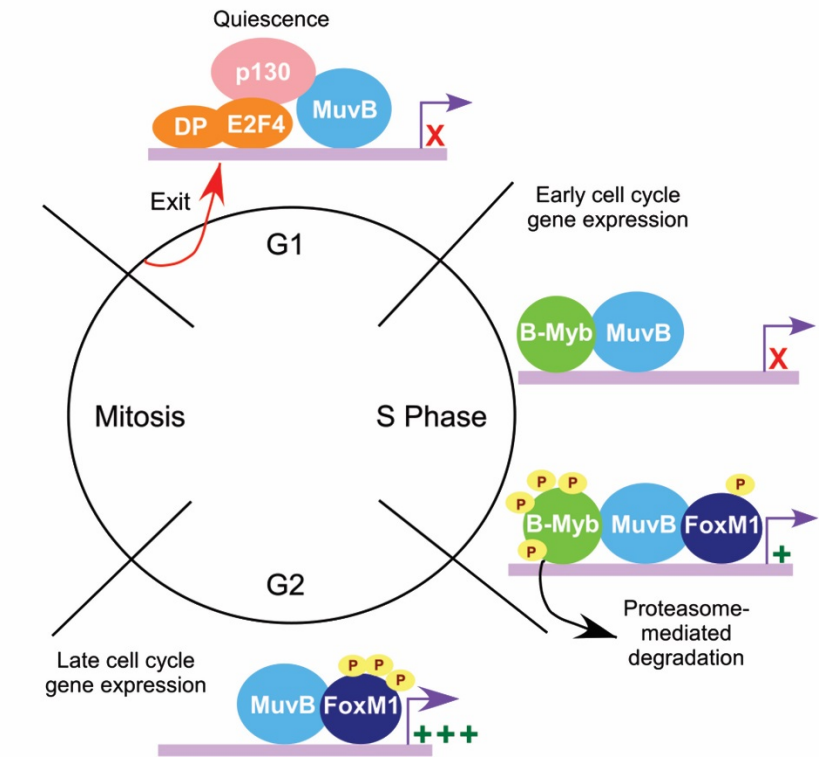
The Forkhead Box M1 (FoxM1) transcription factor, which is a member of the forkhead box (Fox) transcription factor family, is a critical regulator of Cyclin B transcription (86). Fox transcription factors share a highly conserved DNA-binding domain (DBD), which typically bind a conserved (A/C)AA(C/T) sequence, a forkhead box or winged helix domain (WHD) and a transactivation domain (TAD) (308). Fox proteins may also possess repressor domains, nuclear export signals, nuclear localization signals, and in the case of FoxM1, LXL motifs, which allow interaction with CDKs (236). FoxM1 is a 763 amino acid protein which autorepresses its own activity through an N-terminal repressive domain, which is alleviated by Cyclin A-CDK binding to two RXL / LXL motifs in the C-terminus (241). Once FoxM1 is derepressed, it requires further phosphorylation to become hyperphosphorylated and activate a bound promoter (236). CDK1, CDK2, CDK4, CDK6, Chk2, ERK1, ERK2, and PLK1 have all been reported to phosphorylate FoxM1 at the following residues: Ser-251, Ser-331, Ser-361, Ser-264, Thr-596, Thr-600, Thr-611, Ser-678, Thr-704 (16, 68, 138, 241, 272, 405). CDK4/6 and PLK1 phosphorylation of the

Figure 1-12: Regulation of mitotic entry and mitotic gene transcription by FoxM1

Top: The activity of the Cyclin B-CDK1 complex is required for entry into mitosis. The Cyclin B-CDK1 complex also acts in a positive feedback loop by activating the transcription factor, FoxM1. FoxM1 in turn transcriptionally upregulates a number of key mitotic genes including Cyclin B1, Polo-like kinase 1 (PLK1), which phosphorylates Cyclin B1 mediating its nuclear import, and Aurora Kinase B and Bub1, which are critical for chromosome segregation and spindle assembly. Adapted from (86).

Bottom: The multisubunit DREAM complex is composed of DP (p130-E2F4 Dimerization Partner), RB-like p130 or p107, the repressive E2F4 and MuvB (multi-vulval class B). The DREAM complex represses all cell cycle gene expression during G0 by binding to gene promoters. As cells enter and progress through the cell cycle the repressive DP, p130 or p107 and E2F4 are released from the complex and MuvB successively recruits B-Myb and the transcription factor FoxM1. FoxM1 is phosphorylated by several kinases and becomes transcriptionally active as it becomes hyperphosphorylated. Adapted from (370).


Cdk1
CBP
Cyclin B
P
FoxM1
Transcription
Transcription
Cdc25B
S
Cdk1
Cyclin B
P
G1
G2
Plk1
Aurora B
CENP-F
Kinetochore localization
BubR1
Mad1
Mad2
CENP-E
Spindle assembly checkpoint proteins
M
Cdk1
Cyclin B
Degradation
Cdc20 APC/C
Inhibited by spindle assembly checkpoint


Quiescence
p130
DP
E2F4
MuvB
X
Exit
G1
Early cell cycle gene expression
B-Myb
MuvB
X
Mitosis
S Phase
P
B-Myb
MuvB
FoxM1
+
G2
Proteasome-mediated degradation
Late cell cycle gene expression
MuvB
FoxM1
+++

FoxM1 TAD have been shown to be critical for its activity (16, 138). In addition to Cyclin B, FoxM1 is a critical transcription factor for other G2/M genes, including Aurora Kinase B, CDC25B, CENP-F and PLK1 (86) (See figure 1-12 top).

Unsurprisingly, given their importance to cell cycle transcriptional regulation, viruses have evolved the capacity to target several proteins involved in CHR transcriptional regulation. HPV's early E7 protein interacts with the pocket proteins p107 and p130 and targets them for degradation, potentially derepressing the DREAM complex (358). HPV E7 also interacts with B-Myb, FoxM1 and Lin9 (MuvB component), allowing E7 to directly activate cell cycle gene transcription independent of pocket proteins (334). Adenovirus E1A and SV40 large T-antigen can bind a weaker LxSxExL motif in Lin52 (MuvB component), which the authors suggest enables the DREAM complex to be disassembled by competing, stronger binding, viral oncoproteins (158). Hepatitis B, instead of targeting the DREAM complex, upregulates FoxM1 promoting tumor metastasis and hepatocellular carcinoma through its X protein (435).

VIRAL REGULATION OF G2/M CELL CYCLE ARREST

Viruses of all types, both DNA and RNA, have been shown to induce a G2/M cell cycle arrest using very diverse methods, either during infection and/or *via* expression of viral proteins (109). JC polyomavirus and HPV-type 1 (HPV1) inhibit or delay the activation of Cyclin B-CDK1 by inducing p21 with the JC polyomavirus agnoprotein or by utilizing the HPV1 E4 protein to maintain CDK1 inhibitory phosphorylation (108, 223). HPV16's E4 protein is able to sequester the Cyclin B-CDK1 complex to the cytoplasm,

thus preventing its nuclear localization and activity (110). Chicken anemic virus (CAV) and HSV-1 allow cells to enter mitosis, but then prevent mitotic exit by inhibiting the APC with apoptin protein or interfering with kinetochores with Vmw110 and ICP0, respectively (127, 260, 410). HIV VPR induces a well-studied G2 arrest with VPR, but the mechanism remains unclear (109). Finally, parvoviruses, specifically AAV2 and MVM, have been shown to induce a G2 block from the presence of the viral genome alone (96, 330, 354).

G2/M ARREST AND THE VIRAL LIFECYCLE

Although the methods utilized by viruses to induce and maintain a G2/M block have been relatively well-studied, less work has been targeted at understanding *why* some viruses go to such great lengths to induce G2/M arrest. One prediction is that simply preventing new cell production itself is beneficial to the virus (109). Some viruses, such as HSV1, HPV6 and HIV seem to be more transcriptionally active during G2. As an extension, there are a number of viruses, such as SV40, HPV31, parvoviruses and baculoviruses that seem to prefer replicating in an extended, pseudo-S phase (109). In this work, we show that MVM induces a pre-mitotic cell cycle block by preventing FoxM1 binding, and as a result RNA polymerase II, to the Cyclin B promoter.

2 Efficient parvovirus replication requires CRL4^{Cdt2}-targeted depletion of p21 to prevent its inhibitory interaction with PCNA

2.1 Introduction

Minute Virus of Mice (MVM) is an autonomously-replicating parvovirus which induces a DNA damage response resulting in substantial p53 activation which persists throughout the course of viral replication (7). p53 is a well-established activator of p21$^{WAF1/Cip1}$ (hereafter referred to as p21) expression. Transient expression of the MVM NS1 protein alone also leads to an increase in p21 levels (10, 331). However, surprisingly, while these signals lead to an increase in p21 RNA accumulation, p21 protein levels remain low throughout the course of infection, including during the prolonged G2 phase in which the viral genome is replicated (10). p21 can be a potent antiviral factor and possesses several potentially inhibitory activities including cyclin-dependent kinase (CDK) inhibition and repression of E2F1-mediated expression (1). In addition, p21 has been shown to be an effective inhibitor of the DNA polymerase δ cofactor PCNA (64, 65, 419), and it has been shown to inhibit MVM replication *in vitro* (31). p21 depletion during MVM infection was shown to be proteasomally mediated, suggesting that an E3 ubiquitin ligase was involved in targeting p21 for degradation (10).

Viruses often make use of the ubiquitin conjugation machinery to target for degradation cellular proteins that might otherwise negatively affect viral replication (357). The Cullin-RING Ligase (CRL) CRL4^{Cdt2} consists of the scaffold protein

Cullin 4 and the homo-trimeric protein DDB1 which serves as an adaptor for the putative substrate recognition protein Cdt2. This ligase has been shown to program the ubiquitination and subsequent degradation of p21 in response to DNA damaging agents such as UV treatment in order to ensure low p21 levels during S-phase (3, 219, 317). Upon DNA damage or S-phase entry $CRL4^{Cdt2}$ is recruited to chromatin *via* PCNA interaction where it targets substrate proteins for degradation (172).

We show here that efficient MVM replication in S/G2 arrested cells required the targeting for proteasomal degradation of p21 by the $CRL4^{Cdt2}$ E3-ubiquitin ligase which was re-localized to viral chromatin within active MVM replication centers. PCNA provides a molecular platform that aids substrate recognition by the $CRL4^{Cdt2}$ E3-ubiquitin ligase, and p21 targeting to this ligase during MVM infection required its interaction with PCNA. PCNA is also an important co-factor for DNA polymerase δ-dependent MVM replication which can be antagonized by p21 *in vitro*. Expression of a stable p21 mutant that retained interaction with PCNA inhibited MVM replication, while a stable p21 mutant which could no longer interact with PCNA did not. Introduction of a p21-derived peptide that bound to PCNA also substantially decreased viral replication. Our results suggest that interaction with PCNA was important for targeting p21 to the re-localized $CRL4^{Cdt2}$ubiquitin ligase, yet subsequent depletion of p21 was required to prevent its sustained interaction with PCNA which otherwise inhibited efficient viral replication.

2.2 RESULTS

The CRL4^{Cdt2} ligase mediates p21 degradation during MVM infection

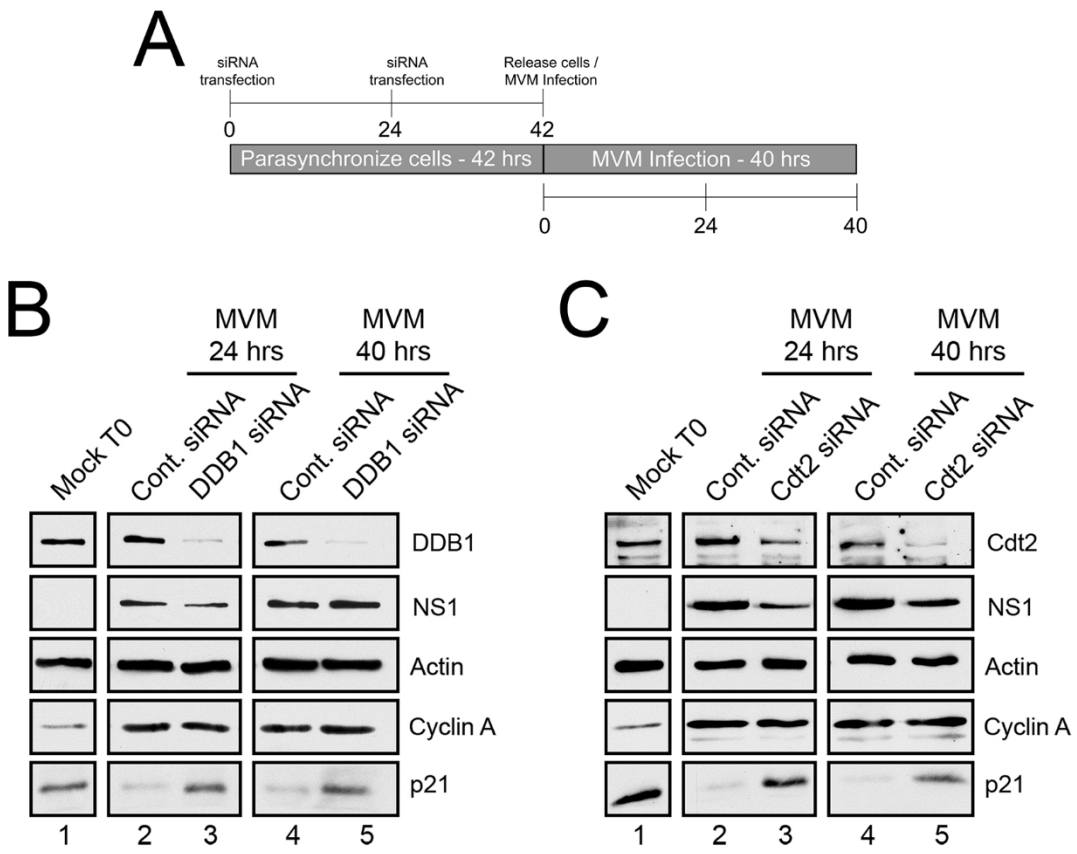
The CRL4^{Cdt2} E3 ubiquitin ligase has been implicated in targeting p21 for proteasomal degradation upon S-phase entry and after cellular DNA damage (3, 219, 317). Was this ubiquitin ligase also enlisted to target p21 at late times during MVM infection when cells were blocked at the G2/M border? To test this possibility, DDB1 and Cdt2, components of this ligase which are not present in other E3 ubiquitin ligases known to modify p21 (172, 395), were targeted *via* RNAi in the protocol illustrated in Figure 1A.

For these experiments cells were parasynchronized prior to infection to maximize the number of cells progressing uniformly through S-phase. This both synchronized infection and the characterization of p21 depletion. At the time of release from the synchronization procedure (as the cells progressed from G0 to G1) there were high levels of p21 expression (Mock T0, Figures 1B and 1C, lanes 1), which were reduced 24 hr post MVM infection (Figures 1B and 1C, lanes 2), and remained low up to 40 hr pi (Figures 1B and 1C, lanes 4). Targeting of endogenous DDB1 (panel 1B) or Cdt2 (panel 1C) by RNAi, which led to significant depletion of these proteins (Figure 1B and 1C, lanes 3 and 5), substantially prevented the loss of p21 both at 24 hr pi (Figures 1B and 1C, lanes 3), and also 40 hr pi (Figure 1B and 1C, lanes 5) – the latter being a point well past S-phase when infected cells are known to be arrested in G2 (7). Expression of cyclin A

Figure 2-1: p21 degradation is mediated by the CRL4^{Cdt2} ligase complex.

A) Schematic illustrating the experimental protocol for siRNA knockdown of ligase components in Figures 1B and 1C.

B and C) ***p21 degradation requires DDB1 (B) and Cdt2*** *(C)*. Murine A9 cells were targeted with control siRNA or siRNA to DDB1 (B) or Cdt2 (C) as depicted in Figure 1A. Uninfected control cells were harvested at the time of release (Mock T0). Infections were done at the time of release at an MOI of 10 before harvest at 24 and 40 hr pi. Western blots were performed using antibodies against the indicated proteins.


A
siRNA transfection
siRNA transfection
Release cells / MVM Infection
0
24
42
Parasynchronize cells - 42 hrs
MVM Infection - 40 hrs
0
24
40
B
MVM 24 hrs
MVM 40 hrs
Mock T0
Cont. siRNA
DDB1 siRNA
Cont. siRNA
DDB1 siRNA
DDB1
NS1
Actin
Cyclin A
p21
1
2
3
4
5
C
MVM 24 hrs
MVM 40 hrs
Mock T0
Cont. siRNA
Cdt2 siRNA
Cont. siRNA
Cdt2 siRNA
Cdt2
NS1
Actin
Cyclin A
p21
1
2
3
4
5

indicated unperturbed entry into S-phase (Figures 1B and C, lanes 2–5), and expression of the viral NS1 protein indicated that the initiation of viral infection was unaffected by the RNAi protocol (Figures 1B and C, lanes 2–5). Similar results were also obtained following knockdown of Cullin 4A, a component of the $CRL4^{Cdt2}$ligase also present in several other ubiquitin ligases (data not shown). Taken together, these results indicated that the $CRL4^{Cdt2}$ ligase was responsible for p21 degradation throughout virus infection.

$CRL4^{Cdt2}$ also targets Set8 and Cdt1 for degradation during S-phase and in response to DNA damaging agents (2, 58, 203). We also observed loss of Set8 in response to MVM infection (Figure S1). Cdt1 levels were not reduced for reasons not yet clear.

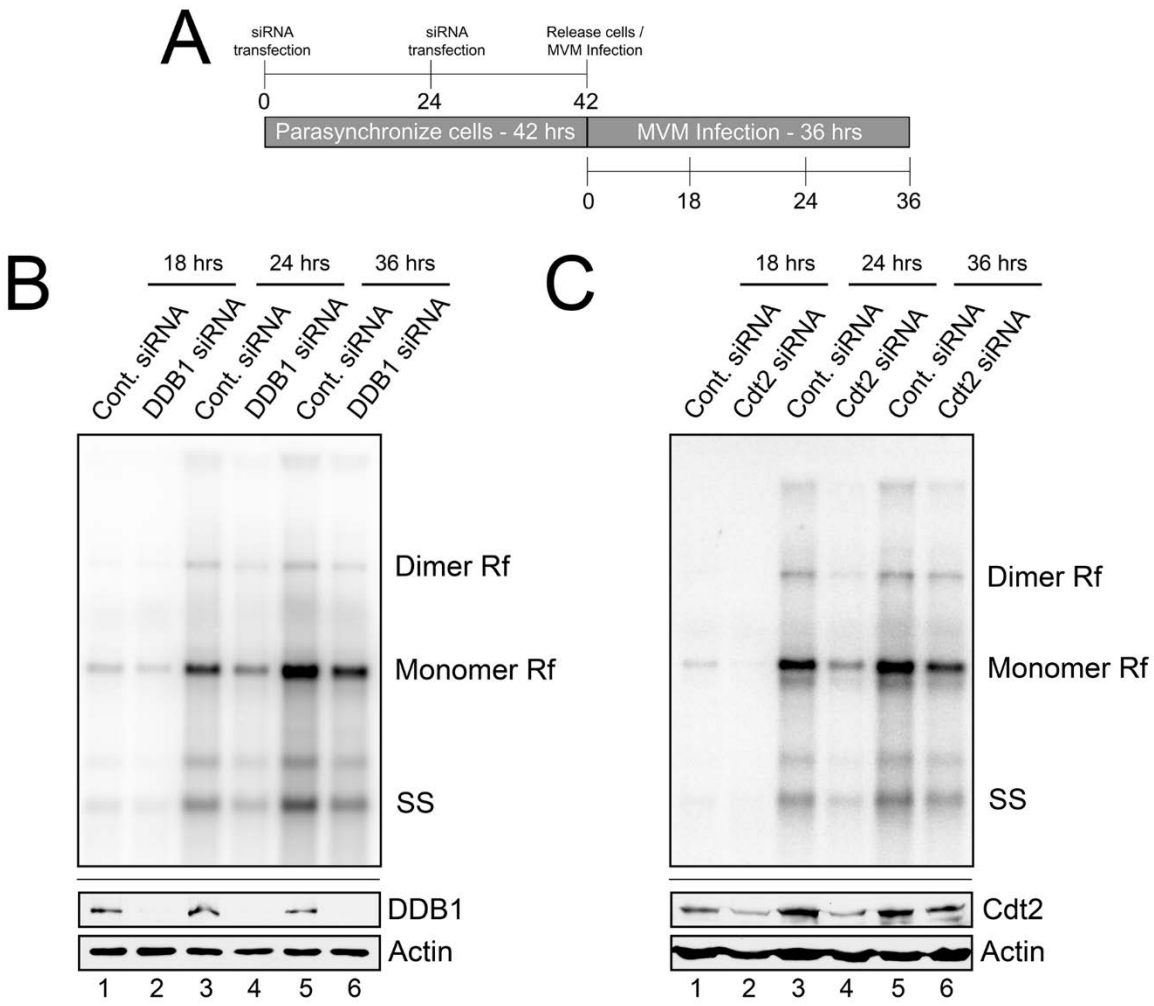
$CRL4^{Cdt2}$ ligase function is required for efficient MVM replication

We next examined the functional consequence of $CRL4^{Cdt2}$ E3 ligase depletion for viral replication using the protocol illustrated in Figure 2A. DDB1 knockdown resulted in an approximate 2-fold decrease in accumulated viral replicative forms at each time point compared to application of negative control siRNA (Figure 2B, compare lanes 1 to 2, 3 to 4, 5 to 6). Cdt2 knockdown during infection resulted in an approximate 2.5-fold decrease in accumulated viral replicative forms at each time point when compared to negative control siRNA (Figure 2C, compare lanes 1 to 2, 3 to 4, 5 to 6). Importantly, as mentioned above, expression of NS1 (Figure 1), and flow cytometric analysis (Figure S2), confirmed normal entry into S-phase following this synchronization and RNAi protocol which thus did not affect the S-phase dependent initiation of infection. These results

Figure 2-2: The CRL4^{Cdt2} E3 ligase complex is important for MVM replication.

