Genomes of the Autonomous Parvovirus Minute Virus of Mice Induce Replication Stress
Through RPA Exhaustion

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ABSTRACT

The oncolytic autonomous parvovirus Minute Virus of Mice (MVM) establishes infection in the nuclear environment by usurping host DNA Damage Response (DDRs) in the vicinity of cellular DNA break sites. MVM replication induces a global cellular DDR that is dependent on signaling by the ATM kinase and inactivates the cellular ATR-kinase pathway. However, the mechanism of how MVM generates cellular DNA breaks remains unknown. Using single molecule DNA Fiber Analysis, we have discovered that MVM infection leads to a shortening of host replication forks as infection progresses, as well as induction of replication stress prior to the initiation of virus replication. Ectopically expressed viral non-structural proteins NS1 and NS2 are sufficient to cause host-cell replication stress, as is the presence of UV-inactivated non-replicative MVM genomes. The host single-stranded DNA binding protein Replication Protein A (RPA) associates with the UV-inactivated MVM genomes, suggesting MVM genomes might serve as a sink for cellular stores of RPA. Overexpressing RPA in host cells prior to UV-MVM infection rescues DNA fiber lengths and increases MVM replication, confirming that MVM genomes deplete RPA stores to cause replication stress. Together, these results indicate that the presence of ssDNA in the nucleus generated by MVM genomes and viral proteins induces replication stress in the host cell through RPA exhaustion, rendering the host genome vulnerable to additional DNA breaks.
50  AUTHOR SUMMARY
51  Paroviruses are used in the clinic to design recombinant gene therapy vectors and as oncolytic agents. The autonomous parovirus MVM utilizes the host cell’s DNA damage response machinery to replicate in host cells and cause additional DNA breaks. However, the mechanism of MVM-induced DNA damage remains unknown. We have discovered that MVM sequesters the host DNA repair protein RPA, which normally associates with single stranded DNA in the nucleus, rendering the host genome susceptible to replication stress. Our study provides insights into the mechanisms utilized by single-stranded DNA viruses to amplify host-cell DNA damage.
INTRODUCTION

Parvoviruses are small, non-enveloped, single-stranded DNA viruses with a linear genome that are used as gene therapy vectors and oncolytic agents in the clinic (1-3). Unlike Adeno-Associated Viruses (AAV) that are used to design gene therapies and require a coinfecting helper virus (Adenovirus, Herpesvirus or Papillomavirus) to replicate, the oncolytic protoparvovirus Minute Virus of Mice (MVM) replicates autonomously in host cells in S phase (4-6). This replication is carried out by an alternating strand displacement and rolling circle pathway referred to as “rolling hairpin” replication, that originates at the palindromic Inverted Terminal Repeats (ITRs) flanking the 5 kb viral genome (5). Since it does not encode its own polymerases, MVM is completely dependent on host replication proteins, primarily pol-alpha and delta, to amplify its genome (6). MVM replication critically depends on its non-structural protein NS1 that transcriptionally activates the expression of the viral capsid genes, functions as a helicase and a nickase to cleave dimer MVM genomes during replication and transports the viral genome to cellular sites of DNA damage (7, 8). The other non-structural protein encoded by MVM, NS2, is only required for replication in mice but not in transformed human cells and functions in viral genome export in murine cells (9).

Infection of host cells by DNA viruses elicit nuclear DNA Damage Response (DDR) signals that mirror those initiated upon induction of cellular DNA breaks (10, 11). Designed to maintain the fidelity of the host genome from genotoxic stress, these cellular DDR signals can impede or facilitate the life cycle of viral pathogens (12). Therefore, viruses have evolved distinct mechanisms to modulate the host DDR pathways, as is the case for Adenovirus, that degrades the DDR protein MRE11 using its oncoproteins E1B55K and E4ORF6 to overcome the suppressive effect of the host DDR (13-16). SV40 and Polyomaviruses on the other hand utilize the host DDR pathways for their benefit through their Large T Antigen (LTA) proteins, that interact with DDR factors to enhance their ability to replicate (17-19). We have previously discovered that MVM utilizes its non-structural protein NS1 to bind and localize the viral genome.
to pre-existing cellular sites of DNA damage that contain host replication and repair proteins, presumably to jumpstart virus replication (8, 20). MVM establishes replication centers at these nuclear milieu, forming virus replication factories dubbed APAR (Autonomous Parvovirus Associated Replication) bodies. APAR bodies coincide with the viral genome and non-structural proteins NS1 and NS2; as well as host replication and DDR proteins such as DNA polymerase delta, MRE11, RPA and many more (6, 21-25). Although MVM replication requires signals from DDR proteins in the ATM kinase pathway, at late stages it inactivates the ATR signaling pathway, the cell’s primary responder to single-stranded DNA breaks generated by replication stress (21, 22, 26). It is speculated that inactivation of the ATR signaling pathway serves to inactivate the proteins responsible for detecting single-stranded MVM genomes, thus facilitating viral pathogenesis (21). MVM infection eventually leads to a potent pre-mitotic cell cycle block at the G2/M border mediated by degradation of cellular p21 and transcriptional repression of Cyclin B1 during which the host genome is also fragmented (27-30).

MVM genomes localize to cellular sites that are rich in DDR proteins even in the absence of viral infection. Chemical induction of replication stress using hydroxyurea (HU) preferentially enriches DDR proteins at these same cellular sites (20). We have previously dubbed these cellular sites where MVM establishes replication centers in the nuclear milieu as Virus Associated Domains (VADs), which also colocalize with Early Replicating Fragile (ERF) sites, where transcription-replication conflicts take place at higher frequency leading to a high basal level of DDR signals (20, 31, 32). Interestingly, in addition to transporting the viral genomes to the cellular VAD sites, ectopically expressed NS1 proteins also associate with cellular VADs (8). As virus replication amplifies, MVM genomes spread along the host genome to induce further DNA damage beyond the VADs, causing the recruitment of additional DDR proteins. Induction of additional cellular replication stress leads to enhanced MVM replication, suggesting a causal link exists between replication stress genome-wide and virus pathogenesis (20).
MVM infection inactivates signaling through the ATR-CHK1 kinase pathway, responsible for maintaining host genome integrity in response to replication stress (21, 26). ATR activation is initiated by the binding and autophosphorylation of single-stranded DNA molecules by Replication Protein A (RPA). The ssDNA-RPA subunits sequentially recruit ATRIP, the checkpoint clamp complex RAD9-HUS1-RAD1 (9-1-1) and TOPBP1, leading to phosphorylation of ATR (26). MVM infection inactivates the ATR pathway at the level of TOPBP1, although earlier members of the ATR signaling cascade continue to associate with APAR bodies (21). In doing so, MVM inactivates the downstream CHK1 kinase, normally required to counter new origin firing in response to replication stress. However, it remains unknown how MVM induces replication stress in host cells and whether this is exacerbated by ATR-CHK1 inhibition. Since paroviral replication utilizes many host DDR proteins that are normally at cellular replication forks, it additionally remains possible that stoichiometric competition for host DDR proteins exacerbates replication fork instability and eventual fork collapse. Some of these host factors usurped by MVM could include those that have been historically found associated with MVM APAR bodies, including RPA and PCNA, which are essential for MVM replication and associate with NS1 during ectopic expression (20, 25, 33).

In this study, we show that MVM infection starts to induce host replication stress during early S phase soon after initiation of virus replication, even when infected with replication incompetent UV-inactivated MVM. In addition, MVM induces replication stress by modulating host origin firing, as well as by serving as a sink for cellular RPA molecules. MVM-induced replication stress and origin misfiring can be rescued by inhibiting CDC7 activity, as well as by overexpressing exogenous RPA during infection. Together, these results indicate that MVM genomes induce cellular replication stress through RPA exhaustion, contributing host genome instability and induction of cellular DNA breaks.
RESULTS

MVM infection induces cellular replication stress that precede DDR signals.