A) Schematic showing the experimental protocol for figures 2B and 2C.

B and C) *Upper panels:* murine A9 cells treated with siRNA as shown in 2A were infected at an MOI of 0.5, harvested at the indicated time points and processed for Southern blotting using an MVM genomic probe. Rf - replicative forms. SS - single stranded genomic DNA. Representative Southern Blots are shown; quantifications in the text reflect two DDB1 and three Cdt2 separate knockdown experiments. *Lower panels:* western blots show knockdown of DDB1 and Cdt2 done in parallel experiments under identical conditions to replication assays.


A
siRNA transfection
siRNA transfection
Release cells / MVM Infection
0
24
42
Parasynchronize cells - 42 hrs
MVM Infection - 36 hrs
0
18
24
36
B
18 hrs
24 hrs
36 hrs
Cont. siRNA
DDB1 siRNA
Cont. siRNA
DDB1 siRNA
Cont. siRNA
DDB1 siRNA
Dimer Rf
Monomer Rf
SS
DDB1
Actin
1 2 3 4 5 6
C
18 hrs
24 hrs
36 hrs
Cont. siRNA
Cdt2 siRNA
Cont. siRNA
Cdt2 siRNA
Cont. siRNA
Cdt2 siRNA
Dimer Rf
Monomer Rf
SS
Cdt2
Actin
1 2 3 4 5 6

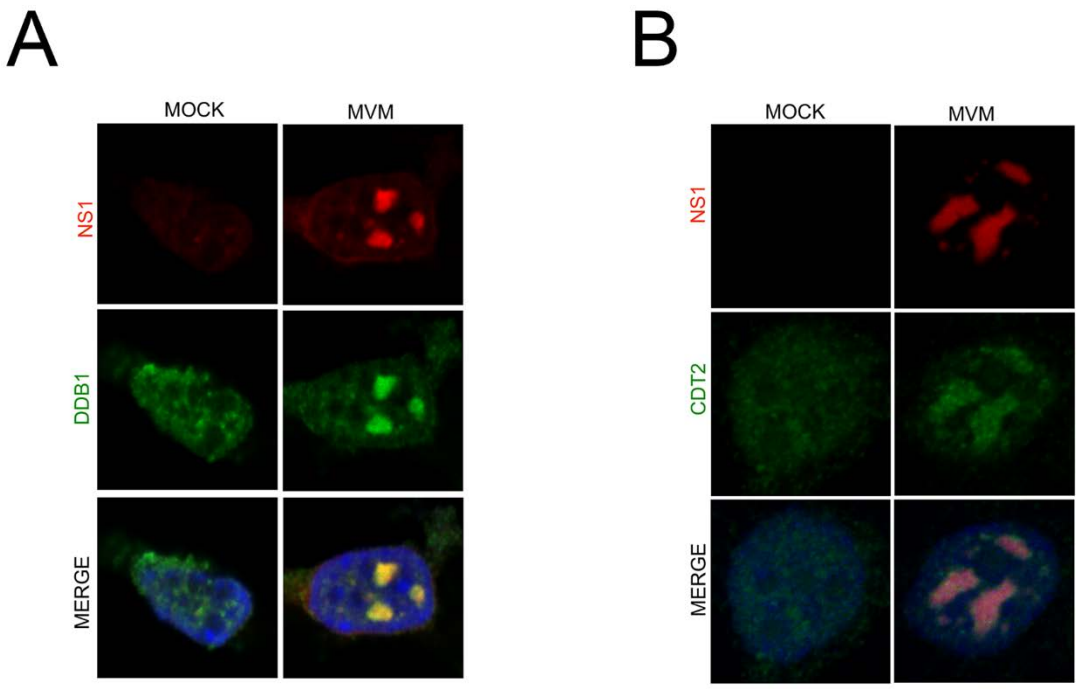
demonstrated that the activity of the $CRL4^{Cdt2}$ E3 ubiquitin ligase was necessary for efficient viral replication.

The $CRL4^{Cdt2}$ ligase is recruited to viral replication compartments

MVM replicates in nuclear compartments termed autonomous parvovirus associated replication (APAR) bodies which have been shown to be enriched for cellular proteins such as DNA polymerase δ, RPA, cyclin A, and PCNA, which are also essential for parvovirus replication (32). These nuclear bodies have been shown, *via* BrdU staining, to serve as sites of ongoing viral replication in infected cells and can be visualized by staining for the viral replicator protein NS1 (105). Importantly, whereas punctate staining distributed throughout the nucleus was observed in mock treated cells, we detected recruitment of both DDB1and Cdt2 to NS1-containing viral replication compartments. Resistance to detergent pre-extraction prior to immunofluorescence (Figure 3A and 3B, note the merged images for each) also suggested that it may be bound to viral chromatin. These results indicated that MVM infection redirected the viral replication-enhancing $CRL4^{Cdt2}$ ligase complex to APAR bodies. The recruitment was specific for the $CRL4^{Cdt2}$ ligase because the APC/C^{Cdc20} E3 ligase, which targets p21 for degradation after mitotic entry (15), was not similarly recruited (Figure S3). This is the first demonstration of the specific recruitment of a cellular E3 ubiquitin ligase to parvoviral replication compartments.

Figure 2-3: The $CRL4^{Cdt2}$ ligase is recruited to viral replication compartments.

A and B) Murine A9 cells were mock infected or infected with MVM at an MOI of 10. At 24 hr pi cells were pre-extracted using cytoskeletal buffer and processed for IF using antibodies against NS1 and DDB1 (A) or Cdt2 (B).


A
MOCK
MVM
NS1
DDB1
MERGE
B
MOCK
MVM
NS1
CDT2
MERGE

Depletion of p21 during MVM infection requires its interaction with the CRL4^{Cdt2} ligase and PCNA

The DNA polymerase δ cofactor PCNA is known to target the CRL4^{Cdt2} E3 ubiquitin ligase to cellular chromatin *via* its interaction with Cdt2 during normal cell division (170, 172). Ubiquitin modification of p21 by the CRL4^{Cdt2} ligase requires Cdt2 interaction with the p21 degron motif, as well as interaction between p21 and PCNA *via* its PCNA-interaction (PIP) box (171). p21 is recruited to UV-induced DNA lesions *via* interaction with PCNA, and a p21 mutant defective in binding to PCNA was resistant to degradation following UV treatment (3, 317).

To investigate the importance of these interactions for the MVM-dependent targeting of p21 by the CRL4^{Cdt2} ligase we generated stable murine cell lines *via* lentivirus transduction that conditionally expressed FLAG-tagged wild-type or mutant p21 (p21^{WT}, p21$^{\Delta Degron}$, p21$^{\Delta PCNA}$, mutations shown in Figure 4A) in a doxycycline-responsive manner. As expected, MVM infection of a p21^{WT} expressing cell line resulted in degradation of the tagged p21 (Figure 4C and 4E, compare lanes 2 to 4), which could be prevented by treating cells with the proteasome inhibitor MG132 (Figure S4, panel A), or *via* siRNA knockdown of CRL4^{Cdt2} components (Figure S4, panels B and C). These results suggested that the depletion of p21 in these cell lines occurred *via* a similar mechanism to that of the endogenous p21. The lysine at the p21 +4 position, 3' to its PIP box, has been reported to be required for interaction of p21 with the CRL4^{Cdt2} ligase complex *via* Cdt2.

Figure 2-4: p21 degradation during MVM requires interaction with PCNA and the CRL4^{Cdt2} ligase complex.

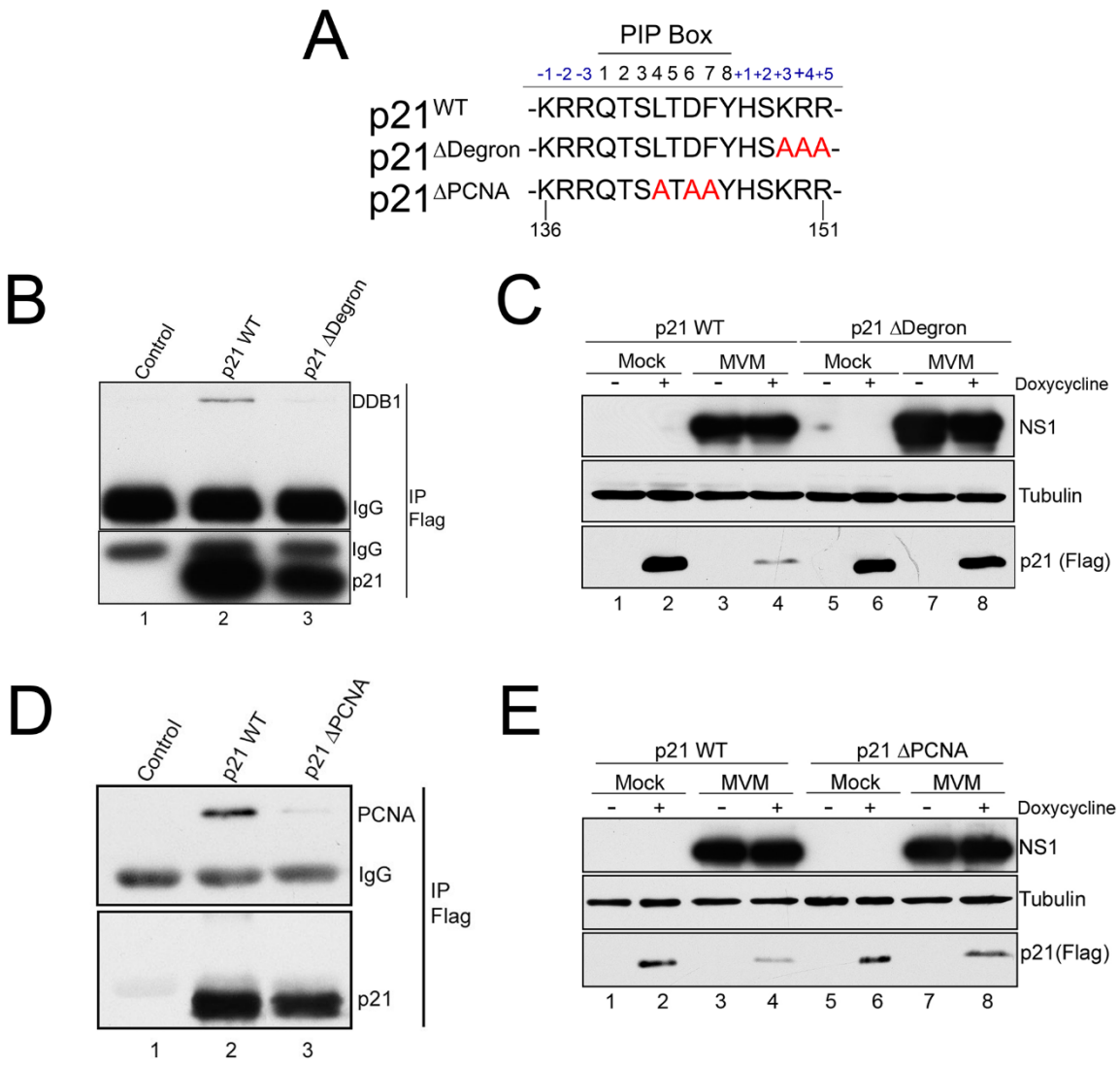
A) Illustration of the p21 PIP/degron region (amino acid 136 to 151) of wild-type murine p21 (p21^{WT}), p21$^{\Delta degron}$, and p21$^{\Delta PIP}$. Mutations are shown in red.

B) ***p21$^{\Delta degron}$ does not interact with the CRL4^{Cdt2} complex***. 293T were cells transfected with constructs expressing FLAG-tagged p21^{WT} (lane 2), p21$^{\Delta degron}$ (lane 3) or control plasmid (lane 1). Cells were harvested at 48 hr. Lysates were immunoprecipitated using with FLAG antibody and blotted against the indicated proteins.

C) ***MVM degradation of p21 requires its interaction with the CRL4^{Cdt2} ligase complex***. Murine A9 cell lines stably expressing p21^{WT} and p21$^{\Delta degron}$ were generated as described. Cells were parasynchronized, released into complete media and mock-infected or infected with MVM at an MOI of 10. At 20 hr pi cells were treated with doxycycline to induce p21 expression. Cells were harvested 6 hrs later and processed for western blotting using the indicated antibodies.

D) ***p21$^{\Delta PCNA}$ does not interact with PCNA***. Experiment performed as for Figure 4B.

E) ***MVM degradation of p21 requires its interaction with PCNA***. Experiment performed as for 4C.



Experiments in figures 4B & 4D were performed by Richard Adeyemi.

Mutation of the +3 to +5 amino acids KRR to AAA ($p21^{\Delta degron}$) abolished p21 interaction with the $CRL4^{Cdt2}$ complex following its transient transfection as reflected by loss of interaction with DDB1 (Figure 4B, compare lanes 2 to 3). Murine cell lines that expressed the $p21^{\Delta degron}$ mutant were generated, and when infected with MVM, in contrast to cell lines expressing wild-type p21 ($p21^{WT}$), the $p21^{\Delta degron}$ protein was resistant to degradation (Figure 4C, compare lanes 6 and 8 to lanes 2 and 4). This suggested that MVM-induced p21 degradation required interaction of p21 with the $CRL4^{Cdt2}$ ubiquitin ligase complex.

The $CRL4^{Cdt2}$ ligase also requires PCNA as a cofactor for targeting of its substrates (172). Mutation of the p21 PIP box ($p21^{\Delta PCNA}$, Figure 4A) abrogated interaction with PCNA (Figure 4D, compare lane 3 to 2), and the $p21^{\Delta PCNA}$ protein expressed in murine cells was not degraded as efficiently as the wild-type protein ($p21^{WT}$) following infection (Figure 4E, compare lanes 8 and 6 to 4 and 2). These results suggested that PCNA-binding was also essential for effective $CRL4^{Cdt2}$ targeting of p21 during MVM infection.

Stable p21 that retains interaction with PCNA inhibits MVM replication

p21 interaction with PCNA has been shown to interfere with DNA polymerase δ−mediated cellular DNA replication (64, 65). PCNA is an important cofactor for MVM replication; it contributes to MVM replication *in vitro* (31), and is recruited to APAR bodies during infection. Thus, we investigated whether MVM-dependent depletion of

p21 during infection, mediated by the $CRL4^{Cdt2}$ E3 ubiquitin ligase, promoted efficient replication of the MVM genome by abrogating its inhibition of PCNA.

Unexpectedly, the $p21^{\Delta Degron}$ mutant, although stable, interacted poorly with PCNA for reasons not yet clear (data not shown). As a result, we could not use cell lines expressing this mutant to determine whether stabilized, p21 affected MVM replication *via* PCNA binding. Thus we generated a murine cell line conditionally expressing a mutant p21 in which all seven lysines in p21 were changed to arginine [$p21^{K7R}$, a similar mutation has been reported by others (33)]. Similar to the $p21^{\Delta Degron}$ mutant, the $p21^{K7R}$ protein was resistant to degradation following MVM infection (Figure 5A, compare lanes 6 and 8 to 2 and 4), yet retained substantial interaction with PCNA in transient transfection assays (Figure 5B, compare lane 3 to lanes 2 and 4). Whereas induction of $p21^{WT}$ expression for 8 hrs after infection had little effect on MVM replication (Figure 5C, compare lanes 1 and 2), $p21^{K7R}$ expression reduced replication by up to 3 fold (Figure 5C, compare lanes 3 and 4). Importantly, in contrast, the $p21^{\Delta PCNA}$ mutant-expressing cell line, which expressed a stable p21 which no longer could interact with PCNA (Figure 4D and 4E), failed to inhibit MVM replication upon induction (Figure 5D, compare lanes 3 and 4). This was also the case with the $p21^{\Delta Degron}$ mutant-expressing cell lines (data not shown). Furthermore, cell lines expressing a mutant of $p21^{K7R}$ in the PIP box-mutated background ($p21^{K7R\Delta PIP}$) (see Figure 5B, lane 4) also failed to inhibit MVM replication (Figure S5), demonstrating that absent PCNA binding, the K7R mutation itself had no deleterious effect on MVM

Figure 2-5: A stabilized p21 that binds to PCNA inhibits MVM replication.

A) ***p21 degradation by MVM requires a ubiquitin-modifiable lysine***. Murine A9 cell lines stably expressing HA-tagged wild-type p21 ($p21^{WT}$) or a mutant in which all seven lysines have been mutated to arginines ($p21^{K7R}$) were generated. p21 degradation assay was performed as described in 3C.

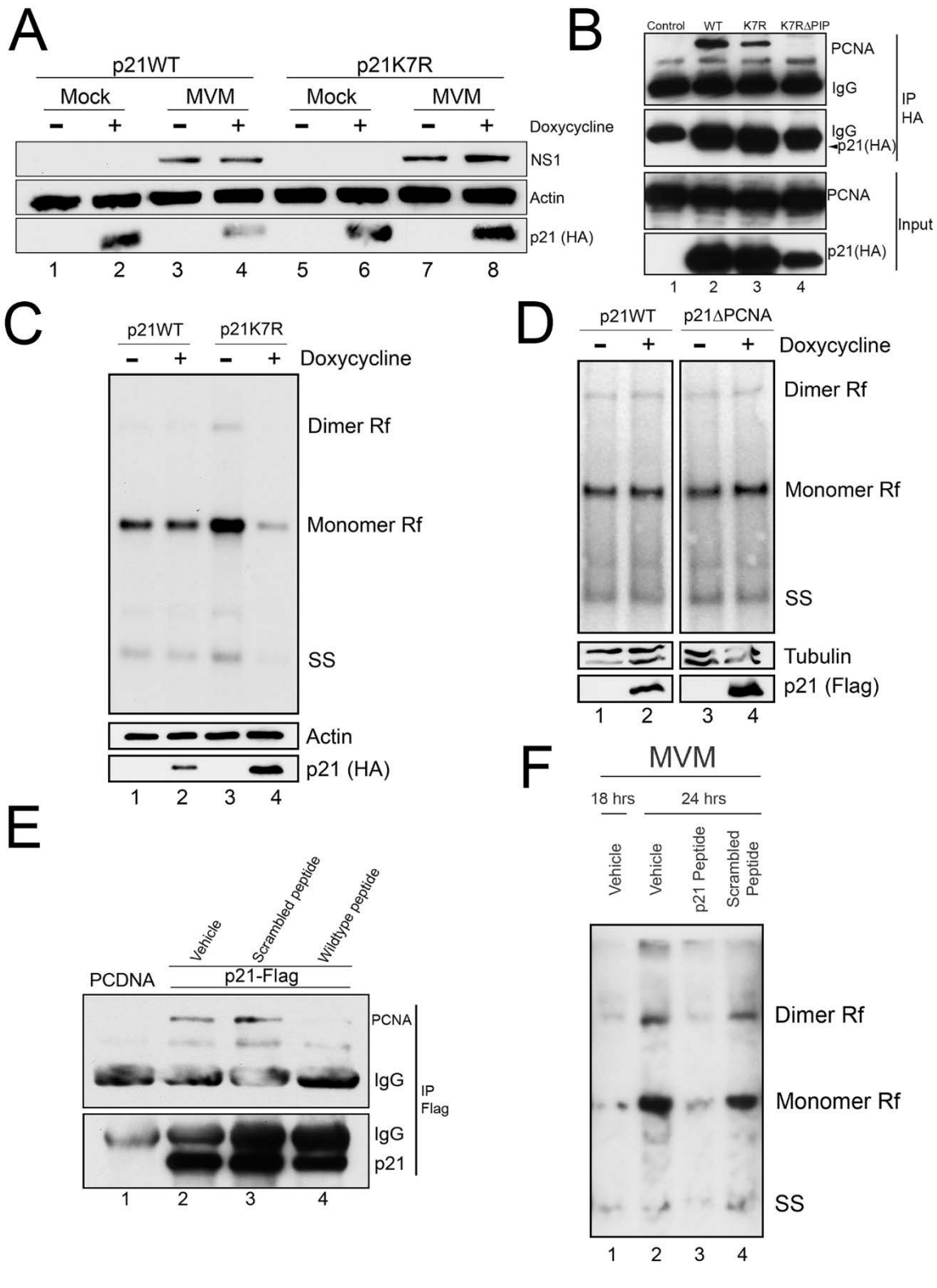
B) ***$p21^{K7R}$ retains interaction with PCNA***. Control plasmid (lane 1), $p21^{WT}$ (lane 2), $p21^{K7R}$ (lane 3), and $p21^{K7R}$ with additional mutations that disrupt PCNA interaction ($p21^{K7R\Delta PIP}$, lane 4) were transfected into 293T cells. At 48 hr cells were lysed, immunoprecipitated with HA antibodies and blotted using the indicated antibodies. The $p21^{K7R\Delta PIP}$ was expressed at lower levels in this experiment however interaction with PCNA was not detected even upon longer exposure.

C) ***$p21^{K7R}$ inhibits MVM replication***. $p21^{WT}$ and $p21^{K7R}$ cell lines were parasynchronized, released and infected with MVM at an MOI of 0.5. At 16 hr pi cells were treated with doxycycline to induce p21 expression and harvested 8 hrs later. Cells were processed for Southern blotting (top panel), or for western blotting using the indicated antibodies (bottom panels). A representative experiment is shown; quantifications in the text reflect three independent experiments.

D) ***$p21^{\Delta PCNA}$ does not inhibit MVM replication***. $p21^{WT}$ and $p21^{\Delta PCNA}$ cells were treated, processed and standardized as in 4C.

E) ***A p21 peptide competitively binds to PCNA***. 293T cells were transfected with a construct expressing FLAG-tagged p21 (lanes 2 to 4) or control plasmid (PCDNA, lane 1) and processed for co-immunoprecipitation as in 4D. Treatment with wild-type but not scrambled peptide reduced interaction of p21 with PCNA.

F) ***A p21-derived peptide inhibits MVM replication***. Murine A9 cells were parasynchronized, released and infected with MVM at an MOI of 1. At 18 hr pi control cells were harvested (lane 1) and remainder were treated with vehicle (lane 2), wild-type peptide (lane 3) or scrambled peptide (lane 4) for 6 hrs. At 24 hr pi cells were harvested and processed for Southern blotting. A representative experiment is shown; the experiment was done three times.



Experiments in figures 5B, 5E & 5F were performed by Richard Adeyemi.

replication. All the mutants tested were recruited to APAR bodies during MVM infection (Figure S6).

To confirm that the p21-PCNA interaction mediated the inhibitory role of p21 during infection, we made use of a previously described peptide containing 20 residues derived from sequences comprising the p21 PIP box (424) fused to a 16-mer penetratin motif to facilitate cellular entry (118). A scrambled version of the p21 peptide linked to the penetratin peptide was used as control. Following application to murine cells, the wild-type (Figure 5E, lane 4), but not the scrambled version (Figure 5E, lane 3), disrupted the interaction between over-expressed FLAG-tagged p21 and endogenous PCNA, demonstrating that the peptide could competitively interact with PCNA. Subsequently, while treatment of cells with the scrambled peptide had no effect on MVM replication (Figure 5F, compare lanes 2 and 4), treatment of cells with the wild-type p21 PCNA-binding peptide significantly inhibited MVM replication (Figure 5F, compare lanes 2 and 3). These results indicated that a stabilized p21 interaction with PCNA was detrimental to viral replication. Thus, while interaction with PCNA was important for targeting p21 to the re-localized $CRL4^{Cdt2}$ ligase, efficient viral replication required subsequent depletion of p21 and consequent abrogation of its inhibition of PCNA.

2.3 Discussion

Parvoviruses and other small DNA viruses rely on host polymerases to replicate their genomes. How the replication machinery is exploited to sustain parvovirus replication in G2-arrested cells, which normally contain potentially inhibitory cellular proteins such as p21, is not fully understood. p21 levels are high in G1, low in S-phase, restored in G2 phase, and are regulated by proteasomal degradation during cell cycle progression (1). We and others have previously reported that expression of the parvoviral NS1 protein leads to increases in p21 levels (10, 331). Additionally, p53, a transcriptional activator of p21, is significantly up-regulated and activated throughout MVM infection. Remarkably however, while these signals lead to increased p21 RNA accumulation, p21 protein levels remain low throughout infection (10). Here we have identified the mechanism by which p21 was degraded upon parvovirus infection, and identified the consequence of this for virus replication.

Degradation of p21 during S-phase and in response to DNA damaging agents such as UV treatment is programmed by the $CRL4^{Cdt2}$ ubiquitin ligase (172) and in this manuscript we have demonstrated that the same ligase targets p21 for degradation during MVM infection. The circumstances surrounding p21 degradation and the signals leading to it in the context of parvoviral infection, however, differ from how it occurs during S-phase. During MVM infection p21 loss persists for extended periods of time while virus is replicating in G2 arrested cells in the presence of high amounts of activated p53 and NS1 (10). Additionally, whereas ATR activity is important for p21

degradation in response to various DDR-inducing agents (33, 248), the ATR substrate Chk1 is not activated during MVM replication (8), suggesting that p21 degradation during infection may occur independently of ATR activity.

During MVM infection the $CRL4^{Cdt2}$ ligase is recruited to viral replication centers. Recently, the $CRL4^{Cdt2}$ ligase was shown to be recruited to cellular chromatin *via* direct PCNA interaction (170). It is not yet clear whether similar mechanisms mediate $CRL4^{Cdt2}$ recruitment to MVM APAR bodies; however, it appears that PCNA recruitment to MVM chromatin may represent a critical step leading to viral hijacking of the $CRL4^{Cdt2}$ ligase. Neither interaction with PCNA nor the ligase was required for recruitment of p21 to replication centers, as all the mutants tested were relocalized to APAR bodies.

Stabilization of p21 *via* $CRL4^{Cdt2}$ depletion led to reduced MVM replication subsequent to the initiation of genome replication following S-phase entry. This is the first published demonstration of the requirement and re-localization to replication centers of a specific cellular ubiquitin ligase during autonomous parvovirus replication. p21 is a potent inhibitor of CDKs and PCNA. Exactly how stabilized p21 inhibited MVM replication is not fully clear; however, interaction with PCNA mediated its inhibitory role. Using inducible cell lines expressing wild-type and mutant p21 proteins we demonstrated that p21 degradation during infection required motifs that mediate interaction with both Cdt2 and PCNA. PCNA is recruited to MVM chromatin (32) and is essential for parvovirus replication (74, 75). Overexpression of a stable mutant p21 that retained interaction with PCNA, but could not be targeted for degradation by the

$CRL4^{Cdt2}$ ligase, led to reduced virus replication. Stable p21 mutants unable to bind PCNA did not affect MVM replication, indicating that other potential inhibitory functions of p21, such as CDK binding and promoter repression, were not detrimental to viral replication absent PCNA interaction. Additionally, introduction of a p21-derived peptide which maintained PCNA interaction abolished viral replication. Thus, p21 interaction with PCNA (and Cdt2) was necessary for targeting of p21 to the co-localized $CRL4^{Cdt2}$ ligase, and its subsequent depletion also prevented its sustained interaction with PCNA that would otherwise be inhibitory to viral replication. Although our earlier work had suggested that Cdk2 activity was required for MVM replication and that p21 degradation might be necessary to prevent inhibition of CDKs (10), we have recently shown that Chk2 activation during MVM infection results in CDC25A degradation leading to partial CDK2 inhibition, independent of p21 (9). Thus, while some level of CDK activity is required for MVM replication, prevention of CDK inhibition is not likely to be the critical reason for p21 degradation.

p21 has been shown to inhibit MVM replication *in vitro*, and this effect was shown to be overcome by the addition of increasing amounts of PCNA (31). p21 binds to PCNA *via* its PIP box, a conserved motif shared by substrates of the $CRL4^{Cdt2}$ ligase, as well as cellular proteins such as DNA polymerase δ, that are essential for replication but escape ubiquitin targeting due to the absence of a PIP degron (172). The p68 subunit of DNA polymerase δ binds to PCNA *via* a similar hydrophobic pocket recognized by the p21 PIP box. Thus, while the mechanism of p21 inhibition of the DNA polymerase δ/PCNA complex has not been clearly resolved, a stabilized high-affinity interaction of

p21 with the DNA polymerase δ binding pocket within PCNA could directly inhibit viral replication by competing with DNA polymerase δ for binding to its cofactor (294).

Due to its myriad effects on cell cycle progression and cancer, several viruses make use of different strategies to inactivate p21 during infection. For example, several oncogenic DNA viruses indirectly inhibit p21 by targeting p53 (146). Additionally, papillomaviruses HPV E7 proteins sequester p21 thereby preventing its interaction with PCNA and CDKs (140, 205). KSHV encodes a microRNA that down-regulates p21 in order to prevent cell cycle arrest (156). p21 restricts HIV in myeloid cells of certain patients (63, 371), and a recent report demonstrated that this restriction may occur *via* ribonucleotide reductase-2 repression resulting in inhibition of dNTP biosynthesis (14). Thus, cellular components required for viral genomic replication, such as dNTPs and polymerase cofactors, may be critically dependent on p21 depletion. Our findings present first a novel mechanism of p21 antagonism by a virus, namely, the use of a cellular E3 ligase recruited to viral replication factories. Further, we shown that efficient virus replication depends on the depletion of p21 to prevent its inhibition of PCNA. PCNA is an important cofactor for the replication of several DNA viruses. PCNA-mediated degradation of p21 in the context of viral infection may emerge as an important paradigm for allowing sustained viral replication of DNA polymerase-δ dependent viruses in infected cells.

2.4 Materials and Methods

Cell lines and drug treatments

Murine A9 and Human 293T cell lines were propagated as previously described (69, 314). Stable doxycycline-inducible A9 cell lines were generated by infection of A9 cells with pseudotyped virus using the pINDUCER20 lentiviral system (284). Cell lines were selected with 800 μg/ml of geneticin (Gibco) and maintained like regular A9s except for addition of geneticin. A9 cells were parasynchronized in G0 by isoleucine deprivation as previously described (7). pINDUCER20 lentiviral transformed cell lines were induced with 500 ng/mL doxycycline hydrochloride (MP Biomedical). MG132 (Calbiochem) was added at a final concentration of 10 μM.

Viruses and infections

Wild-type MVMp was propagated and titered in murine A9 cell lines as described (7). Pseudotyped viruses were generated by co-transfecting equal concentrations of HIV Gag/Pol, VSV-G and pINDUCER20 plasmids into 293T cells. Supernatants were collected and used to infect A9 cells.

Plasmids and DNA transfection

Murine wild-type p21 cDNA (Origene) was cloned into p3XFLAG-CMV 7.1 (Sigma). Additionally, p21 was tagged with a 3× HA tag by PCR mutagenesis. $p21^{K7R}$ was generated by PCR mutagenesis. $p21^{\Delta PCNA}$, $p21^{\Delta degron}$ and $p21^{K7R\Delta PIP}$ (Q139A, L142A, F145A, Y146A) mutants were generated by site-directed mutagenesis (Agilent). FLAG and HA-tagged wild-type and mutant p21 were cloned into pDONR221 (Invitrogen) and

pINDUCER20 using BP and LR clonase kits (Invitrogen) respectively. pINDUCER reagents were a gift from Guang Hu at NIH/NIEHS. DNA transfection was performed using LipoD293 (Signagen) or Lipofectamine (Invitrogen).

RNA interference

A9 cells were transfected twice with 40 nM Control siRNA (Qiagen #1022076), Cdt2 (DTL) siRNA (Dharmacon #L-045921-01-0005), Cul4A (Dharmacon #L-052208-00-0005) or DDB1 siRNA (Dharmacon #L-043146-01-0005). siRNA transfections were performed using HiPerfect (Qiagen).

Peptides

Wild-type p21 (KRRQTSMTDFYHSKRRLIFSRQIKIWFQNRRMKWKK) and scrambled p21 (KSTARHTKLSAQRYIRSFARRQIKIWFQNRRMKWKK) were purchased from Peptide 2.0 Inc. The peptides consist of p21-derived or scrambled sequences fused to penetratin – a 16 amino acid nuclear internalization sequence derived from the Antennapedia homeodomain (118). Peptides were added to cells at 25 μM.

Antibodies

Commercially available antibodies used in this study were obtained from Bethyl (Cdc20, cat# A301-180A), Cell Signaling (Set8, cat# 2996S), Invitrogen (DDB1, cat# 399901; p21, cat# 556430), Millipore (Cdt1, cat# 06-1295; PCNA, cat# CBL407), Novus (Cullin 4A, cat# NB100-2267), Pierce (Actin, cat# MA515739), Sigma (FLAG, cat# F1804; HA, cat# H9658; Tubulin, cat# T4026), and Upstate (Cyclin A, cat# 06-138). Cdt2 antibody was a generous

gift from Anindya Dutta (University of Virginia). All secondary antibodies were from Invitrogen. NS1 (CE10) and NS1/2 (M55) were previously described (7).

Immunoblot analysis

Immunoblots were performed as described previously (7). Protein concentrations were quantified by Bradford assay and equal amounts of lysates were run.

Co-immunoprecipitation

FLAG and HA-tagged p21 was immunoprecipitated from 293T cells as previously described (317). FLAG beads (Sigma) and HA antibodies were used for IP.

Immunofluorescence (IF)

For IF, A9 cells were grown on glass coverslips in 35 mm dishes and infected with MVMp at an MOI of 10. At 24 hr pi, cells were washed twice with cold phosphate-buffered saline (PBS) solution and then with cytoskeleton buffer [10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA]. Afterwards cells were pre-extracted for in cytoskeletal buffer containing 0.5% Triton X-100, protease and phosphatase inhibitors for 3 minutes on ice, washed, fixed with 4% paraformaldehyde and stained for the indicated proteins. Nuclei were visualized by staining with To-Pro-3 (Invitrogen). The coverslips were mounted in Fluoromount-G (Southern Biotech) and images were acquired using a Zeiss LSM 510 META confocal microscope. All images were captured using an objective of 63×.

Analysis of viral DNA

Southern blots were carried out as previously described (69), using whole MVM genome probes. Loading of DNA samples was normalized using a nanodrop spectrophotometer. This procedure was verified using probes on Southern blots against mitochondrial DNA. Unless otherwise indicated, infections were carried out at a low MOI in order to maximize effects of siRNA knockdowns and overexpression analyses on viral replication. Representative Southern Blots are shown; quantifications in the text reflect multiple knockdown experiments.

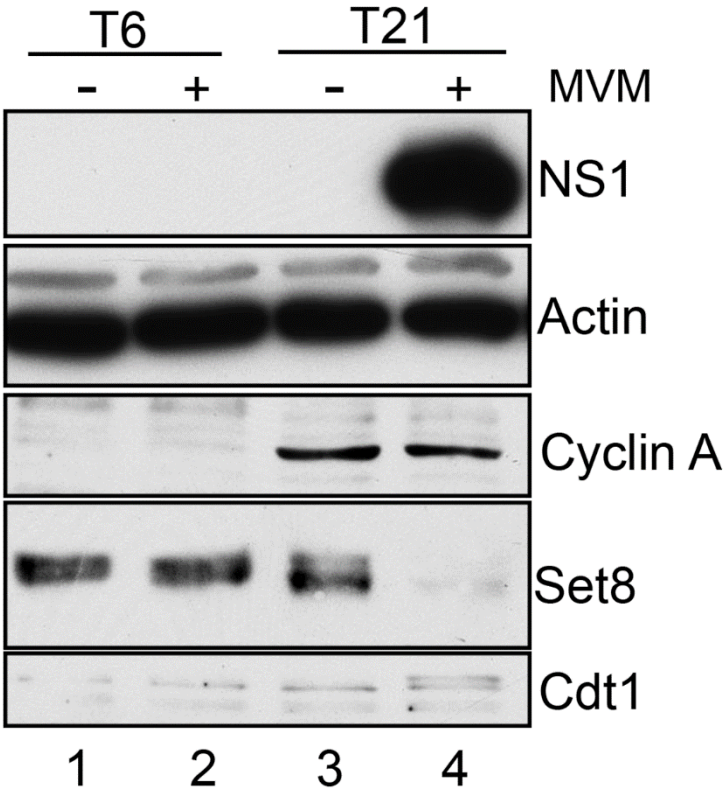
Cell cycle analysis

A9 cells were fixed in 70% ethanol overnight at 4°C. Cells were then pelleted, washed in PBS and resuspended in PBS containing 0.2 mg/ml RNAse A for 1 hr at 37°C, then propidium iodide was added at 40 μg/ml. Flow cytometry was performed using FACScan (BD biosciences). Data were analyzed using Summit software (Beckman Coulter).

2.5 Supporting Information

Figure 2-S1: Set8 but not Cdt1 is degraded during MVM infection.

Parasynchronous A9 cells were infected with MVM at an MOI of 10. Western blots were performed against the indicated proteins. MVM infection resulted in reduction in Set8 protein levels. Cdt1, whose expression levels were not altered, appeared to run as a doublet upon MVM infection.



Experiment in supplemental figure 1 was performed by Richard Adeyemi.

Figure 2-S2: Cdt2 knockdown does not significantly affect S-phase entry.

A) Schematic illustrating the experimental protocol for S1B.

B) Murine A9 cells were treated with siRNA as shown in S1A and were processed for flow cytometry as described in Experimental Procedures. Mock T0 cells were processed at time of release from parasynchronization and show a majority of cells in G0/G1. siRNA-transfected T16 cells were processed at 16 hr post-release and showed the expected reduction in G0/G1 levels compared to mock T0 cells. There was no significant reduction in S-phase accumulation upon Cdt2 knockdown compared to control siRNA treatment; however, the G2/M to S ratio under these conditions varies between experiments. PI stands for propidium iodide.

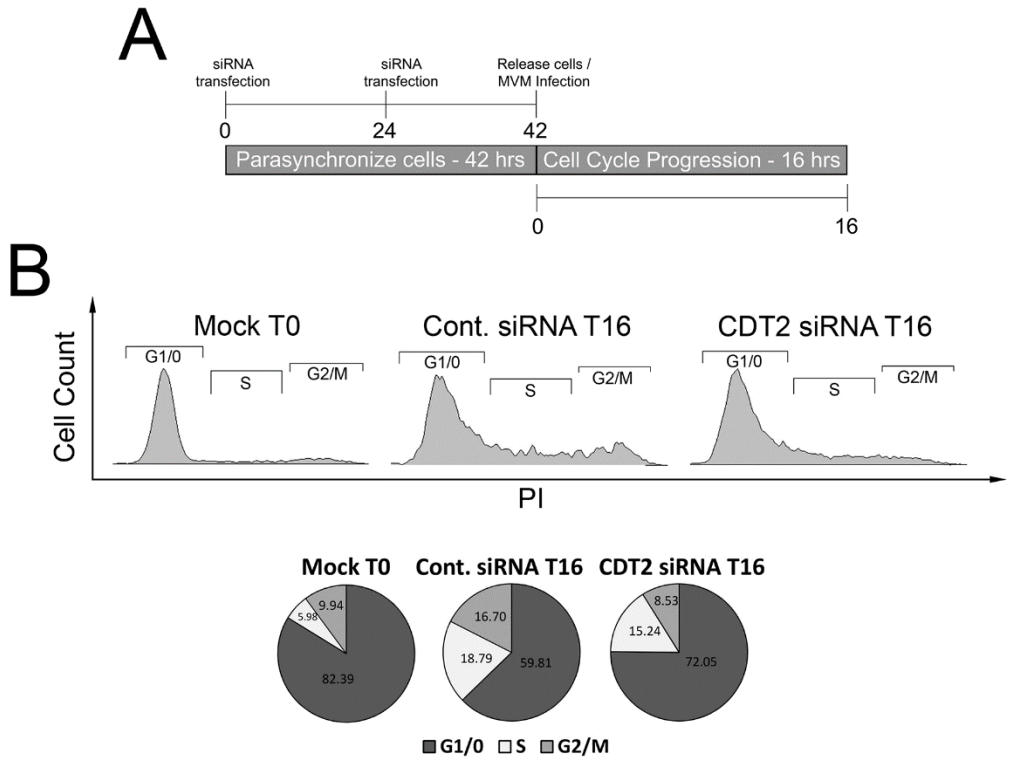

A
siRNA transfection
siRNA transfection
Release cells / MVM Infection
0
24
42
Parasynchronize cells - 42 hrs
Cell Cycle Progression - 16 hrs
0
16
B
Mock T0
Cont. siRNA T16
CDT2 siRNA T16
G1/0
S
G2/M
Cell Count
PI
Mock T0
Cont. siRNA T16
CDT2 siRNA T16
9.94
5.98
82.39
16.70
18.79
59.81
8.53
15.24
72.05
G1/0
S
G2/M

Figure 2-S3: APC/C E3 ubiquitin ligase is not recruited to APAR bodies.

Murine A9 cells were mock infected or infected with MVM at an MOI of 10. At 32 hr pi cells were processed for immunofluorescence as described in experimental procedures, without detergent pre-extraction, using antibodies against NS1 and Cdc20.

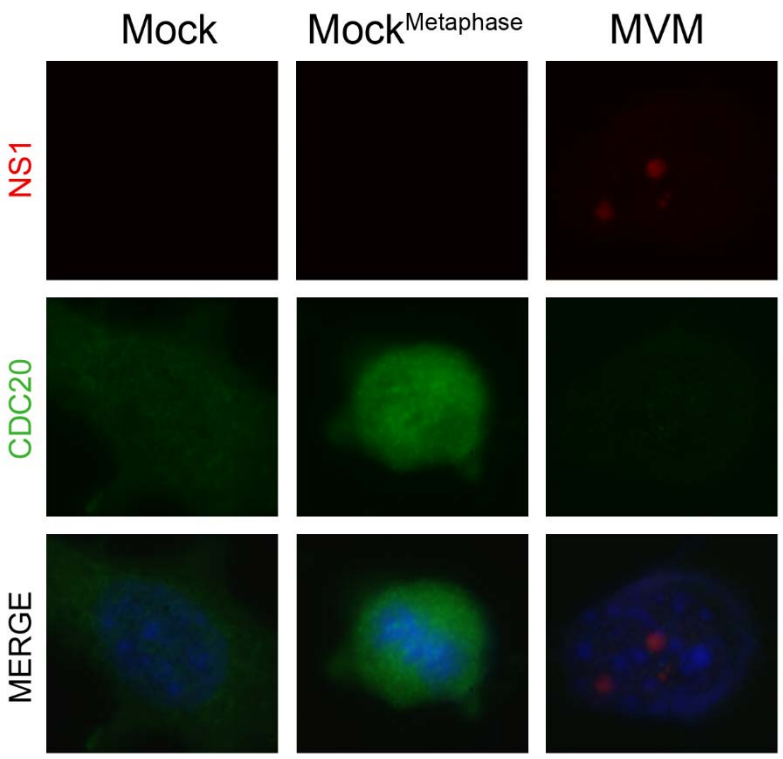
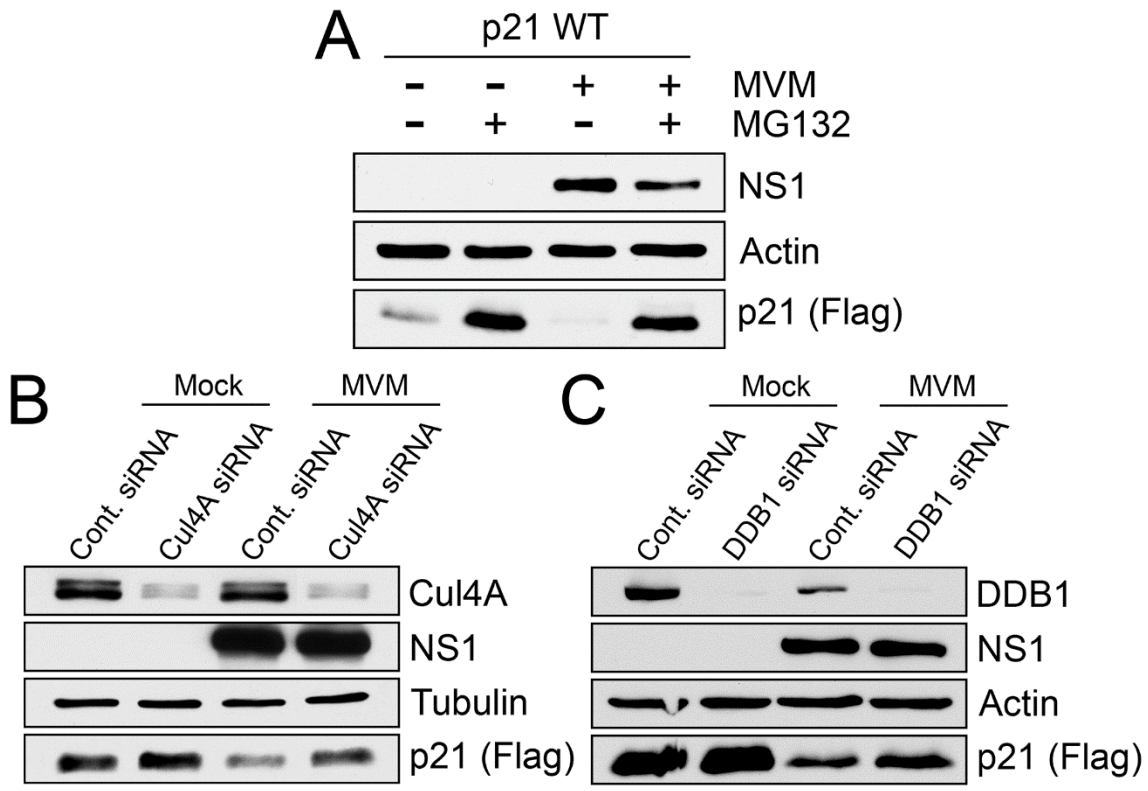

Mock
Mock Metaphase
MVM
NS1
CDC20
MERGE

Figure 2-S4: Overexpressed p21 is degraded in a proteasome and CRL4^{Cdt2} -dependent manner following MVM infection.