To determine how MVM infection induces cellular DNA damage, we performed single-molecule DNA Fiber Analysis (DFA) in para-synchronized murine A9 cells infected at high multiplicity of infection (MOI of 25) with the prototype strain MVMP. DFA experiments were performed by sequential pulses of IdU and CldU for 20 minutes each at the indicated timepoints of MVM infection prior to slide fixation (Fig. 1A). In our synchronization protocol, the host cells enter S phase approximately 10 hours post release (and post infection). As shown in Figs. 1B and 1C, the lengths of the DNA fibers in cells pulsed with either IdU or CldU were decreased in MVM-infected samples at 12 hours post-infection (hpi) when compared with uninfected cells which were in the same stage of the cell cycle (i.e. early S phase). As replication progressed, we observed a progressive shortening of replication fibers at 18 hpi and 24 hpi, comparable to that of fibers undergoing HU induced replication stress (Fig. 1B and 1C; representative fibers shown in Fig. 1D). To independently corroborate the impact of replication fiber shortening with induction of cellular DDR signals, we measured the cellular levels of phosphorylated H2AX (\(\gamma\)H2AX) alongside those of the viral non-structural phosphoproteins NS1, a marker for virus replication (Fig. 1E). Interestingly, although we had observed a shortening of host replication fibers at 12 hpi, we did not detect significant levels of \(\gamma\)H2AX at this timepoint (Fig. 1E, lane 2). This observation suggested that replication stress precedes the induction of cellular DDR signals during MVM infection. We categorized the DNA fibers associated with replication forks at different stages over the time-course of MVM infection, observing a large percentage of new origins firing at 24 hpi (Fig. 1F; green fractions). Since MVM infected cells at 24 hpi are in G2 phase of cell cycle during which new origins are not expected to fire, this suggested that MVM infection may modulate the machinery used by the host cell to control replication origin firing. To determine the generalizability of our findings of MVM-induced replication stress, we measured
cellular DNA fibers in U2OS cells infected with MVM and the NS2-expression mutant of MVM (MVM\textsuperscript{\textDeltaNS2}) at 24 hpi, which infects only transformed human cells. These measurements revealed that both wild-type MVM and MVM\textsuperscript{\textDeltaNS2} infection induce replication stress at equivalent levels in U2OS cells (Fig. 1G and 1H). Taken together, our studies on the impact of MVM infection in A9 and U2OS cells revealed that MVM induces replication stress that precedes the induction of cellular DDR, leading to increased new-origin firing at late stages of infection. 

\textit{MVM-induced replication stress is reversible by Cdc7 inhibition.}

Licensing of the host replication complex during DNA replication is carried out by phosphorylation of the Minichromosome Maintenance (MCM) helicase complex by the serine/threonine kinase CDC7 (34). The ATR-CHK1 pathway stabilizes CDC7 during replication stress to prevent replication fork collapse (35). However, MVM infection inactivates the ATR/CHK1 pathway at late stages, suggesting that early in infection (12 hpi and 18 hpi) timepoints there are sufficient CHK1 levels to respond to replication stress and unscheduled origin firing. However, we saw major dysregulation of new origin firing during the late stages of MVM infection (Fig. 1F). Therefore, we hypothesized that ATR/CHK1 inactivation at late stages of infection dysregulates CDC7 and its ability to regulate new origin firing. To test this hypothesis, we treated MVM infected A9 cells with the CDC7 inhibitor PHA 767491 at 20 hpi (iCDC7; as illustrated in Fig. 2A), which led to the rescue of DNA fiber shortening at 24 hpi (Fig. 2B, 2C), correlating with a decrease in new origin firing in the host cells (Fig. 2D). In addition, treating with the CDC7 inhibitor resulted in lowered levels of NS1 and \(\gamma\)H2AX in MVM infected A9 cells, as detected via western blot (Fig. 2E, compare lanes 3 and 4). The diminished levels of \(\gamma\)H2AX in the presence of iCDC7 support the rescue of replication fiber length observed in Figs. 2B and 2C, indicating lower levels of DNA damage and replication stress. This decrease of MVM replication was further corroborated by smaller nuclear APAR bodies monitored by NS1 staining in MVM-infected A9 cells at 24 hpi (Fig. 2F). Taken together, these findings suggested
that MVM infection at late stages of infection induces replication stress through the
dysregulation of the replication kinase CDC7.

Ectopically expressed MVM Non-structural proteins NS1 and NS2 cause replication fork
shortening.