A) Parasynchronized murine A9 cell lines stably expressing FLAG-tagged p21^{WT} were mock infected or infected with MVM at an MOI of 10. At 18 hr pi cells were treated with doxycycline to induce p21 expression and treated with MG132 as indicated. Cells were harvested 6 hrs later and processed for western blotting using the antibodies indicated.

B and C) p21^{WT} cell lines were treated with control siRNA or siRNA targeted to Cul4A (B) or DDB1 (C), as indicated, during parasynchronization. Cells were released and mock infected or infected with MVM at an MOI of 10. At 18 hr pi cells were treated with doxycycline to induce p21 expression. Cells were harvested at 24 hr pi and processed for western blotting using the antibodies indicated.



Experiment in supplemental figure 2B was performed by Richard Adeyemi.

Figure 2-S5: $p21^{K7R\Delta PIP}$ does not inhibit MVM replication.

$p21^{WT}$ and $p21^{K7R\Delta PIP}$ cell lines were parasynchronized, released and infected with MVM at an MOI of 0.5. At 16 hr pi cells were treated with doxycycline to induce p21 expression and harvested 8 hrs later. Cells were processed for Southern blotting (top panel), or for western blotting using the indicated antibodies (bottom panels).

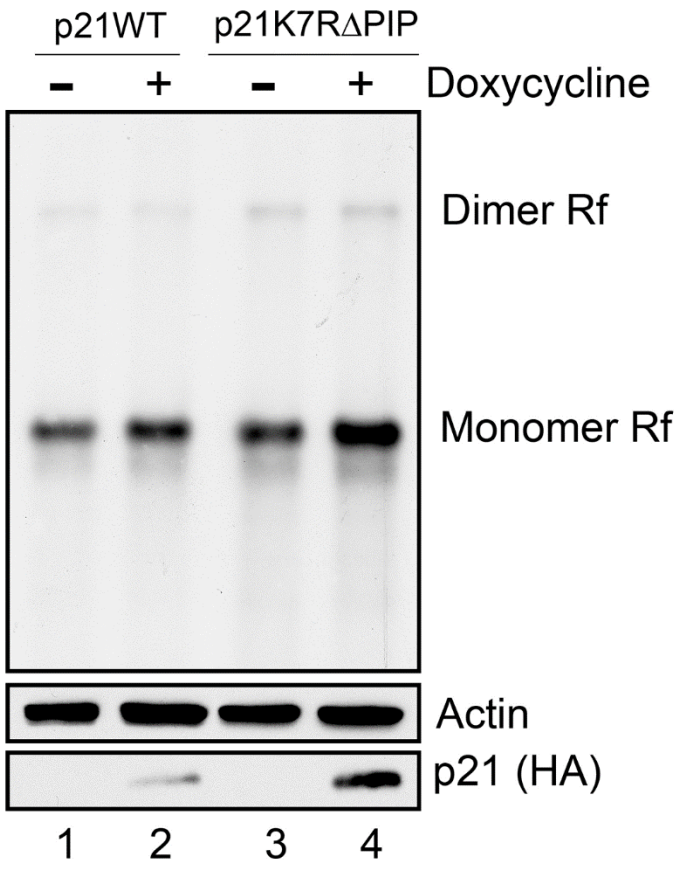
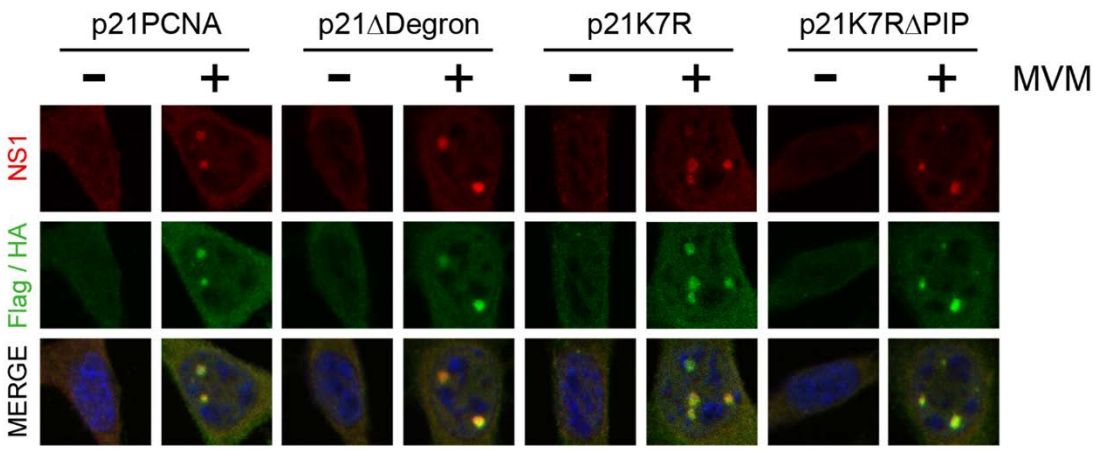

p21WT
p21K7RΔPIP
-
+
-
+
Doxycycline
Dimer Rf
Monomer Rf
Actin
p21 (HA)
1
2
3
4

Figure 2-S6: p21 mutants are recruited to MVM replication compartments.

Murine A9 cell lines stably expressing FLAG-tagged $p21^{PCNA}$, $p21^{\Delta Degron}$ or HA-tagged $p21^{K7R}$ or $p21^{K7R\Delta PIP}$ were mock infected or infected with MVM at an MOI of 10. At 18 hr pi cells were treated with doxycycline to induce p21 expression. At 24 hr pi cells were processed for IF using antibodies against NS1 and FLAG or HA.


p21PCNA
p21ΔDegron
p21K7R
p21K7RΔPIP
- + - + - + - +
MVM
NS1
Flag / HA
MERGE

This work (Efficient parvovirus replication requires $CRL4^{Cdt2}$-targeted depletion of p21 to prevent its inhibitory interaction with PCNA) was completed in collaboration with Richard Adeyemi. Richard and I both conceived of and performed the experiments and completed the writing, in conjunction with David Pintel.

Credit for each figure is outlined below:

Figure 1 – Work completed by Matthew Fuller

Figure 2 – Work completed by Matthew Fuller

Figure 3 – Work completed by Richard Adeyemi and Matthew Fuller

Figure 4 – Work completed by Richard Adeyemi and Matthew Fuller

Figure 5 – Work completed by Richard Adeyemi and Matthew Fuller

Figure S1 – Work completed by Richard Adeyemi

Figure S2 – Work completed by Matthew Fuller

Figure S3 – Work completed by Matthew Fuller

Figure S4 – Work completed by Richard Adeyemi and Matthew Fuller

Figure S5 – Work completed by Matthew Fuller

Figure S6 – Work completed by Matthew Fuller

3 Minute virus of mice inhibits transcription of the cyclin B1 gene during infection

3.1 Introduction

Minute virus of mice (MVM) is an autonomously replicating parvovirus which induces a robust ATM-dependent DNA damage response (DDR) (7). Inhibition of this response significantly reduces viral replication (7). The virally-induced DDR results in the phosphorylation of several proteins important for DNA repair including H2AX, p53, Chk2, NBS1 and RPA32. Numerous DDR proteins colocalize within viral replication centers, termed autonomous parvovirus associated-replication bodies (APAR), suggesting that MVM may be recruiting many of these proteins either for direct usage or to sequester them. Taken together, our observations suggest that the DDR plays an important role during MVM replication.

MVM infection results in an extended pre-mitotic cell cycle block during which viral replication proceeds. We have previously shown that this cell cycle block correlates with the activation of Chk2 and the loss of CDC25A phosphatase activity (9). Experiments designed to monitor cell cycle progression following infection revealed that the G2/M block imposed by MVM prevents entry of infected cells into mitosis as measured by the lack of Ser-28 phosphorylation of histone H3. Normally, the entry into mitosis is tightly regulated and requires the cyclin B1/CDK1 complex to reach threshold levels of activity (253). Importantly, the kinase activity of the cyclin B1/CDK1 complex in MVM infected cells was dramatically reduced compared to the activity of this complex in cells trapped in early mitosis by nocodazole. These observations thus suggested that

low cyclin B1/CDK1 activity was an important factor in maintaining the pre-mitotic cell cycle block during MVM infection. Surprisingly, we found that although the inhibitory phosphorylation of CDK1 at Tyr-15 was lost during infection – an indicator typically associated with activity of the cyclin B1/CDK1 complex - cyclin B1 and its encoding RNA were substantially depleted (9). These results suggested that MVM was regulating the stability or production of cyclin B1 RNA to prevent cyclin B1/CDK1 activity, and in this way affecting this critical pre-mitotic checkpoint.

The nine intron-containing cyclin B1 gene is regulated in a complex manner during cell cycle progression (184, 253). It has at least five transcriptional promoter elements that regulate its transcriptional activity. These include CCAAT-boxes, GC-boxes, E-boxes, p53 responsive elements, and the cell cycle genes homology region (CHR), which bind NF-Y, SP1, Myc, p53 and the Myb-MuvB (MMB) complexes, respectively (59, 80, 139, 195, 229, 254, 285, 306, 442). The CHR, and its protein-binding partners, are important contributors to the intricate regulation of the cyclin B1 gene (426). Many cell cycles genes in addition to cyclin B1 are regulated *via* CHR sites, including cyclin B2, CDKD1, CDC25 and Polo-like kinase-1 (PLK1) (305). CHR-regulated genes are typically active during G2/M and are repressed during other stages of the cell cycle. The DREAM complex both represses the transcriptional activity of these CHR genes during G0 and G1, and helps activate them as cells transit from late G1 phase into S and progress towards the mitotic border (369). As cells progress through late S and into G2/M, the DREAM complex dissociates from a cellular promoter, MuvB remains and successively recruits both B-Myb, forming the MMB complex, and the forkhead

transcription factor FoxM1 (370). Phosphorylation of five to seven sites that reside within the C-terminal transactivation domain is critical for FoxM1 function (16). These phosphorylation events, some of which depend upon the kinase activity of the Cyclin A/CDK complex, are thought to activate FoxM1 by relieving auto-repression caused by the interaction of the C-terminus with an N-terminal repressive domain (241). The expression of the cyclin B1 gene can also be regulated at the level of its RNA stability (274). Interactions of the cellular RNA binding protein HuR within the 3' UTR have been shown to affect the stability of cyclin B1 RNA (398, 422).

In this report we characterize how MVM infection reduces expression of the RNA encoding cyclin B1 during infection. In brief, we show that MVM infection resulted in a reduced level of nascent cyclin B1 RNA which was, in turn, associated with a significantly reduced occupancy of RNA polymerase II (RNA pol II) on the cyclin B1 promoter. Binding of the cyclin B1 transcription factor FoxM1 to the cyclin B1 promoter was also significantly reduced during infection, as was detection, *via* Western blot, of the active forms of phosphorylated FoxM1. Gain of function experiments in which the FoxM1 transactivation domain was targeted to the cyclin B1 promoter, using dead-Cas9-FoxM1 fusion proteins in the presence of cyclin B1 promoter guide RNAs, increased both cyclin B1 protein and RNA expression in infected cells. These results implicated FoxM1 as a critical target for cyclin B1 inhibition by MVM, which contributes to the pre-mitotic block evoked during viral infection.

3.2 Materials and Methods

Cell lines, viruses and virus infections

Murine A9 and human NB324K cells were propagated as previously described (7). Wild-type MVMp was propagated following infection in A9 cells and titered by plaque assay on A9 cells as previously described (7). MVM1989 was propagated in 324K cells and titered by plaque assay as previously described (56). Infections were carried out at an MOI of 10 unless otherwise indicated. Pseudotyped viruses were generated by co-transfecting equal concentrations of HIV Gag/Pol, VSV-G and pINDUCER20 plasmids into 293T cells. Stable doxycycline-inducible A9 cell lines were generated by infection of A9 cells with pseudotyped pINDUCER20 virus. Cell lines were selected with 800 µg/mL of geneticin (Gibco). pINDUCER20 lentiviral transformed cell lines were induced with 500 ng/mL doxycycline hydrochloride (MP Biomedical).

Cell synchronization and drug treatments

A9 cells were parasynchronized in G0 by isoleucine deprivation as previously described (380), and viral infections were typically done at the time of release. Entry into S phase occurs approximately 12 hours post-release into complete media (6). Virally infected cells harvested at 24 hours thus indicates an approximately 12 hour transit into S phase. Doxorubicin (Sigma) was added at a concentration of 200 nM at 15 hours post-release from isoleucine deprivation.

Antibodies

Commercially available antibodies obtained from Abcam against the indicated proteins were: H3K4Me2 (Cat# ab32356), H3K9Me3 (Cat# ab8898), RNA polymerase II – S2 (Cat#

ab5095), RNA polymerase II – S5 (Cat# ab5408). Antibodies obtained from Cell Signaling were: Rabbit IgG (Cat# 2729S), Mouse IgG (Cat# 5415S). Antibodies obtained from Millipore were: Cyclin B1 (Cat# 05-373). Antibodies obtained from Pierce were Beta-Actin, (Cat# MA515739). Antibodies obtained from Proteintech were: Beta-Actin (Cat# 60008-1-Ig), B-Myb (Cat# 18896-1-AP), FoxM1 (Cat# 19147-1-AP), NF-YA (Cat# 12981-1-AP). Antibodies obtained from Sigma were: FLAG (Cat# F1804). NS1/2 (M55) was previously described (7).

Plasmids

Murine wild-type Cyclin B1 cDNA (Origene) was cloned into p3XFLAG-CMV 7.1 (Sigma). FLAG-tagged wild-type Cyclin B1 were cloned into pDONR221 (Invitrogen) and pINDUCER20 using BP and LR clonase kits (Invitrogen), respectively. pINDUCER reagents were a gift from Guang Hu at NIH/NIEHS (6, 284). pcDNA-dCas9-HA was a gift from Charles Gerbach (Addgene plasmid #61355) (182). pgRNA-Humanized was a gift from Stanley Qi (Addgene plasmid #44248) (242). pCMV-CD4 was a gift from Marc Johnson.

Immunoblot analysis

Immunoblots were performed as previously described (7). Protein concentrations were quantified by Bradford assay and equal amounts of lysates were run. Loading controls were included in all assays.

Cell cycle analysis

A9 cells were fixed in 70% ethanol overnight at 4°C. Cells were then pelleted, washed in PBS and resuspended in PBS containing 0.2 mg/ml RNase A for 1 hour at 37°C, then

propidium iodide was added at 40 μg/mL. Flow cytometry was performed using an Accuri C6 (BD Biosciences). Data were analyzed using FlowJo software (FlowJo, LLC).

Chromatin Immunoprecipitation (ChIP)

A9 cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature, and quenched with glycine (0.125M). Cells were collected and lysed using ChIP lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8, protease inhibitors). The lysates were sonicated using a Diagenode Bioruptor for 60 cycles (30 seconds on and 30 seconds off). The lysate was immunoprecipitated with the indicated antibodies and isolated with protein A dynabeads (Invitrogen). Following elution, chromatin-antibody complexes and input DNA were reverse crosslinked by heating and proteinase K digestion at 65°C overnight. The DNA was purified using the Qiagen DNA purification kit. ChIP assays were analyzed by quantitiative PCR with iTAQ universal SYBR green master mix (Biorad). Data are presented as fold enrichment over input. Relative occupancy was calculated as previously described (370).

Ribonuclease protection assay

Total RNA was prepared using the TRIzol reagent (Invitrogen) and RNase protection was performed as previously described (132). Murine cyclin B1 cDNA was obtained from Origene. Nucleotide 1 to 180 of murine cyclin B1 was cloned into pGEM3Z to make an antisense probe (9). 3XF-Cyclin B1 was cloned into pGEM3Z (Promega) to make an antisense probe. Murine beta-actin was cloned into pGEM3Z to make an antisense probe. Murine GAPDH was cloned into pGEM3Z to make an antisense probe.

Nascent and stability assay

For nascent RNA assays, 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) was added at 40 μg/mL, as previously described (380), at 24 hours post-infection for 3 hours. Following DRB treatment, cells were washed twice with PBS and incubated in complete media for indicated time points (390) prior to extraction.

For RNA stability assays, DRB was added at 40 μg/mL at 20 hours post-infection. Cells were harvested every 2 hours, as indicated.

Following indicated time points, total RNA was prepared using the TRIzol reagent (Invitrogen) and cDNA was generated from 1 μg of RNA using the m-MLV Reverse Transcriptase (Promega). Quantitiative PCR with iTAQ universal SYBR green master mix (Biorad). Nascent RNA generations data are presented as relative expression change to T0. Stability data were plotted on a semi-logarithmic scale and are presented as percent mRNA relative to T0, as previously reported (422). 18S rRNA was used to normalize qPCR data.

dCas9-FoxM1 Fusion Assay

Guide RNAs to the cyclin B1 promoter were designed at crispr.mit.edu and cloned into pgRNA-Humanized. The FoxM1 transactivation domain was fused to the C-terminus of dCas9. Cells were co-transfected with cyclin B1 guide RNAs or empty vector, dCas9-FoxM1-Transactivation Domain and pCMV-CD4 during parasynchronization. Following parasynchronization cells were released into complete media and infected at an MOI of 10. 30 hours post-infection cells were harvested and positively selected for CD4 expression.

3.3 Results

Cyclin B1 protein and RNA are reduced during MVM infection.

At late times during MVM infection (para-synchronization by isoleucine deprivation and infections performed as described in Materials and Methods), when cells are arrested prior to mitosis, levels of cyclin B1 and its encoding RNA are reduced (9). Depletion of cyclin B1 in murine A9 cells can again be seen in Figure 1A (compare lane 6 to 5). Treatment with the topoisomerase II inhibitor doxorubicin (318) blocks cells prior to mitosis, similar to MVM; however, in contrast to MVM, treatment with doxorubicin induced high levels of cyclin B1 (Fig. 1A, lanes 7-9). This result suggested that the depletion of cyclin B1 seen was MVM specific, and not merely a general characteristic of cells prevented from mitotic entry. A typical cell cycle profile of MVM-infected and doxorubicin-treated para-synchronized A9 cells is shown in Fig. S1.

Cyclin B1 is degraded as cells cycle from mitosis into G1. However, both proteasome and protease inhibitors had only a negligible ability to restore cyclin B1 levels during infection (data not shown). Additionally, efficient siRNA knock-down of the CDC20 E3 Ubiquitin ligase, as previously reported (6), which targets cyclin B1 for ubiquitination during G1 also had no effect on the accumulation of cyclin B1 in MVM infected cells (data not shown). However, analysis of RNA from the 18hr and 24hr samples from the experiment shown in Fig. 1A demonstrate that cyclin B1 RNA is significantly depleted by this time point (Fig. 1B, lane 4).

A similar depletion of cyclin B1 can be seen following MVM infection of human NB324K cells, both at the protein level (Fig. 1C) and the RNA level (data not shown).

Figure 1 – Cyclin B1 protein and RNA are reduced during MVM infection

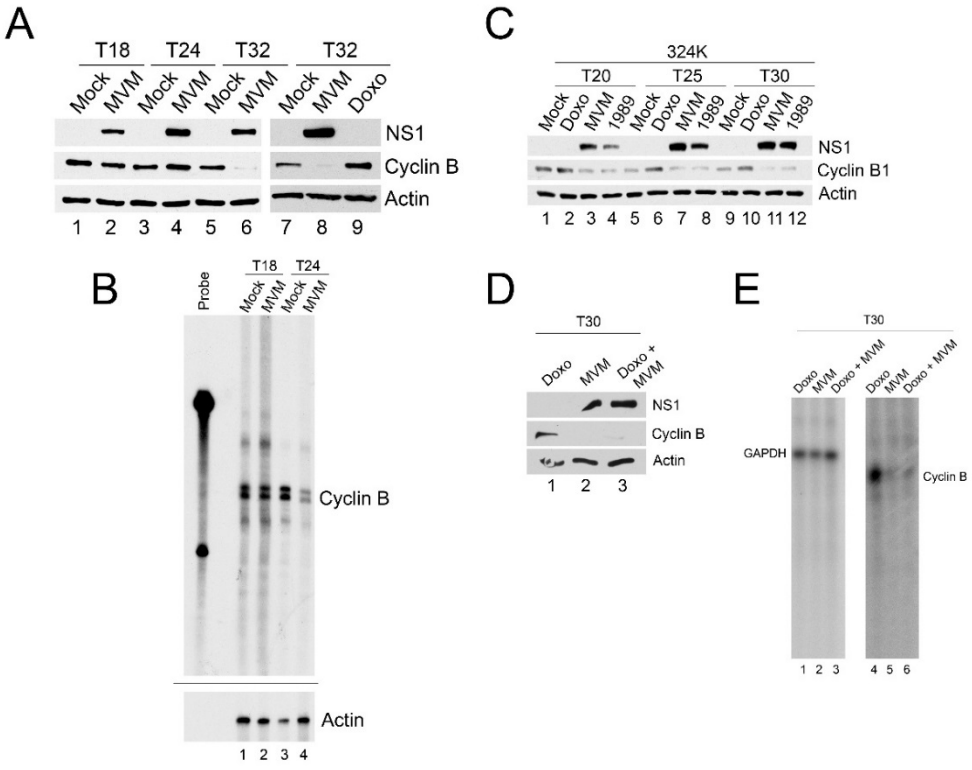
(A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. In a separate experiment, 15 hours post-release, cells were treated with 200 nM doxorubicin as indicated. Cells were harvested at indicated time points and RIPA lysates were Western blotted using antibodies directed against the indicated proteins. Entry into S phase occurs approximately 12 hours post-release into complete media. Virally infected cells harvested at 24 hours thus indicates an approximately 12 hour transit into S phase.

(B) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. Cells were harvested at indicated time points and total RNA was isolated using TRIzol reagent. Samples were processed for RNAse protection assay (RPA) as described in Materials and Methods using probes against cyclin B1 or actin.

(C) Human NB324K cells were parasynchronized by isoleucine deprivation and infected with MVM or MVM-1989 at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. Cells were harvested at indicated time points and RIPA lysates were Western blotted using antibodies directed against the indicated proteins.

(D) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. Cells were harvested 30 hours post-release and RIPA lysates were Western blotted using antibodies directed against the indicated proteins.

(E) Murine A9 cells were treated as described in (D). Cells were harvested 30 hours post-release and total RNA was isolated using TRIzol reagent. Samples were processed for RPA using probes against cyclin B1 or GAPDH.


A
T18
T24
T32
T32
Mock
MVM
Mock
MVM
Mock
MVM
Mock
MVM
Doxo
NS1
Cyclin B
Actin
1 2 3 4 5 6 7 8 9
B
Probe
T18
T24
Mock
MVM
Mock
MVM
Cyclin B
Actin
1 2 3 4
C
324K
T20
T25
T30
Mock
Doxo
MVM
1989
Mock
Doxo
MVM
1989
Mock
Doxo
MVM
1989
NS1
Cyclin B1
Actin
1 2 3 4 5 6 7 8 9 10 11 12
D
T30
Doxo
MVM
Doxo + MVM
NS1
Cyclin B
Actin
1 2 3
E
T30
Doxo
MVM
Doxo + MVM
Doxo
MVM
Doxo + MVM
GAPDH
Cyclin B
1 2 3
4 5 6

MVM mutants that lack the viral NS2 protein can replicate in NB324K cells, and infection with such a mutant (NS2-1989) also results in the loss of cyclin B1, indicating that NS2 is not involved in this loss during infection of these cells (Fig.1C).

Interestingly, MVM infection greatly reduces the high levels of cyclin B1 protein and RNA normally induced by doxorubicin treatment (Fig. 1D, compare lane 3 to 1; Fig. 1E, compare lane 6 to 4). These results suggested MVM can play a dominant role in this regard, consistent with our conclusion that MVM's role is specific.

Nascent cyclin B1 RNA production is reduced during infection.

Cyclin B1 mRNA levels vary across the cell cycle. In addition to being subjected to degradation as discussed above, in normal cycling cells cyclin B1 expression is also controlled at the levels of transcription initiation and RNA stability. Extensive characterization of cyclin B1 RNA during MVM infection, when cyclin B1 protein levels were reduced, revealed no significant changes to the stability of cyclin B1 RNA (Fig. S2). We next chose to determine whether the cyclin B1 promoter or 3'-end (UTR and polyadenylation site) might confer responsiveness to MVM infection. For this analysis we examined the effect of MVM infection on the cyclin B1 cDNA representing only its ORF (from AUG to TAA) driven by the heterologous tet-responsive element inducible promoter (TRE2) and utilizing a SV40 polyadenylation signal, as expressed from an integrated lentivirus vector. The cloned cyclin B1 ORF was tagged with a triplet FLAG sequence to allow detection of the ectopically expressed protein. In addition, we developed an RNase protection probe across the tagged region so that we could detect both the endogenous and ectopically-expressed cyclin B1 RNAs using a single probe in

the same sample. As can be seen in Fig. 2A, while the endogenous cyclin B1 RNA was significantly reduced by MVM 32 hr post-infection, expression of cyclin B1 RNA from the ectopically expressed construct was reduced only slightly (Fig. 2A, compare lanes 5 and 6 to lanes 7 and 8). These results were recapitulated at the level of protein accumulation (Fig. 2B, compare lane 3 to lane 2; in this figure the ectopically expressed protein represents only a fraction of the total cyclin B1 – it's triple tag is readily detected by the anti-FLAG antibody, but is below the level of detection by antibody to cyclin B itself). These results confirmed that the primary effect of MVM on cyclin B1 expression was at the RNA rather than the protein level, and suggested that it was mediated by either its natural promoter, 3' UTR, or polyadenylation site.

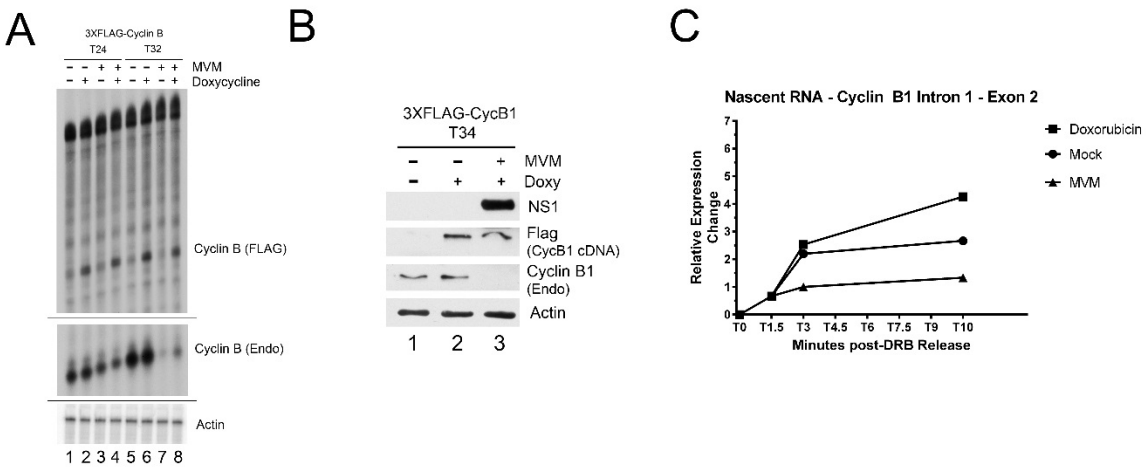
We suspected MVM might be affecting transcriptional initiation of the cyclin B1 gene, and so we chose to compare the generation of nascent pre-mRNA from the endogenous cyclin B1 gene in uninfected and infected cells. As can be seen in Fig 2C, within 3 minutes following resumption of transcription following DRB treatment, MVM infection resulted in a significant decrease in RNA elongation into the 5'-end of exon 2 of cyclin B1, approximately 700 nucleotides downstream of the transcription start site, relative to mock infected cells. As expected, doxorubicin treated cells showed a substantial increase in nascent cyclin B RNA generation over mock. The decreased nascent transcript accumulation in MVM infected cells remained considerably depressed over the 10-minute time course of the experiment. These quantitative real-time PCR results suggest a decreased rate of transcription compared to mock infected cells, and an even greater difference in rates relative to doxorubicin treated cells that

Figure 2 – Cyclin B1 driven from a heterologous promoter and 3'UTR is stable during MVM infection. Nascent cyclin B1 RNA production is reduced during infection.

(A) Murine A9 cells lines inducibly expressing 3XF-cyclin B1 were generated as described in Materials and Methods. Cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. Cells were induced as indicated with doxycycline at time of release. Cells were harvested at indicated time points and total RNA was isolated using TRIzol reagent. Samples were processed for RNAse protection assay (RPA) using probes against 3XF-cyclin B1 (which separately detects FLAG-tagged and endogenous cyclin B1 RNA) or actin.

(B) Inducible murine A9 cell lines described in (A) were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. Cells were induced as indicated with doxycycline at time of release. Cells were harvested 34 hours post-infection and RIPA lysates were Western blotted using antibodies directed against the indicated proteins.

(C) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) was added at 40 μg/mL 24 hours post-infection for 3 hours. Cells were then washed with PBS and incubated in complete media for indicated time points. Total RNA was then isolated using TRIzol and samples were assayed by RT-qPCR as described in Materials and Methods. Data are presented as relative expression change from two independent experiments. 18S rRNA was used for normalization of signals.


A
3XFLAG-Cyclin B
T24
T32
MVM
Doxycycline
Cyclin B (FLAG)
Cyclin B (Endo)
Actin
1 2 3 4 5 6 7 8
B
3XFLAG-CycB1
T34
MVM
Doxy
NS1
Flag
(CycB1 cDNA)
Cyclin B1
(Endo)
Actin
1 2 3
C
Nascent RNA - Cyclin B1 Intron 1 - Exon 2
Relative Expression
Change
Minutes post-DRB Release
T0 T1.5 T3 T4.5 T6 T7.5 T9 T10
Doxorubicin
Mock
MVM

are at a similar stage of the cell cycle as infected cells. These results focused further attention on a role for MVM in the transcription initiation of the cyclin B gene, although a contribution of the 3' end of the gene to its regulation during infection has not been ruled out under some circumstances.

MVM infection reduces occupancy of RNA polymerase II on the cyclin B1 promoter, although the local chromatin architecture is consistent with an open configuration.

Repression of cell cycle genes may be caused by changes in local chromatin accessibility (12). However, the chromatin profile of the cyclin B1 promoter region during infection did not show a correlation with selected chromatin repressive markers. As can be seen in Fig. 3A, repressive H3K9Me3 methylation was found at similarly low levels at the transcription start site of the cyclin B1 gene in either MVM infected, doxorubicin-treated, or mock cells. Reciprocally, H3K4Me2 methylation, a histone modification associated with active chromatin, was found over the same region in infected, treated and mock cells (Fig. 2B). Taken together, these results suggested that cyclin B1 transcription is not substantially altered by perturbations in local chromatin accessibility.

However, while the chromatin profile of the cyclin B1 promoter remained in an open configuration during MVM infection, we detected a significant difference in RNA pol II occupancy in infected cells compared to either mock or doxorubicin-treated cells. ChiP assays using antibodies to either the promoter associated (paused) form of RNA pol II phosphorylated on the carboxy-terminal domain (CTD) at serine 5 (S5, Fig. 3C), or the

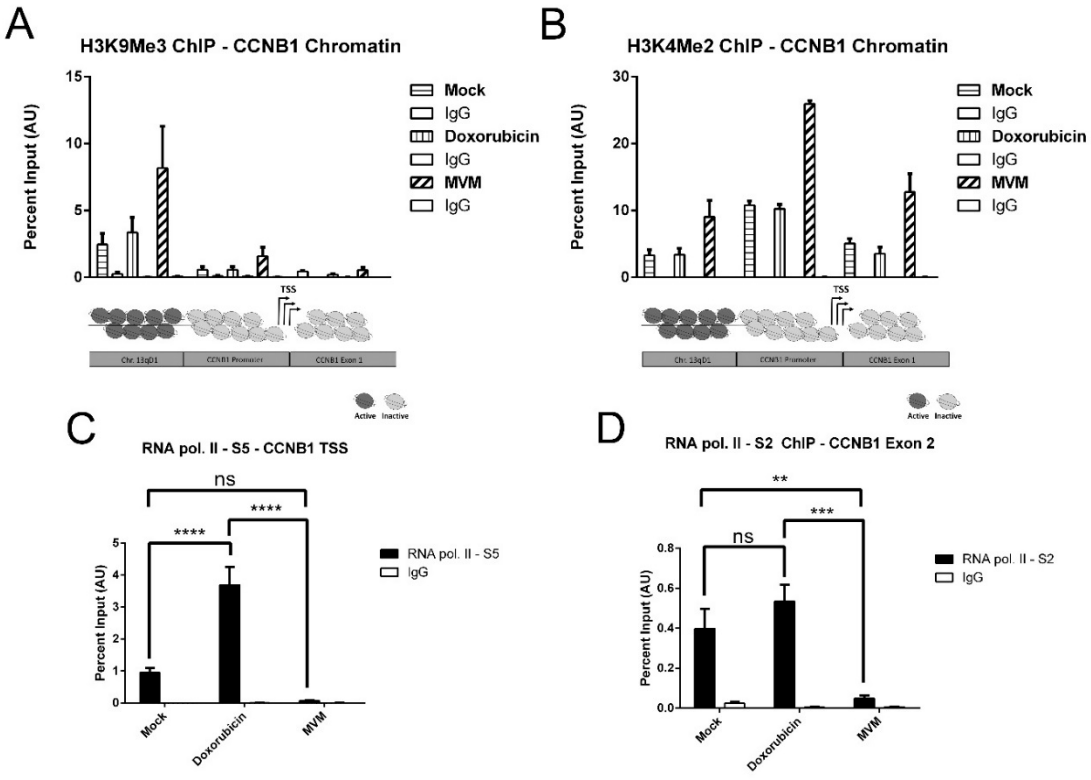
Figure 3 – MVM infection reduces occupancy of RNA polymerase II on the cyclin B1 promoter, although the local chromatin architecture is consistent with an open configuration.

(A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. 24 hours post-infection cells were processed for chromatin immunoprecipitation (ChIP) as described in Materials and Methods using antibodies directed against the repressive chromatin mark H3K9ME3. Samples were analyzed by qPCR as described in Materials and Methods. Data are presented as mean values for percent input signal from at least three independent experiments (± SEM). Note the differences in the scale of the X-axes for panel A and B.

(B) Murine A9 cells were treated and analyzed as described in (A) using antibodies directed against the active chromatin mark H3K4Me2. Data are presented as mean values for percent input signal from at least three independent experiments (± SEM). Note the differences in the scale of the X-axes for panel A and B.

(C) Murine A9 cells were treated and analyzed as described in (A) using antibodies directed against the paused, promoter associated (CTD S5)-phosphorylated RNA pol II. Data are presented as mean values for percent input signal from at least three independent experiments (± SEM). Significant differences between treatments are denoted as **** $p \leq 0.0001$ (t-test). NS denotes no significant difference. Note the differences in the scale of the X-axes for panel C and D.

(D) Murine A9 cells were treated and analyzed as described in (A) using antibodies directed against the elongating (CTD S2)-phosphorylated RNA pol II. Data are presented as mean values for percent input signal from at least three independent experiments (± SEM). Significant differences between treatments are denoted as ** $p \leq 0.01$ (t-test) or *** $p \leq 0.001$ (t-test). NS denotes no significant difference. Note the differences in the scale of the X-axes for panel C and D.


A
H3K9Me3 ChIP - CCNB1 Chromatin
Percent Input (AU)
Mock
IgG
Doxorubicin
IgG
MVM
IgG
TSS
Active
Inactive
B
H3K4Me2 ChIP - CCNB1 Chromatin
Percent Input (AU)
Mock
IgG
Doxorubicin
IgG
MVM
IgG
TSS
Active
Inactive
C
RNA pol. II - S5 - CCNB1 TSS
ns
****
****
Percent Input (AU)
RNA pol. II - S5
IgG
Mock
Doxorubicin
MVM
D
RNA pol. II - S2 ChIP - CCNB1 Exon 2
**
***
ns
Percent Input (AU)
RNA pol. II - S2
IgG
Mock
Doxorubicin
MVM

elongating, CTD-S2 phosphorylated RNA pol II (S2, Fig. 3D), showed significant reductions in occupancy during infection. This suggested that the decrease in nascent RNA seen in Fig. 2 was caused by a failure of RNA pol II to engage the cyclin B1 promoter. In preliminary experiments that expand upon this observation we have found that during infection, the expression of numerous cellular genes are reduced, while for many, expression is increased or unchanged (data not shown). This will be the object of additional study.

The transcription factor FoxM1 exhibits reduced occupancy on the cyclin B1 promoter during MVM infection.

Expression of the cyclin B1 gene is governed by multiple transcription factors including the heterotrimer NF-Y, SP1, Myc, p53, the MMB complex and the DREAM complex. As previously mentioned, CHR-regulated genes are typically active during G2/M and are repressed during other stages of the cell cycle. The DREAM complex both represses the transcriptional activity of these CHR genes during G0 and G1, and helps activate them as cells transit from late G1 phase into S and progress towards the mitotic border. As cells transition from G1 to S phase, the repressive DREAM complex is dissociated, leaving MuvB bound to the promoter at CHR sites, which then successively recruits B-Myb, forming the MMB complex, as well as the transcription factor FoxM1 (370). FoxM1 must be activated *via* hyperphosphorylation, mostly concentrated in its transactivation domain, to stimulate transcription of the cyclin B1 gene by RNA pol II (16, 68, 138, 236, 241, 370).

p53 has been shown to prevent entry into mitosis by regulating cyclin B1 expression (194). Although p53 is activated during MVM infection (7), efficient knock-down of p53 in infected cells had no detectable effect on cyclin B1 expression (data not shown). Transcriptional activation of CHR regulated genes has also been shown to require the binding of the NF-Y transcription factor (59, 305, 426), perhaps acting as a pioneer factor (136). Focused ChIP assays, however, showed that there was no significant difference in NF-YA occupancy at the cyclin B1 promoter in MVM infected cells compared to doxorubicin-treated and mock cells (Fig. 4A). Similarly, there was no significant difference in B-Myb occupancy in MVM-infected cells compared to mock, and only a minor difference compared to doxorubicin-treated cells (Fig. 4B). However, there was a significant difference in the binding of FoxM1 to the cyclin B1 promoter in MVM infected cells compared to cycling mock-infected cells, and a highly significant difference compared to doxorubicin-treated cells residing at a similar same stage of the cell cycle (Fig.4C). The occupancy of FoxM1 to the cyclin B1 promoter reduced progressively as infection proceeded (Figs. 4D and S3). These results suggested that a reduction in FoxM1 engagement with the cyclin B1 promoter mediated MVM repression of cyclin B1 gene expression.

Levels of phosphorylated forms of FoxM1 are reduced during MVM infection. Targeting of the transactivation domain of FoxM1 to the cyclin B1 promoter partially restores cyclin B1 expression.

The transcriptional activity of FoxM1 depends upon phosphorylation of at least five to seven key residues within the C-terminal transactivation domain (16). We

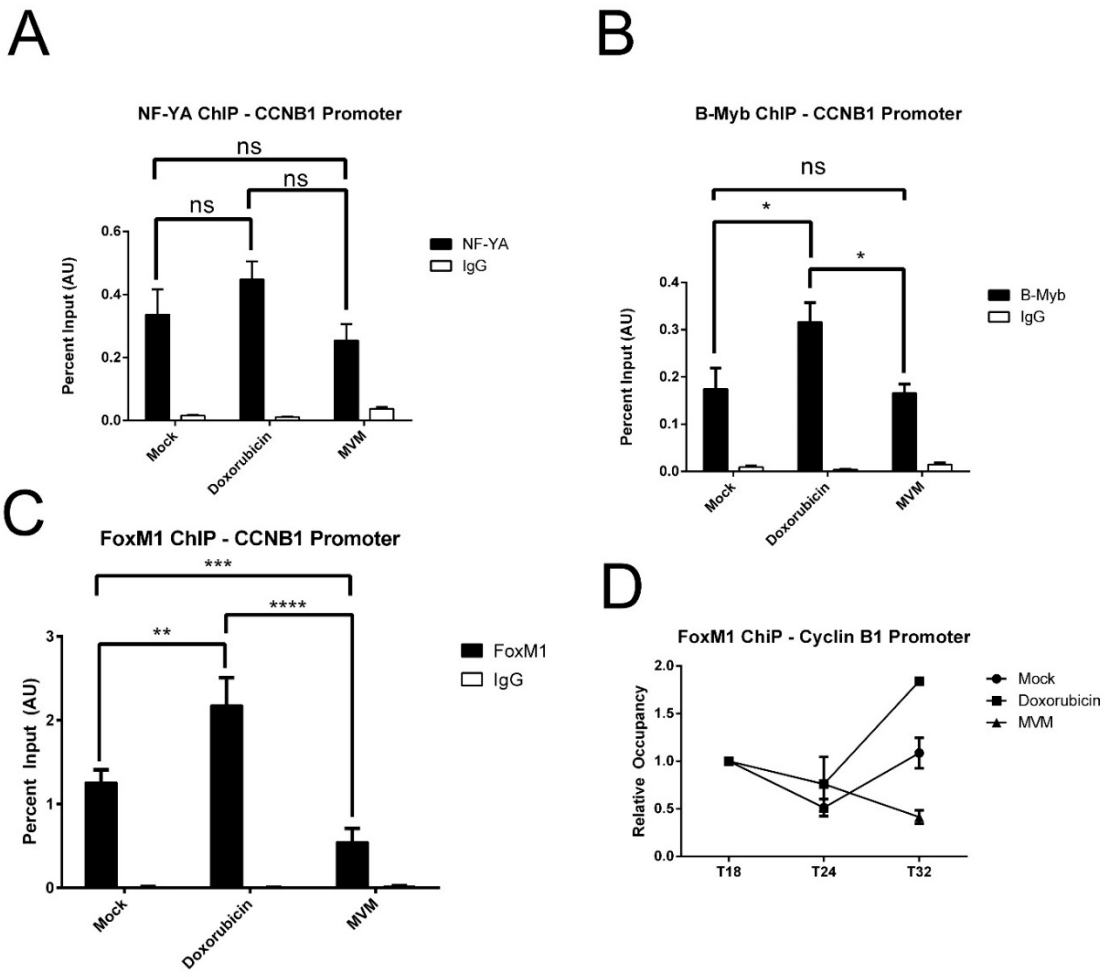
Figure 4 – The transcription factor FoxM1 exhibits reduced occupancy on the cyclin B1 promoter during MVM infection

(A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. 24 hours post-infection cells were processed for chromatin immunoprecipitation (ChIP) as described in Materials and Methods using antibodies directed against NF-YA. Samples were analyzed by qPCR as described in Materials and Method. Data are presented as mean values for percent input signal from at least three independent experiments (± SEM). NS denotes no significant difference.