Characterization of the cellular DDR during MVM infection has previously determined
that the MVM-induced DDR requires ongoing virus replication (22). Since host cell replication
stress precedes virus replication, we attempted to determine which viral elements are sufficient
to host-cell replication stress. To assess the contribution of MVM non-structural proteins to host-
cell replication stress, we transfected plasmids expressing NS1 and NS2 into A9 cells [which
have been previously shown to not induce cellular \( \gamma \)H2AX; (22)] and assessed their impact on
host replication forks by DNA Fiber Assays at 24 hours post-transfection (Fig. 3A). Ectopic
expression of both NS1 and NS2 were sufficient to induce shortening of host-cell replication
forks, observed by decreasing length of both IdU and CldU (Fig. 3B, 3C). Since our observation
of NS1-induced replication stress might be generated downstream of cytopathic cellular effects,
we overexpressed an ATP-ase mutant of NS1 which is incapable of binding to DNA [labelled as
NS1\(^{K405S}\); (36, 37)]. Overexpression of NS1\(^{K405S}\) in A9 cells did not lead to an impact on host-cell
replication forks, suggesting the DNA binding function of NS1 is sufficient to induce host cell
replication stress (Fig. 3B, 3C; NS1\(^{K405S}\) samples). Although the NS1 overexpression did not
impact the types of replication fibers, NS2 overexpression led to a slight increase in stalled
replication forks in addition to shortening of both IdU and CldU tracks (Fig. 3B-3D). Taken
together, these results suggested that individual MVM proteins are sufficient to induce
replication stress without the induction of additional cellular DDR signals.

The non-replicating MVM genome binds cellular RPA and induces replication stress.

Since MVM infection induced replication stress in host cells entering S phase at the
earliest timepoints prior to detectable NS1 (Fig. 1B, 1C, 1E; 12 hpi timepoints) we reasoned that
NS1 might not be sufficient to cause replication stress in host cells early in infection. Therefore,
we examined the ability of the non-replicating MVM genome to induce replication stress in the
host using UV-inactivated MVM, which has been previously shown to not induce cellular γH2AX
(22). As shown in Fig. 4B and 4C, UV-MVM infection was sufficient to cause shortening of host
replication forks, comparable to that on replicating MVM. Categorization of the replication fiber
types revealed that UV-MVM genomes induced new origin firing in the same way as productive
MVM infection (Fig. 4D). These findings with UV-inactivated MVM genomes indicated to us that
the mere presence of the ssDNA viral genome is enough to induce replication stress on the host
cell, as well as dysregulate new origin firing. Because the UV-MVM genome is largely made up
of ssDNA, we hypothesized that the presence of the viral genome may serve as a sink for RPA
molecules in the cell, preventing it from properly protecting the host genome, leaving it
vulnerable to replication stress-induced fork collapse. To determine if RPA binds to the
replication incompetent UV-MVM genome, we performed ChIP-qPCR on the MVM genome on
the P4 promoter, finding that RPA associated with the UV-inactivated MVM genome at
substantially higher levels than non-specific IgG (Fig. 4E). Since cellular replication stress
generates single stranded DNA at host replication forks that are subsequently coated with RPA,
we imaged the location of RPA molecules relative to APAR bodies. Strikingly, we found that
both total RPA and phospho-RPA molecules colocalized with MVM replication centers (Fig. 4F,
top) whereas γH2AX signals surrounded the APAR bodies (Fig. 4F, bottom), consistent with
prior observations of MVM localizing to cellular DDR sites (8). Taken together, these findings
suggested that the MVM genome might be a substrate for RPA exhaustion, depleting the
cellular stores of this critical single-stranded DNA binding protein and rendering the host
genome vulnerable to replication stress.

RPA overexpression rescues MVM-induced replication stress.

To confirm that depletion of cellular RPA levels leads to MVM-induced replication stress,
we overexpressed phospho-RPA32 ectopically by transient transfection in A9 cells during
parasynchronization [designated as pRPA (38) and schematized in Fig. 5A]. Upon infection with UV-inactivated MVM, the presence of extra molecules of phospho-RPA in the nuclear compartment was sufficient to rescue host replication fibers, as measured by both IdU and CldU lengths (Fig. 5B and 5C). However, overexpression of phospho-RPA in host cells did not impact the fraction of DNA fiber types in host cells (Fig. 5D) and led to a modest increase in MVM replication (Fig. 5E). Taken together, our findings indicate that the MVM genome serves as a sink for cellular stores of RPA to induce replication stress in host cells.
**DISCUSSION**