(B) Murine A9 cells were treated and analyzed as described in (A) using antibodies directed against B-Myb. Significant differences between treatments are denoted as * $p \leq 0.05$ (t-test). NS denotes no significant difference.

(C) Murine A9 cells were treated and analyzed as described in (A) using antibodies directed against FoxM1. Significant differences between treatments are denoted as ** $p \leq 0.01$ (t-test), *** $p \leq 0.001$ (t-test) or **** $p \leq 0.0001$ (t-test). NS denotes no significant difference. Note the differences in the scale of the X-axes for panel C compared to panels A and B.

(D) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. At indicated time points (18, 24 or 32 hours post-infection) cells were processed for chromatin immunoprecipitation (ChIP) as described in Materials and Methods using antibodies directed against FoxM1. Samples were analyzed by qPCR as described in Materials and Method. Data are presented as relative occupancy as previously described (370), calculated from three experimental data points (± SEM).


A
NF-YA ChIP - CCNB1 Promoter
ns
ns
ns
Percent Input (AU)
0.6
0.4
0.2
0.0
Mock
Doxorubicin
MVM
NF-YA
IgG
B
B-Myb ChIP - CCNB1 Promoter
ns
*
*
Percent Input (AU)
0.4
0.3
0.2
0.1
0.0
Mock
Doxorubicin
MVM
B-Myb
IgG
C
FoxM1 ChIP - CCNB1 Promoter
***
****
**
Percent Input (AU)
3
2
1
0
Mock
Doxorubicin
MVM
FoxM1
IgG
D
FoxM1 ChiP - Cyclin B1 Promoter
Relative Occupancy
2.0
1.5
1.0
0.5
0.0
T18
T24
T32
Mock
Doxorubicin
MVM

observed a significant reduction in the levels of forms of phosphorylated FoxM1 (PP-FoxM1) in MVM infected cell at T25 and T30 hours post-infection compared to mock infected or doxorubicin-treated controls (Fig 5A compare lanes 4-6 and 7-9). The total levels of FoxM1 were not significantly altered during infection, consistent with the phosphorylated form being the transcriptionally active form, as shown by others (16, 138, 241).

To test whether FoxM1 was sufficient to reverse the MVM-induced depletion of cyclin B1, we fused the FoxM1 transactivation domain (TAD) to a catalytically-inactive Cas9 nuclease (dCas9). Recruitment of ectopically expressed dCas9-FoxM1-TAD to the cyclin B1 promoter led to substantial upregulation of both cyclin B1 protein (Fig. 5B) and mRNA (Fig. 5C) in MVM infected cells. These results suggested that FoxM1 was a critical target for cyclin B1 inhibition by MVM.

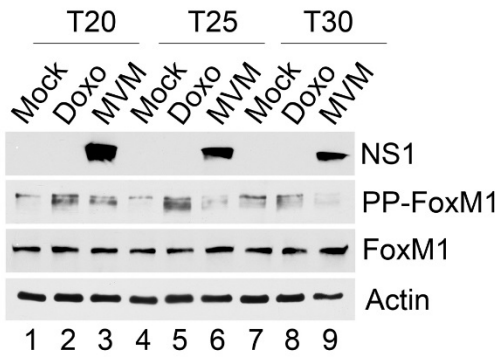
Figure 5 – Levels of phosphorylated FoxM1 are reducing during MVM infection. Targeting of the transactivation domain of FoxM1 to the cyclin B1 promoter partially restores cyclin B1 expression.

(A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. Cells were harvested at indicated time points and RIPA lysates were Western blotted using antibodies directed against the indicated proteins.

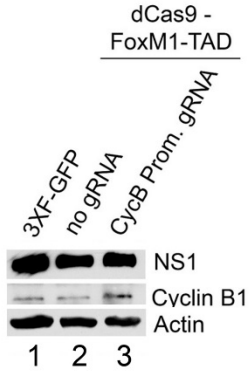
(B) Murine A9 cells were transfected during parasynchronization by isoleucine deprivation with plasmids containing no guide RNAs or cyclin B1 promoter targeting guide RNAs, catalytically-inactive Cas9 fused to the FoxM1 transactivation domain and human CD4 as indicated. Cells were released into complete medium and infected with MVM at an MOI of 10. Transfected cells were positively selected by CD4 as described in Materials and Methods. Whole cell lysates were assayed by Western blotting using antibodies directed against the indicated proteins.

(C) Total RNA was isolated with TRIzol reageant in murine A9 cells from (B). Samples were analyzed by qPCR as described in Materials and Methods. Data are presented as fold change normalized to beta-Actin and calculated as $\Delta\Delta$-C_t.

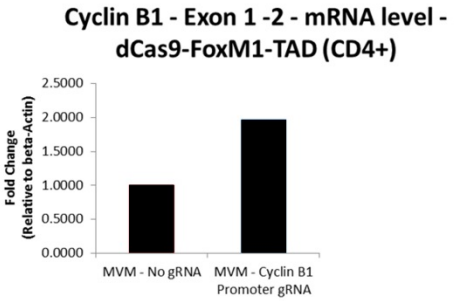
A


T20
T25
T30
Mock
Doxo
MVM
Mock
Doxo
MVM
Mock
Doxo
MVM
NS1
PP-FoxM1
FoxM1
Actin
1 2 3 4 5 6 7 8 9

B


dCas9 -
FoxM1-TAD
3XF-GFP
no gRNA
CycB Prom. gRNA
NS1
Cyclin B1
Actin
1 2 3

C


Cyclin B1 - Exon 1 -2 - mRNA level -
dCas9-FoxM1-TAD (CD4+)
Fold Change
(Relative to beta-Actin)
2.5000
2.0000
1.5000
1.0000
0.5000
0.0000
MVM - No gRNA
MVM - Cyclin B1
Promoter gRNA

3.4 Discussion

Parvoviruses induce a potent pre-mitotic cell cycle block during which replication continues for an extended period of time. MVM utilizes an unusual strategy to impose this restriction, one which involves inhibiting transcription of cyclin B1. A key feature of this block is the MVM-induced depletion of forms of phosphorylated FoxM1, a key transcription factor regulated by the DREAM/MMB complexes. Targeting the transactivation domain of FoxM1 to the cyclin B1 promoter during MVM infection was able to restore significant expression of cyclin B1, implicating FoxM1 as being directly involved in MVM's depletion of cyclin B1 expression.

Our initial studies showed that the proteasome inhibitor MG132 could only recover cyclin B1 levels during MVM infection to a small degree – not greater than would be expected in cells that had evaded our blocking procedure (data not shown). Together with the observation that levels of cyclin B1 mRNA were reduced in infection, these results focused our attention on mechanisms that might account for depletion of cyclin B1 mRNA. In our first experiments we monitored effects of MVM infection on transiently transfected reporter genes engineered to either be driven by heterologous promoters of contain heterologous 3' ends. These experiments gave equivocal results, perhaps due to the overwhelming nature of the transfected reporter, and the differences in transfection vs infection efficiencies. However, examination of the cyclin B1 RNA from integrated lentivirus vectors suggested that differences in cyclin B1 RNA levels during infection were modulated by either the 5'- or 3'- ends of the gene. That these experiments also were reflected in the level of cyclin B1 protein, supported the

notion that protein stability was not the primary cause of depletion. We detected little difference in the stability of cyclin B1 RNA in infected cells; however, examining the production of nascent RNA directly from the endogenous cyclin B1 gene during infection suggested that MVM inhibited the production of cyclin B1 RNA. These results were supported by ChIP assays which demonstrated a significant reduction in RNA pol II occupancy during MVM infection. These results did not preclude possible additional effects attributable to the cyclin B1 3' UTR at specific points during infection. Initiation of transcription of the cyclin B gene is complex, and the DREAM complex has been shown to be critical for its cell cycle regulation. MVM induces the depletion of forms of phosphorylated FoxM1, a critical transcription factor regulated by the DREAM/MMB complexes, and we detected less occupancy of both RNA pol II and FoxM1 at the cyclin B1 promoter during infection. Targeting of the transactivation domain of FoxM1 to the cyclin B1 promoter during MVM infection regained a significant amount of cyclin B1 expression, supporting a role for FoxM1 in the regulation of cyclin B1 by MVM.

Importantly, the reduction of cyclin B1 RNA during infection is likely not the result of global repression of cellular transcription. We have recently begun an investigation of the expression levels of multiple cellular genes during infection. The results are complex, but include genes whose expression is reduced, increased, and those not significantly changed.

How MVM reduces phosphorylation of FoxM1 is not yet clear. Cyclin A/CDK activity is required for alleviation of FoxM1 auto-repression, and Chk2 activity is important for regulating FoxM1 levels during a DDR (241, 405). Importantly, both cyclin

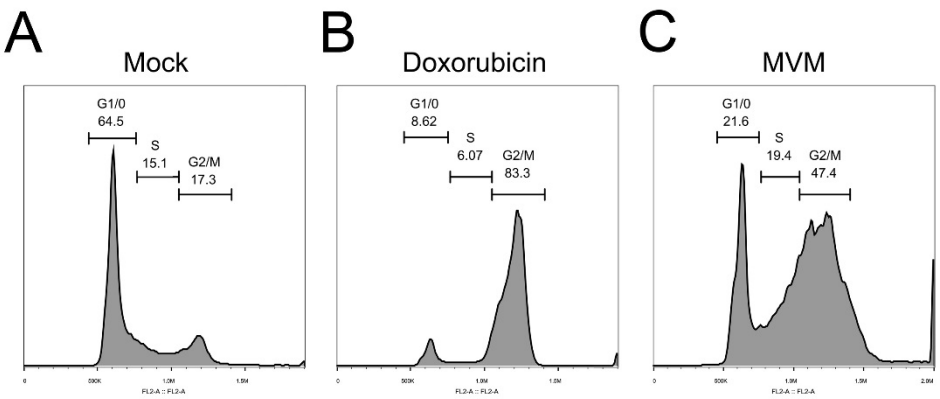
A and Chk2 are recruited to APAR bodies during MVM infection (9, 32), opening the possibility that viral sequestration of cyclin A or Chk2 prohibits activation of FoxM1. In addition, CDK1 has been shown to phosphorylate FoxM1, which is critical for the interaction of PLK1 and FoxM1 (138). This interaction allows for direct phosphorylation and activation of FoxM1 by PLK1. Potentially, the lack of CDK1 kinase activity during MVM infection may prevent the activation of the known positive-feedback loop required for strong FoxM1 activation and execution of the mitotic program. It will be interesting to determine if MVM prevents the interaction of PLK1 and FoxM1. Although reconstitution of FoxM1 to the cyclin B1 promoter *via* dCas9 targeting elevated cyclin B1 RNA and protein levels during infection, it is not yet clear how the FoxM1 transactivation domain fused to dCas9 escapes MVM inhibition. It is possible that MVM prevents alleviation of FoxM1 autorepression, which could be circumvented by introduction of the transactivation domain without the presence of endogenous regulatory domains. It is also unclear how the dCas9-fused FoxM1 transactivation domain becomes activated during MVM infection, which may suggest that MVM specifically targets earlier FoxM1 regulatory steps. Attempts at pushing MVM infected cells into mitosis by supplying activated cyclin B1 have not yet been successful, which suggests that elevating cyclin B1 levels alone may not be sufficient to execute the mitotic program.

Numerous other viruses have been shown to affect the cell cycle during infection. HIV VPR inhibits the activation of the cyclin B1/CDK1 complex, possibly by binding and altering the activity of the CDC25C phosphatase which is required to

remove CDK1's inhibitory phosphorylation (155, 173, 359). HPV1, reoviruses and SV40 also prevent activation of CDK1 by maintaining the inhibitory phosphorylation of CDK1 (223, 343, 378). HIV VPR alternatively upregulates the CDK-inhibitor p21 which helps maintain the inactive state of the cyclin B1/CDK1 complex (71). The agnoprotein of JC polyomavirus also induces p21 to inhibit the activation of the cyclin B/CDK1 complex (108). HIV can also utilize its Tat protein to stimulate the expression of cyclin B1, which is thought to promote apoptosis, and then targets cyclin B1 for proteasomal degradation by binding to the N-terminus of cyclin B1 (448). HPV16's E4 protein is able to sequester the cyclin B/CDK1 complex to the cytoplasm, thus preventing its nuclear localization and activity (110). In the studies presented here we demonstrate that MVM uses a different approach to prevent the activation of the cyclin B/CDK1 complex by depleting both the cyclin B1 protein and, preceding that loss, the cyclin B1 RNA.

3.5 Supplemental Material

Supplemental Figure 1 – Cell cycle profile of murine A9 cells after specific treatments
Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. 24 hours post-infection cells were then processed for flow cytometry as described in Materials and Methods. Data were processed in FlowJo (FlowJo, LLC) and are presented as percent total per cell cycle phase.


A
Mock
G1/0
64.5
S
15.1
G2/M
17.3
B
Doxorubicin
G1/0
8.62
S
6.07
G2/M
83.3
C
MVM
G1/0
21.6
S
19.4
G2/M
47.4

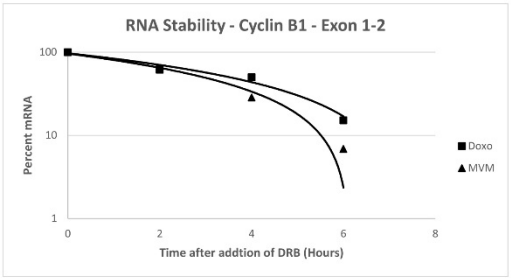
Supplemental Figure 2 – Cyclin B1 RNA stability is not significantly different in doxorubicin-treated and MVM infected cells

(A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) was added at 40 μg/mL 20 hours post-infection. Cells were harvested every two hours as indicated. Total RNA was isolated using TRIzol and samples were assayed by RT-qPCR as described in Materials and Methods utilizing primers for cyclin b1 exons 1-2. Data were plotted on a semi-logarithmic graph and are presented as percent mRNA from two independent experiments. 18S rRNA was used for normalization of signals.

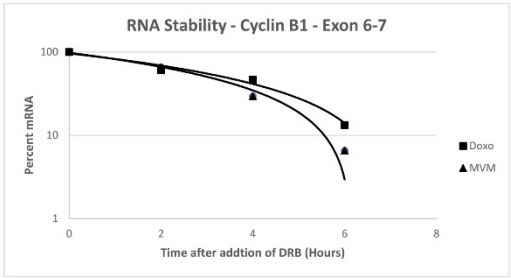
(B) Murine A9 cells were treated as described in (A). Samples were assayed by RT-qPCR as described in Materials and Methods utilizing primers for cyclin b1 exons 6-7.

(C) Semi-logarithmic graphs from (A) and (B) were utilized to calculate mRNA half-life as previously described (422).

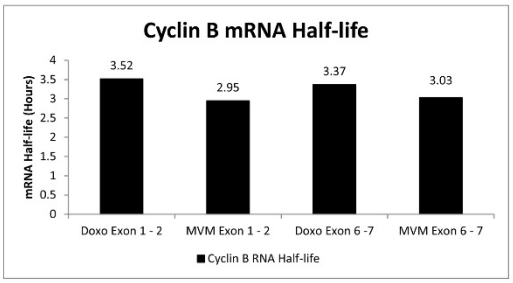
A


RNA Stability - Cyclin B1 - Exon 1-2
Percent mRNA
100
10
1
0
2
4
6
8
Time after addtion of DRB (Hours)
Doxo
MVM

B

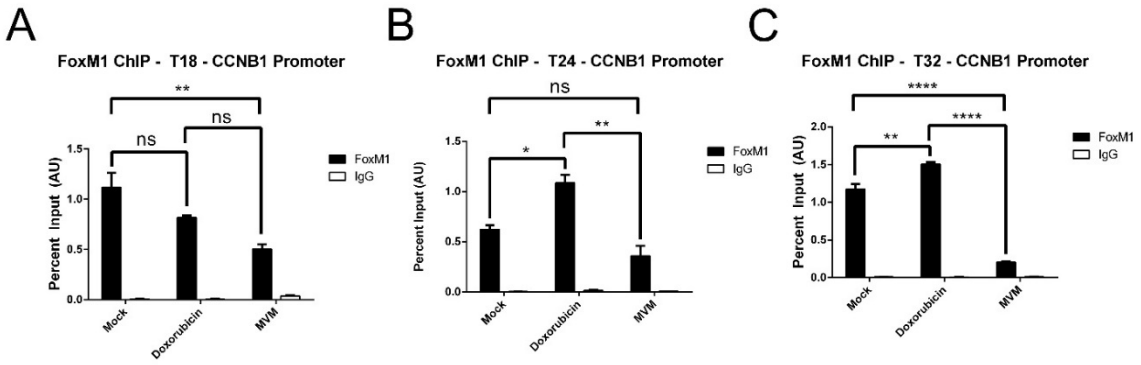

RNA Stability - Cyclin B1 - Exon 6-7
Percent mRNA
100
10
1
0
2
4
6
8
Time after addtion of DRB (Hours)
Doxo
MVM

C


Cyclin B mRNA Half-life
mRNA Half-life (Hours)
4
3.5
3
2.5
2
1.5
1
0.5
0
3.52
2.95
3.37
3.03
Doxo Exon 1 - 2
MVM Exon 1 - 2
Doxo Exon 6 -7
MVM Exon 6 - 7
Cyclin B RNA Half-life

Supplemental Figure 3 – FoxM1 occupancy on the cyclin B1 promoter decreases during MVM infection

Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-release cells were treated with 200 nM doxorubicin as indicated. At indicated time points (18, 24 or 32 hours post-infection) cells were processed for chromatin immunoprecipitation (ChIP) as described in Materials and Methods using antibodies directed against FoxM1. Samples were analyzed by qPCR as described in Materials and Method. Data are presented as mean values for percent input signal from at least three independent experiments (± SEM). Significant differences between treatments are denoted as * $p \leq 0.05$ (t-test), ** $p \leq 0.01$ (t-test) or **** $p \leq 0.0001$ (t-test). NS denotes no significant difference.


A
FoxM1 ChIP - T18 - CCNB1 Promoter
Percent Input (AU)
FoxM1
IgG
Mock
Doxorubicin
MVM
ns
ns
**
B
FoxM1 ChIP - T24 - CCNB1 Promoter
Percent Input (AU)
FoxM1
IgG
Mock
Doxorubicin
MVM
ns
*
**
C
FoxM1 ChIP - T32 - CCNB1 Promoter
Percent Input (AU)
FoxM1
IgG
Mock
Doxorubicin
MVM
**
****
****

4 Minute virus of mice relocalizes the RNA-binding protein HuR during infection

4.1 Introduction

Infection of the parvovirus minute virus of mice (MVM) induces an ATM-dependent DNA damage response (DDR), which is required for replication (7). The virally-induced DDR facilitates a critical pre-mitotic G2/M block *via* Chk2 activation and depletion of the key mitotic cyclin, cyclin B1, by targeting the production of cyclin B1 RNA (9). The activity of cyclin A, an important S phase cyclin, is required alongside RFC, PCNA and polymerase δ for efficient viral replication (31). Importantly, the RNA-binding protein HuR is known to play a key role in regulating the DDR and modulating the RNA stability of key cell cycle regulators cyclin A and cyclin B1 (215, 422). Given its ability to regulate several known key players involved in MVM replication, we sought to characterize the role of HuR in parvoviral infection.

HuR is a member of the highly conserved ELAV/Hu family of RNA-binding proteins (389). This protein family contains four members: HuB, HuC and HuD, which are neuron-specific and reside mostly within the cytoplasm, and HuR, which is a predominantly nuclear protein (183). HuR binds AU-rich elements within pre-mRNAs and mature mRNAs, typically in intronic regions or the 3'UTR, which stabilizes those transcripts (245, 304). It has been shown in several cases that microRNAs and HuR compete for binding within these regions which affords HuR the ability to regulate diverse cellular processes including cellular proliferation, apoptosis, differentiation and immune responses (156, 303, 346, 398). In addition, HuR can regulate the transcription

of certain mRNAs *via* nucleo-cytoplasmic shuttling and competition with destabilizing RNA-binding proteins (4, 54, 144).

HuR function is regulated at several layers including abundance, localization and RNA binding (157). Transcription of HuR is regulated by NF-κB and protein abundance is regulated by a HuR-mediated positive-feedback loop and other RNA-binding proteins binding to HuR mRNA (210, 349, 441). HuR binds target RNAs *via* three RNA recognition motifs (RRMs) (213) and phosphorylation of certain residues at or near these regions has been shown to alter HuR's affinity for RNA binding (379). In addition, HuR contains a hinge region responsible for subcellular localization (157). Residues within this hinge region can be phosphorylated by multiple kinases including cyclin-dependent kinases, CDK1 (216) and CDK5 (134), checkpoint kinase, Chk2 (5), and several members of the protein kinase C (PKC) family (157). It is important to note that several of these kinases can phosphorylate multiple residues within HuR and thus potentially, simultaneously regulate localization, RNA binding, protein stability and splicing.

In this report we characterize the relocalization of HuR during MVM infection and the potential implications of this modulation. We show that MVM infection results in the predominant, but not complete, relocalization of HuR to the cytoplasm which could be detected in both the natural host murine cell as well as permissive human cells. Importantly, this effect was dominant over the effects of the strong DNA damaging agent doxorubicin. Ectopic expression of a triple-FLAG tagged HuR fused to an SV40 nuclear localization signal during MVM infection was also relocalized during infection, suggesting that MVM employs a robust mechanism to modulate HuR subcellular

localization. Overexpression of the viral non-structural protein NS1 triggered cytoplasmic relocalization of HuR, which could also be dominant over doxorubicin treatment. MVM relocalization of HuR was independent of the Crm1 export, which was surprising given Crm1's important role in HuR mRNA export (143, 441) and the strong interaction of MVM non-structural protein NS2 and Crm1 (40, 288). siRNA knockdown of HuR during MVM infection reduced the protein expression of cyclin B1, independent of RNA levels, while overexpression of triple-FLAG tagged HuR elevated cyclin B1 expression. Taken together, the results suggested that HuR may be promoting the translation of cyclin B1 mRNA, and potentially other mRNAs, during MVM infection. HuR was also shown to bind MVM RNA, further suggesting an important role for this RNA-binding protein during MVM infection.

4.2 Materials and Methods

Cell lines, viruses and virus infections

Murine A9 and human NB324K cells were propagated as previously described (7). Wild-type MVMp was propagated following infection in A9 cells and titered by plaque assay on A9 cells as previously described (7). MVM-Crm1(-) and MVM-14-3-3(-) interaction mutant viruses were propagated in 324K cells and titered by plaque assay as previously described (45, 288). Infections were carried out at an MOI of 10 unless otherwise indicated. Pseudotyped viruses were generated by co-transfecting equal concentrations of HIV Gag/Pol, VSV-G and pINDUCER20 plasmids into 293T cells. Stable doxycycline-inducible A9 cell lines were generated by infection of A9 cells with pseudotyped pINDUCER20 virus (284). Cell lines were selected with 800 µg/mL of geneticin (G418) (Gibco). pINDUCER20 lentiviral transformed cell lines were induced with 500 ng/mL doxycycline hydrochloride (MP Biomedical).

Cell synchronization and drug treatments

A9 cells were parasynchronized in G0 by isoleucine deprivation as previously described (6). Doxorubicin (Sigma) was added at a concentration of 200 nM at 15 hours post-release from isoleucine deprivation. Leptomycin B (Cell Signaling) was added at a concentration of 10 nM at 18 hours post-infection for 6 hours (288).

siRNA transfections

ON-TARGET plus SMART pool siRNAs directed against mouse HuR (cat # L-053812-00-0005) was obtained from Dharmacon. Negative control siRNAs were obtained from Qiagen. A9 cells plated in isoleucine-deprived media in 60 mm dishes were transfected

at the day of plating with 40 nm of siRNA using HiPerfect transfection reagent (Qiagen) (6). Transfections were repeated 24 hours later and the next day the cells were released into complete media and processed as described in the figure legends.

Plasmid DNA transfections

1 μg or 2.5 μg of plasmid DNA were transfected into 60 mm or 35 mm dishes, respectively, using LipoD293 transfection reagent (SignaGen) according to manufacturer's specifications.

Antibodies

Commercially available antibodies obtained from Cell Signaling against the indicated proteins were: Rabbit IgG (Cat# 2729S). Antibodies obtained from Millipore were: Cyclin B1 (Cat# 05-373), γ-H2AX (Cat# 05-636) HuR (Cat# 07-1735). Antibodies obtained from Pierce were Beta-Actin, (Cat# MA515739). Antibodies obtained from Sigma were: FLAG (Cat# F1804), HA (Cat# H3663), Tubulin (Cat# T4026). NS1 (CE10/2C9B), NS1 (91W52) and NS1/2 (M55) have been previously described (6, 9, 10).

Plasmids

Murine wild-type HuR (ELAVL1) cDNA (Origene) was cloned into p3XFLAG-CMV 7.1 (Sigma). SV40 large T-antigen NLS was fused to HuR cDNA by overlapping PCR and cloned into p3XFLAG-CMV 7.1 (Sigma). MVM NS1, NS2 or NS1/2 were cloned in pCMV-HA-N (Clontech). NS1 was cloned into pDONR221 (Invitrogen) and pINDUCER20 using BP and LR clonase kits (Invitrogen), respectively. pINDUCER reagents were a gift from Guang Hu at NIH/NIEHS (6, 284).

Immunoblot analysis

Immunoblots were performed as previously described (7). Protein concentrations were quantified by Bradford assay and equal amounts of lysates were run.

Protein fractionation

Cell pellets were subjected to subcellular protein fractionation preparation using the subcellular protein fractionation kit for cultured cells according to the manufacturer's instructions (Thermo Scientific). Western blotting was done loading equal volumes of fractions.

Ribonuclease protection assay

Total RNA was prepared using the TRIzol reagent (Invitrogen) and RNase protection was performed as previously described (9, 132). Murine cyclin B1 cDNA was obtained from Origene. Nucleotide 1 to 180 of murine cyclin B1 was cloned into pGEM3Z to make an antisense probe (9). Murine beta-actin was cloned into pGEM3Z to make an antisense probe (9).

RNA immunoprecipitation (RIP)

RIP assays were completed as previously described (214). Briefly, cells were lysed on ice in polysome lysis buffer (100 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.0), 0.5% NP40, 1 mM DTT) supplemented with RNase inhibitors and protease inhibitors (100 units ml−1 RNase Out, 400 μM VRC, 1X protease inhibitor cocktail) and stored at -80°C for 1 hour to complete lysis. Protein A Dynabeads (Invitrogen) were washed in NT2 buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl2, 0.05% NP40) and incubated with 4 μg HuR or Rabbit IgG antibodies, rotating for 1 hour at 4°C. Lysate was then thawed, cleared at

15,000 g, and added to antibody-bound Dynabeads, which were incubated rotating at 4°C for 2 hours. Ten percent of lysate was saved as total input. Beads were then washed in NT2 buffer and total RNA was prepared using the TRIzol reagent. RIP was followed by quantitiative PCR with iTAQ universal SYBR green master mix (Biorad). Data are presented as fold enrichment over input.

4.3 Results

The RNA binding protein, HuR, is relocalized to the cytoplasm during MVM infection

As previously reported, MVM infection induces a potent G2/M cell cycle block characterized by a significant depletion of cyclin B1 RNA and protein (9). HuR is an important RNA binding protein known to regulate the stability of cyclin A and cyclin B mRNA during cell cycle progression (422). It is also known that the subcellular localization of HuR is dependent on the cell cycle and DNA damage response (DDR) (215, 422). Given the significance of both cell cycle arrest and DDR activation during MVM infection, we first investigated the cellular localization of HuR in infected cells. Surprisingly, we found, *via* immunofluorescence, HuR to be predominantly cytoplasmic during infection while uninfected or doxorubicin-treated cells exhibited both nuclear and cytoplasmic localization, as previously reported (217, 218) (Figure 1A, compare first and second columns to third column).

Cytosolic and nuclear fractionation followed by Western blotting confirmed the predominantly cytoplasmic localization of HuR in MVM infection (Figure 1B, compare lanes 4 and 8). Interestingly, the subcellular localization of HuR during infection appeared to be temporal in nature. At early points in infection the balance between the cytoplasmic and nuclear fractions of HuR in MVM infected cells is relatively comparable to that of mock-treated cells (Figure 1B, compare lanes 1 and 2, 5 and 6). However, by thirty-two hours of infection HuR was relocalized to the cytoplasm, while mock cells exhibited no significant change in HuR distrubution (Figure 1B, compare lanes 3 and 7, 4,

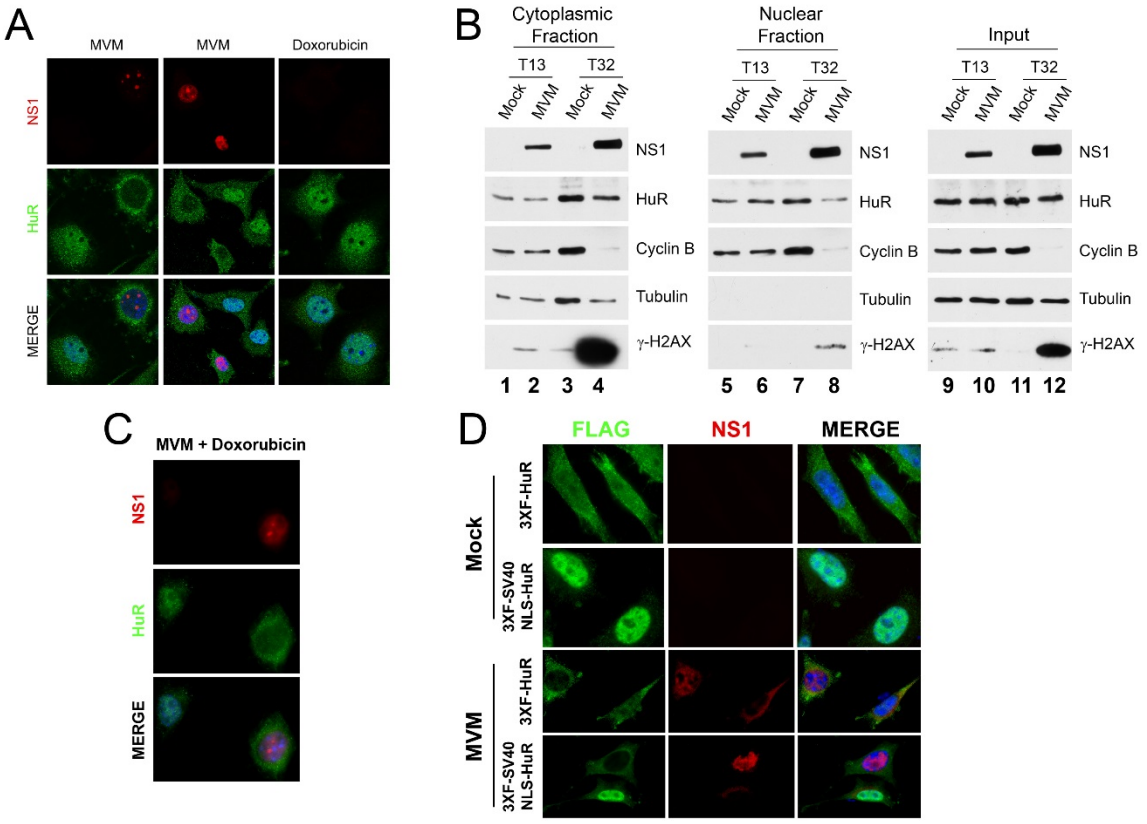
Figure 1 – MVM relocalizes HuR to the cytoplasm during infection

(A) Murine A9 cells were infected with MVM at an MOI of 10 or treated with 200 nM of doxorubicin for 24 hours. At 24 hours cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as described in Materials and Methods using antibodies for NS1 or HuR.

(B) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. At the indicated times cells were harvested and fractionated as described in Materials and Methods. Extracts were assayed by Western blotting using antibodies directed against the indicated proteins.

(C) Murine A9 cells were infected with MVM at an MOI of 10. At 15 hours post-infection cells were treated with 200 nM doxorubicin. At 24 hours cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as described in Materials and Methods using antibodies for NS1 or HuR.

(D) Murine A9 cells were transfected with plasmids expressing 3XF-HuR or 3XF-SV40-NLS-HuR as described in Materials and Methods. 24 hours following transfection cells were mock-treated or infected with MVM at an MOI of 10. At 24 hours post-infection cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as described in Materials and Methods using antibodies for NS1 or FLAG.


A
MVM
MVM
Doxorubicin
NS1
HuR
MERGE
B
Cytoplasmic Fraction
Nuclear Fraction
Input
T13
T32
Mock
MVM
NS1
HuR
Cyclin B
Tubulin
γ-H2AX
1 2 3 4
5 6 7 8
9 10 11 12
C
MVM + Doxorubicin
NS1
HuR
MERGE
D
FLAG
NS1
MERGE
Mock
MVM
3XF-HuR
3XF-SV40 NLS-HuR

and 8). Importantly, the expression level of HuR remained relatively unchanged between thirteen and thirty-two hours in both mock-treated and MVM infected cells (Figure 1B, compare lanes 9-12). These results suggested that protein degradation was unlikely to play an important role in alteration of the balance of HuR localization during MVM infection.

Doxorubicin, a topoisomerase II inhibitor known produce double-stranded breaks and induce a robust DDR (318), has been shown to affect HuR localization in breast cancer cells (243) and HuR is known to be regulated by key DDR proteins (215). In addition, doxorubicin induces a G2/M cell cycle block (25, 385) which is similar to the cell cycle block MVM induces (9). Treatment of cells with doxorubicin resulted in strong nuclear staining *via* immunofluorescence (Figure 1A). Interestingly, doxorubicin treatment of MVM infected cells still resulted in cytoplasmic relocalization of HuR, while an uninfected, doxorubicin-treated cell exhibited typical nuclear and cytoplasmic HuR localization (Figure 1C, compare left and right cells). These results suggest that MVM can play a dominant role over the effects an exogenous DNA damaging agent can impose upon HuR. As doxorubicin treatment and MVM infection result in a similar G2/M cell cycle block, these results also suggest that imposing a pre-mitotic cell cycle block is not sufficient to relocalize HuR, implicating a possible direct viral role in the cytoplasmic relocalization of HuR.

Because MVM was able to exert a dominant effect over a strong DNA damaging agent, we wondered if MVM could overcome the effects of a strong nuclear localization signal (NLS). To investigate this, we utilized a construct in which we cloned the

monopartite SV40 large T-antigen NLS (208, 209) into the N-terminus of a triple-FLAG tagged HuR. First, we confirmed the localization of the ectopically expressed triple-FLAG tagged HuR without the influence of the additional NLS. In mock cells, transient transfection of the triple-FLAG tagged HuR cDNA revealed the localization pattern of the ectopically expressed HuR to be similar to previous results with the endogenous protein (Figure 1D, first row). Importantly, ectopically expressed triple-FLAG tagged HuR was strongly relocalized to the cytoplasm during MVM infection in a manner similar to the endogenous HuR (Figure 1D, third row, compare left and right cells). Fusion of the SV40 NLS to triple-FLAG tagged HuR, transiently expressed in mock-treated cells, demonstrated the efficacy of the NLS tag as we detected strong nuclear localization of the over-expressed protein (Figure 1D, second row). Surprisingly, ectopically expressed triple-FLAG tagged HuR fused to a SV40 NLS was still relocalized to the cytoplasm during MVM infection (Figure 1D, fourth row, compare top and bottom cells). Taken together, these results suggest that the mechanism MVM employs to relocalize HuR during infection is quite robust.

Cytoplasmic relocalization of HuR during MVM infection may be facilitated by the main replicative non-structural protein, NS1.

MVM produces two non-structural proteins during infection: NS1, the main replicative protein with nickase, helicase and ATPase functions which are required for viral replication (102), and NS2, whose function has not yet been fully elucidated but is critical for efficient replication in the natural murine host cell (309). Given the limited arsenal afforded by MVM's small genetic coding capacity to modulate cellular events,

we sought to determine if either of these non-structural proteins aid the relocalization of HuR during viral infection. Surprisingly, transient transfection of HA-tagged NS1 resulted in cytoplasmic relocalization of HuR (Figure 2A, first row, compare left and right cells). In contrast, ectopic expression of HA-tagged NS2 did not alter the balance of HuR localization in NS2-expressing cells (Figure 2A, second row). Transient transfection of a construct expressing both viral non-structural proteins restored the ability to relocalize HuR to the cytoplasm (Figure 2A, third row, compare left and right cells). These results suggest that NS1, but not NS2, may play an important role in HuR relocalization during MVM infection.

Previous results indicated that MVM infection could play a dominant role in HuR relocalization over an exogenous DNA damaging and cell cycle arresting agent. To determine if MVM's non-structural protein NS1 contributes to this dominant role we utilized tetracycline-inducible NS1-expressing A9 cell lines, as previously described (10). Comparable to earlier results, expression of NS1 *via* tetracycline induction in mock-treated cells relocalized HuR to the cytoplasm (Figure 2B, second row, compare middle to adjacent cells). Importantly, uninduced mock-treated cells did not relocalize HuR, suggesting the cytoplasmic relocalization observed in the induced cells was the result of NS1 expression (Figure 2B, first row). Induced expression of NS1 in doxorubicin-treated cells resulted in the cytoplasmic relocalization of HuR (Figure 2B, fourth row, compare left and right cells). As predicted, the uninduced doxorubicin-treated cells exhibited both nuclear and cytoplasmic localization similar to previous results (Figure 2B, third row). Interestingly, induction of NS1 expression at or following doxorubicin treatment

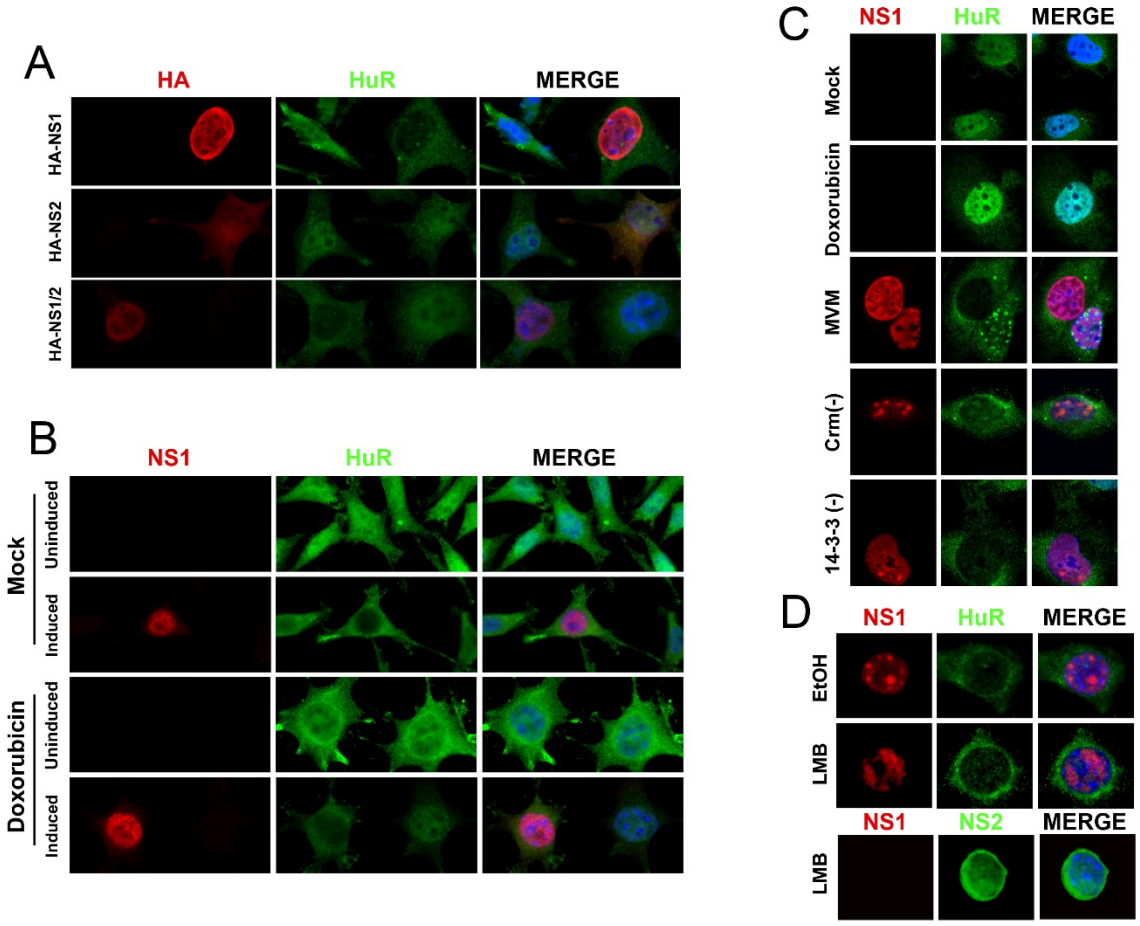
Figure 2 – Ectopic expression NS1 can relocalize HuR to the cytoplasm

(A) Murine A9 cells were transfected with plasmids expressing HA-NS1, HA-NS2 or HA-NS1 + NS2 as described in Materials and Methods. At 24 hours cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as described in Materials and Methods using antibodies for NS1 or HA.

(B) Murine A9 cells lines inducibly expressing NS1 were generated as described in Materials and Methods. Cells were induced for 24 hours with doxycycline. 24 hours post-induction cells were mock-treated or treated with 200 nM of doxorubicin. At 24 hours post-treatment cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as described in Materials and Methods using antibodies for NS1 or HuR.

(C) Human NB324K cells were infected with MVM (wild-type), MVM-Crm(-) or MVM-14-3-3(-) at an MOI of 10 or treated with 200 nM of doxorubicin for 24 hours. At 24 hours cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as described in Materials and Methods using antibodies for NS1 or HuR.

(D) Murine A9 cells were infected with MVM at an MOI of 10. 18 hours post-infection cells were treated with EtOH vehicle or leptomycin B at a concentration of 10 nM. 6 hours post-treatment cells were fixed with 4% paraformaldehyde and processed for immunofluorescence as described in Materials and Methods using antibodies for NS1, NS2 or HuR.


A
HA
HuR
MERGE
HA-NS1
HA-NS2
HA-NS1/2
B
NS1
HuR
MERGE
Mock
Uninduced
Induced
Doxorubicin
Uninduced
Induced
C
NS1
HuR
MERGE
Mock
Doxorubicin
MVM
Crm(-)
14-3-3 (-)
D
NS1
HuR
MERGE
EtOH
LMB
NS1
NS2
MERGE
LMB

both resulted in HuR relocalization, suggesting that the mechanism NS1 utilizes to relocalize HuR is relatively robust (data not shown).