In this study, we show for the first time using single-molecule DNA fiber assays that single-stranded DNA viruses induce replication stress in host cells that precedes virus replication and virus-induced DDR. RPA binds to the UV inactivated MVM genomes and the overexpression of phosphorylated RPA32 molecules leads to rescue of replication fork shortening during MVM infection. Ectopic expression of NS1 and NS2 phosphoproteins are sufficient to induce replication stress in the host genome. We therefore propose that exhaustion of cellular RPA levels contribute to virus-induced host genome instability at the early stages of infection, which is further amplified by the presence of NS1, NS2 and more replicating MVM molecules in the nuclear environment. As MVM replication continues, at 24 hpi (late stages of viral infection), MVM induces aberrantly high firing of replication origins through the CDC7 kinase. Consistent with this finding, CDC7 inhibition rescued host fibers and their propensity to fire replication origins. Taken together, these findings suggest that the viral genome serves as a sink for cellular stores of RPA, causing misfiring of cellular replication forks and contributing to host genome instability which is beneficial for the virus life cycle.

Characterization of the interplay between MVM infection and cellular DDRs have previously shown that MVM induces cellular DDR signals that are detectable by temporal increase of cellular and local $\gamma$H2AX as well as the presence of comet tails indicative of genome fragmentation (20, 22). Interestingly, pulsing MVM infected cells with the replication stress inducing agent hydroxyurea (HU) led to increased virus replication (20). These observations initially suggested that HU-induces the formation of more DNA break sites on the host genome which could serve as potential sites for APAR body formation (8, 20). Our findings in this study build on the proviral nature of host cell replication stress by elucidating the molecular events that drive the cellular DNA break formation at replication forks. Since we detect MVM induced replication stress as early as 12 hours post-release when MVM genomes are yet to amplify vigorously, this suggests that the mere presence of the MVM genome in small quantities can
induce replication stress on the host cell. These findings are further borne out by induction of cellular replication stress even in the absence of MVM replication when infected with UV-inactivated MVM genomes which do not induce cellular γH2AX (22). We propose that this cellular stress is generated via multiple pathways that function at different stages of MVM infection. Prior studies on the late stages of MVM infection (at 24 hpi and beyond) have identified CRL4Cdt2-mediated p21 degradation and transcriptional repression of Cyclin B1 as key events driving cell cycle arrest at the late stages of infection (27-30). Our findings indicate RPA exhaustion as one of the additional host stress pathways which is activated at early stages of MVM infection.

Upon replication stress, the ATR signaling cascade is initiated by RPA binding and subsequent phosphorylation on single stranded DNA. In doing so, single-stranded DNA molecules in the nuclear environment serve as the "lesions" that have driven the need for the evolution of the ATR signaling pathway. The phosphorylated RPA (pRPA) molecules recruit ATRIP and ATR proteins which signal two parallel downstream signaling pathways- (i) mediated by TOPBP1 and RAD9-RAD1-HUS1 complex and (ii) through the protein ETAA1 to autoregulate ATR phosphorylation (39). Interestingly, ETAA1 has a redundant role to that of ATR/ATRIP by binding to pRPA molecules. Prior studies have found that MVM infection inactivates the ATR pathway mediated by TOPBP1 (21). In addition to preventing the activation of ATR signaling on host replication forks, we propose that depleting cellular stores of RPA molecules also depletes ETAA1 proteins from the host, thereby preventing ETAA1-mediated autoregulatory ATR activation signals that protect the host genome. Since ETAA1 signaling functions in parallel to TOPBP1 to maintain genome stability, its absence renders the host cell sensitive to replication stress and formation of cellular DNA breaks. Future studies will elucidate how ETAA1 and RPA molecules induce local signals on the MVM genome and how these interactions may modulate the virus life cycle.
Induction of host-cell replication stress by interfering with RPA function is a common pro-

viral mechanism evolved by multiple viral families. The murine polyomavirus Large T Antigen

associates with RPA using its origin-binding domain to sensitize host cells to DNA damage by

chemicals and UV irradiation (40). Similarly, infection with large DNA viruses such as Herpes