MVM infection can relocalize HuR in human NB324K cells and may be Crm1- and 14-3-3 protein-independent

Given the possibility of NS1's role in modulating HuR localization, we wanted to determine if this modulation occurred in a host cell-type-dependent manner. To this end, we investigated HuR localization during MVM infection of permissive human NB324K cells. Mock-treatment of human NB324K cells revealed subcellular localization of HuR comparable to murine A9 fibroblasts (Figure 2C, first row). In addition, doxorubicin treatment of human NB324K cells resulted in robust nuclear localization of HuR, concurrent with cytoplasmic localization, similar to previous A9 observations (Figure 2C, second row). Interestingly, MVM infection in human NB324K cells resulted in a similar cytoplasmic relocalization of HuR as observed in murine A9 cells (Figure 2C, third row). These result suggest that the relocalization of HuR during MVM infection may be an important hallmark of parvovirus infection, as the effect is likely host-type independent. Closer examination of HuR localization in a limited number of MVM infected NB324K cells revealed that HuR, which remained marginally nuclear following cytoplasmic relocalization, at times formed sizable foci adjacent to viral replication factories, termed autonomous parvovirus-associated replication bodies (APAR) (Figure 2C, third row, bottom cell). This phenomenon could also be observed in some A9 cells under longer immunofluorescence exposure (data not shown). How or why these foci form is currently under investigation. APAR bodies are known to contain the sites of

viral DNA replication and cellular components critical for viral infection including cell cycle proteins, E3 ubiquitin ligases and replication and DDR proteins, among others. It is tempting to speculate that the virus is sequestering HuR for its own use or until it can promote relocalization.

The subcellular localization of HuR is known to be regulated by many factors, which ultimately determine the cellular process utilized for transport (217). HuR binds and exports mRNAs out of the nucleus utilizing either Crm1-dependent or transportin-2-dependent pathways (143). HuR achieves nuclear-cytoplasmic shuttling *via* an internal amino acid sequence, termed the HuR nucleocytoplasmic shuttling sequence (HNS), which employs transportin-1 or transportin-2 as redundant nuclear import factors (129, 159, 360). Importantly, MVM NS2 has been shown to strongly bind Crm1, which is thought to promote nuclear export of NS2 during infection and is critical for nuclear egress of progeny virions (40, 124, 446). MVM NS2 has also been shown to interact with 14-3-3 proteins (40, 45), specifically the epsilon, beta or zeta isoforms (320). 14-3-3 proteins are known to regulate the cell cycle, DDR and apoptosis by controlling the nuclear and cytoplasmic distribution of target proteins (49, 77, 178). The importance of the interaction between NS2 and 14-3-3 isoforms is not yet fully understood. Phosphorylation of HuR at Ser-202 by CDK1, which is known to be inactive during MVM infection (9), has been shown to promote interaction with 14-3-3 proteins and induce nuclear accumulation of HuR (216). We wondered whether NS2 binding to Crm1 could facilitate HuR export during infection or if NS2 association with 14-3-3 proteins could

prevent HuR association with 14-3-3 proteins, thereby promoting cytoplasmic accumulation of HuR.

To determine if Crm1 transport or 14-3-3 interaction played a role in HuR cytoplasmic relocalization, we took advantage of MVM's permissiveness in human NB324K cells and the dispensable nature of NS2 in this system (309). Mutant MVM viruses have been generated in which NS2 does not interact with Crm1 (124, 288) or 14-3-3 proteins (Pintel, unpublished data). Interestingly, each mutant virus expressing NS2 interaction mutations retained the ability to relocalize HuR to the cytoplasm during infection in NB324K cells (Figure 2C, fourth and fifth rows). These results suggest that MVM relocalizes HuR during infection in a Crm1- and 14-3-3 protein-independent manner. Inactivation of CDK1 kinase activity may yet play a role in cytoplasmic accumulation of HuR during infection and remains to be investigated further. Given the importance of Crm1 in nuclear export of HuR, we wanted to further verify if HuR cytoplasmic relocalization during MVM infection was truly Crm1-independent. Leptomycin B has previously been shown to inactivate Crm1 shuttling and induce nuclear accumulation of Crm1 and its associated cargo (230). MVM infected cells treated with leptomycin B still exhibited cytoplasmic HuR relocalization similar to treatment with the ethanol (EtOH) carrier (Figure 2D, compare first and second rows). As an important control, treatment of MVM infected with leptomycin B resulted in strong accumulation of NS2 in the nucleus, as previously reported (40, 288), suggesting that the leptomycin B treatment was effective.

Unexpectedly, infection of NB324K cells with the MVM-1989 mutant virus, which has a mutation in the large intron and does not express NS2 (56), did not result in HuR relocalization in human NB324K cells (data not shown). However, given the severely reduced efficiency of viral infection and replication of this mutant virus, and progression of APAR bodies through the known stages, it is not yet clear if NS2 does indeed play a role in HuR relocalization in human cells. Further, careful studies will be required to elucidate the possibility of NS2's role in or collusion with NS1 for HuR relocalization. It remains possible that the mechanism of relocalization in permissive human cells differs from the mechanism utilized in the host murine cells.

HuR binds MVM RNA and may promote translation of cyclin B1 RNA during MVM infection

HuR has been well characterized as an RNA-binding protein that regulates the stability and nuclear export of numerous target RNAs (421). Following nuclear export, HuR can also promote or inhibit translation of certain RNAs including FoxP1, FoxP2, HIF-1α, Myc, p27 and p53, among others (54, 141, 231, 256, 283, 345). It has been suggested that cyclin B1 translation may be temporally controlled within RNA stress granules. Intriguingly, HuR and cyclin B1 were found, in oocytes, to colocalize within these structures suggesting a possible role for HuR-mediated translational control of cyclin B1 during cellular stress (225). To investigate possible HuR-mediated effects on translation during MVM infection, we utilized HuR-targeting siRNA during infection. At a late time point in infection we recapitulated the previously reported reduction in cyclin B1 expression during MVM infection (Figure 3A, compare lanes 3 and 4) (9). Surprisingly,

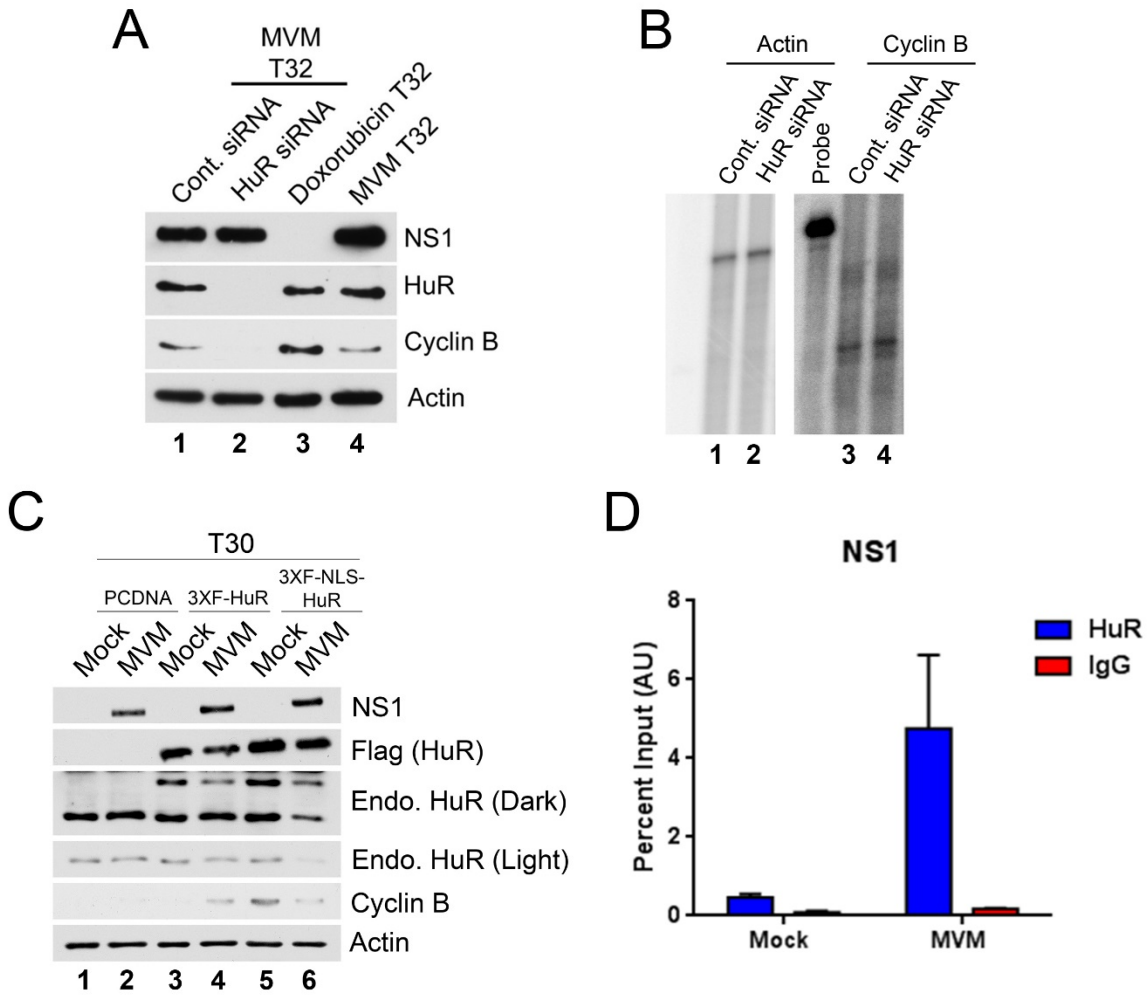
Figure 3 – HuR binds MVM RNA and may promote translation of cyclin B1 RNA during infection

(A) Murine A9 cells were transfected twice during parasynchronization by isoleucine deprivation with control siRNA or HuR siRNA as indicated. Following 42 hours of parasynchronization cells were infected with MVM at an MOI of 10 at the time of release into complete medium. 15 hours post-infection cells were treated with 200 nM of doxorubicin as indicated. 32 hours post-infection cells were harvested and RIPA lysates were assayed by Western blotting using antibodies directed against the indicated proteins.

(B) Murine A9 cells were treated as described in (A). Total RNA was isolated using TRIzol reagent and samples were assayed by RNAse protection assay (RPA) as described in Materials and Methods using probes against murine actin and cyclin B1 (9).

(C) Murine A9 cells were transfected during parasynchronization by isoleucine deprivation with empty vector (pcDNA), 3XF-HuR or 3XF-NLS-HuR as indicated. Following 42 hours of parasynchronization cells were infected with MVM at an MOI of 10 at the time of release into complete medium. 24 hours post-infection cells were harvested and RIPA lysates were assayed by Western blotting using antibodies directed against the indicated proteins.

(D) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. 24 hours post-infection cells were harvested and processed for RIP as described in Materials and Methods using antibodies against HuR or control mouse IgG. Following RIP, total RNA was isolated using TRIzol reagent. Samples were assayed by qPCR as described in Materials and Methods using primers against MVM NS1 genomic region. Data are presented as fold enrichment over input.



Experiment in figure 3D was performed by Kinjal Majumder.

although the control siRNA exhibited reduced cyclin B1 expression, comparable to the untransfected MVM control (Figure 3A, compare lanes 1 and 4), siRNA knockdown of HuR resulted in an even further reduction in cyclin B1 protein (Figure 3A, compare lanes 1 and 2). Monitoring cyclin B1 RNA levels *via* RNase protection assay revealed similarly low levels of cyclin B1 RNA in both control and HuR siRNA knockdown (Figure 3B, compare lanes 3 and 4). These results suggested that HuR might be mediating a translational rather than transcriptional effect on cyclin B1 during MVM infection. Reconstitution of triple-FLAG tagged HuR and SV40-NLS-HuR both resulted in increased cyclin B1 expression in MVM infected cells (Figure 3C, compare lanes 2, 4 and 6). Taken together, one could speculate that the cytoplasmic HuR promotes cyclin B1 RNA translation during MVM infection. MVM relocalization of HuR could prevent nuclear-cytoplasmic shuttling of HuR, thus preventing HuR-dependent transport of nascent cyclin B1 RNA into the cytoplasm and HuR-mediated translation of nascent cyclin B1 RNA, providing another mechanism to keep this critical mitotic cyclin low during infection.

Several viruses are known to facilitate viral mRNA stability, and as a consequence potentially destabilize cellular mRNAs (28), *via* HuR interaction. The Sindbis alphavirus has been shown to relocalize HuR to the cytoplasm and stabilize its viral mRNAs by binding to HuR (120, 393). HuR has also been implicated in enhancement of Hepatitis C virus replication by facilitating interactions of the cellular La protein and polypyrimidine tract binding protein (PTB) with the viral 3'UTR (388). Finally, Human papilloma virus – 1 (HPV-1) has been shown to bind HuR in its 3'UTR and is thought to mediate

posttranscriptional HPV-1 gene expression (394). Given the important role HuR has in multiple viral lifecycles, we speculated that HuR may bind and potentially stabilize MVM mRNA. MVM mRNA, which is very stable with a half-life greater than 6 hours, has been estimated to make up to 25% of the total RNA within the cell during an infection (341, 380). *In silico* analysis revealed two potential consensus HuR binding sites in the 3'UTR of MVM mRNAs (337). Interestingly, RNA immunoprecipitation (RIP) experiments suggest that HuR does bind MVM mRNA during infection (Figure 3D, compare mock to MVM). One could propose that HuR binding of MVM RNA may promote or mediate its robust stability. HuR binding of MVM RNA may also facilitate shuttling of viral RNAs to the cytoplasm and promote their translation. Further experiments must be undertaken to determine if HuR plays a critical role in the regulation of MVM RNA stability or gene expression.

4.4 Discussion

Parvoviruses rely on host machinery for a multitude of functions, including replication of their genome and transcription of their RNA. MVM induces an ATM-dependent DDR during infection, which it then utilizes to impose a pre-mitotic cell cycle block and foster an environment conducive for replication. Many of the proteins involved in these aforementioned processes are recruited to APAR bodies including numerous DDR proteins, transcription factors, RNA polymerase II, PCNA, cyclin A, cyclin B1 and DNA polymerase δ. The RNA-binding protein, HuR, is known to regulate the DDR and modulate the stability of cyclin A and cyclin B1.

Here, we have shown that MVM relocalizes this key regulator which sits at the crux of several important factors in MVM replication. Our data suggests that the main replicative non-structural protein, NS1, likely plays a key role in relocalizing HuR during infection, as ectopic expression of NS1 resulted in the relocalization of HuR. Subcellular fractionation followed by Western blotting demonstrated that the relocalization of HuR during viral infection was temporally regulated. Data collected *via* immunofluorescence was consistent with these results, which revealed that APAR bodies needed to advance beyond the early stages of formation to observe this effect. Taken together, this data suggests that a threshold level of NS1 is required to modulate HuR subcellular localization. It is not yet clear if NS1 directly relocalizes HuR or if the accumulation of NS1 results in the activation or inhibition of a specific kinase or phosphatase, which in turn regulates HuR localization *via* phosphorylation. Identification of the precise mechanism utilized by MVM to achieve HuR relocalization requires further study.

Our data do not yet clearly define the exact nature of the cytoplasmic accumulation of HuR during MVM infection. HuR is known to be exported by the Crm1 and transportin-1 pathways and imported by redundant transportin-1 and transportin-2 pathways. One possibility is that during MVM infection HuR is prevented from re-entering the nucleus following natural nuclear-cytoplasmic shuttling *via* a virally-induced inhibition of the transportin pathways. Another possibility is that HuR is posttranslationally modified in such a way that pre-mature nuclear departure is induced. The modulation of HuR localization and function, including RNA binding and translation, owing to the specific phosphorylation of certain residues has been studied extensively in other systems. To understand the potential outcomes of HuR modulation during MVM infection, a phosphorylation mapping study *via* mass spectrometry analysis will need to be completed. While our data suggest that HuR relocalization during MVM infection is Crm1-independent, we have not yet investigated the possibility of transportin-1's involvement. Additional, careful experiments will need to be undertaken to further delineate the cellular process that MVM exploits to achieve the cytoplasmic relocalization of HuR.

siRNA knockdown of HuR suggested that MVM may further modulate this RNA-binding protein by fine-tuning the translational effects HuR can impose. It is unclear if MVM modifies HuR-mediated translation by preventing the natural nuclear-cytoplasmic shuttling of HuR or if the cytoplasmic relocalization promotes the stability and translation of specific cellular or viral mRNAs. Additional work needs to be undertaken to identify which cellular RNAs are bound by HuR during MVM infection. A combination

of PAR-CLIP and deep sequencing is a promising avenue towards this goal (160, 161). Traditional polysome fractionation experiments will aid in identification of possible HuR translational targets during MVM infection (142).

Although it is interesting to speculate that MVM may modulate a number of cellular RNAs, it is important to note that we have demonstrated binding of HuR to MVM mRNA. Importantly, *in silico* analysis suggested two possible HuR binding sites in the MVM 3'UTR, with additional potential binding sites found throughout the genome (data not shown) (337). Further studies will need to be completed to determine where HuR binds MVM RNA, as these sites may be in intronic regions or the 3'UTR. As previously shown in other systems, HuR may be influencing MVM pre-mRNA processing *via* intronic interactions and stability of these RNAs *via* 3'UTR binding (304). Given the stable nature of MVM RNAs, it is tempting to speculate that the relocalization of HuR is necessary to promote the stability and translation of MVM mRNAs.

Multiple viruses have been shown to harness or modulate HuR to facilitate infection. CRISPR screening revealed that knockout of HuR nearly abolishes HCV replication, consistent with previous reports (356), while other surveyed Flaviviruses were relatively unaffected (276). Closer examination revealed that HuR binds to HCV's 3'UTR and facilitates the binding of host proteins La and PTB, thereby mediating circularization of the genome and stimulating viral replication (388). Sindbis virus was shown to relocalize HuR to the cytoplasm and bind HuR *via* its U-rich 3'UTR to prevent decay of viral RNA (393). Interestingly, co-opting HuR to enhance viral RNA stability results in significant changes to cellular mRNA during Sindbis virus infection, including

destabilization of cellular RNA and defects in alternative splicing and polyadenylation (28). Two other alphaviruses, Ross River Virus and Chikungunya Virus, also relocalize HuR to the cytoplasm during infection, suggesting that this modulation is a hallmark of alphavirus infection (120). Ectopic expression of adenovirus E4orf6 was shown to relocalize pp32/LANP and their associated mRNAs to the cytoplasm, which also resulted in relocalization of pp32/LANP's binding partner, HuR (180). It is important to note that every virus known to utilize HuR during an active infection is an RNA virus. To our knowledge, this report is the first demonstration of a DNA virus relocalizing and binding to HuR during an active infection. Future work will be required to understand how HuR regulates the stability, pre-mRNA processing and translation of both cellular and viral RNAs during MVM infection.

5 Overall summary

Viruses are parasitic entities which must rely on co-opting specific cellular machinery to establish an environment conducive to the requirements of their life cycle. The extent to which a virus depends upon these cellular components varies based on the complexity and capacity of that virus' genome and its encoded gene products. Study of the virus-host interface informs our ability to develop antiviral treatments targeting specific cellular or viral pathways utilized by the virus. Importantly, many cellular pathways have been discovered and numerous critical cellular insights have been reported as a result of studying this interface including alternative splicing, oncogenes, transcriptional enhancers and nuclear localization signals.

Cells face constant insults to their genome and require the ability to repair them, *via* the DNA damage response (DDR), lest they suffer genomic instability or death. The pathways that govern this repair process are multifaceted and intertwined, with crosstalk occurring between different arms of the repair response. These pathways are critical to many forms of life and are conserved, to some extent, between prokaryotic and eukaryotic organisms. Given the critical and ancient nature of this cellular response, it is unsurprising that cells also likely utilize this network as an antiviral system. Naturally, viruses have evolved the ability to inactivate and/or harness this potentially antiviral response. Importantly, cells have integrated this repair process with DNA replication, RNA transcription and cell cycle machinery. This places a virus with the

ability to modulate the DDR at the crux of nearly every cellular process that they may need to exploit to ensure progression through their lifecycle.

In this work, we provide several examples of how a virus can utilize and modulate specific cellular pathways to provide a conducive environment for replication. Infection with minute virus of mice (MVM) introduces a linear single-stranded DNA genome into the host cell nucleus, where it is extended into a duplex form, which then serves as a template for transcription. Transcription and translation of the main viral replicative protein, NS1, ensues and as NS1 accumulates, nascent viral replication bodies form. Within these replication factories the virus relies on cellular proteins, specifically DNA polymerase δ, PCNA, RFC and cyclin A to replicate its genome. As infection progresses, and these replication bodies expand, cell cycle progression is arrested before the onset of mitosis.

Given the activation of ATM, Chk2 and p53 during MVM infection, one might predict that MVM would utilize the CDK inhibitor p21 to induce the observed cell cycle arrest. However, we determined that viral activation of p53, and thereby its downstream target p21, affects the activity of PCNA, which the virus needs to replicate. To provide the activity it requires, MVM recruits and exploits a cellular E3 ubiquitin ligase to target p21 for degradation. This allows MVM to activate ATM and its downstream targets, which is required for replication, yet fine-tune the cellular response to fit its needs.

Though it was surprising that MVM did not utilize p21 to induce the observed pre-mitotic cell cycle arrest, we have shown that the key mitotic cyclin, cyclin B1 is depleted at both an RNA and protein level during infection. To accomplish this feat, MVM targets a key cyclin B1 transcription factor, FoxM1, and reduces its occupancy on the cyclin B1 gene during infection. In turn, this reduces the occupancy of RNA polymerase II on the cyclin B1 gene, culminating in a reduction in the nascent production of cyclin B1 RNA. Although we know that the reduced phosphorylation of FoxM1 we observe in MVM infection is characteristic of an inactive FoxM1, the exact mechanism that MVM utilizes to prevent FoxM1 activation requires further investigation. Importantly, targeting a specific transcription factor during infection permits MVM to fine-tune an infected cell's ability to transcribe RNA, directly and swiftly impacting the environment MVM establishes for itself. Modulation of the subcellular localization of the RNA-binding protein HuR may provide MVM the ability to expertly modulate the translation of the RNAs it has allowed to be transcribed.

Here, we have shown that MVM targets several core cellular processes— protein degradation, RNA transcription, RNA translation and subcellular localization of cellular proteins—to harness and exploit a host cell's resources. While many viruses have been shown to modulate these same cellular functions, MVM does so with an extremely limited genetic arsenal. As investigation continues, it will be interesting to unravel how MVM manages to accomplish the same feats as other viruses which much larger coding capacities.

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Vita

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