Simplex Virus 1 (HSV1) induces activation of ATR and CHK1 that colocalize with ICP4 and

ICP0 proteins in viral replication centers in the nucleus (41). Consistent with these findings, the

gamma-herpesvirus EBV recruits cellular DDR proteins in the homologous recombination repair

pathway such as RPA, RAD51 and RAD52 to the newly synthesized EBV genome in the viral

replication centers (42). This recruitment of DDR proteins to herpesvirus genomes correlates

with the induction of cellular DNA breaks in and around viral replication centers. The Vpr protein

of HIV-1 and HIV-2 also induces replication fork stress on the host genome by inhibiting the

cellular DNA repair pathways (43). In a potentially similar manner, dependoparvoviruses such

as AAV, as well as the derivatives rAAV and UV-inactivated AAV, have been shown to induce

replication stress in the host genome through the ATR/CHK1 pathway, possibly by mimicking

stalled cellular replication forks (44). In line with these observations, RPA has been shown to be

associated with AAV DNA in nuclear foci and associated with AAV replication forks (45, 46).

These findings invite the possibility that AAV and its modified forms could induce global RPA

exhaustion by depleting cellular RPA molecules, leading to replication stress in a similar manner

to MVM. Therefore, RPA exhaustion seems to be an evolutionarily-conserved among viral

pathogens to induce host replication stress by limiting ATR signaling as a means to induce host

genome instability and facilitate virus replication.

Despite their inability to induce substantial levels of cellular DDR (22), ectopic

expression of NS1 and NS2 generate replication stress on the host genome. This NS1-induced

replication stress is dependent on its DNA binding function, as the K405S mutant of NS1 lacking

its helicase and ATP-ase function does not induce replication stress (36, 37). ChIP-seq studies

of ectopically expressed NS1 have previously found these molecules associated with cellular
Therefore, we speculate that soluble NS1 proteins localize to pre-existing cellular DDR sites (many of which are fragile genomic sites) and amplify the genomic stress at these regions by perturbing the replisome at these milieu. However, the mechanism of how ectopic NS2 induces replication stress remains unknown. Since the requirement for NS2 in facilitating MVM replication is species specific, it is unlikely that NS2 plays a redundant role to that of NS1 in facilitating replication stress. MVM-NS2 interacts with CRM1 proteins to facilitate nuclear egress of the virus, likely mediated by interaction with the 14-3-3 subunits c, β and/or ζ (9, 47-49). The 14-3-3 family of proteins regulate a wide variety of cellular processes, including apoptosis and cell cycle progression (50). Out of these protein subtypes, the 14-3-3 variants γ, β, and ζ are known interactors of CHK1 (50). Indeed, 14-3-3γ allows CHK1 to phosphorylate Cdc25A (51) and 14-3-3β/ζ binds CHK1 to retain CHK1 in the nucleus to promote cellular checkpoints (52). We speculate that NS2-mediated sequestration of 14-3-3 γ, β, and ζ is a redundant pathway utilized by wild-type MVM during infection to inactivate CHK1 signaling in murine hosts. Future studies will elucidate the connection between these redundant cellular stress response pathways that are usurped by MVM and single-stranded DNA viruses for efficient pathogenesis. Taken together, our findings indicate that parvoviruses exploit the host cell’s single-stranded DDR machinery for their benefit and facilitate viral pathogenesis.
MATERIALS AND METHODS

Cell Lines

Male murine A9 fibroblasts and female human U2OS osteosarcoma cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, high glucose; Gibco) supplemented with 5% Serum Plus (Sigma Aldrich) and 50 μg/ml gentamicin (Gibco). For synchronous infection experiments, cells were blocked in isoleucine deficient media as previously described (53). Cells were cultured in 5% CO2 at 37 degrees Celsius. Cell lines are routinely authenticated for mycoplasma contamination and background levels of DNA damage by γH2AX staining.

Virus and viral infection

MVMp virus was produced in A9 and 324K cells as described previously (22). MVMp infection was carried out at a Multiplicity of Infection (MOI) of 25 unless otherwise noted. MVM was irradiated in a UV-crosslinker (Stratagene) to generate UV-inactivated MVM virus as previously described (22).

Plasmids, Transfections, and Inhibitors

RPA overexpression vector has been previously generated (38). NS1, NS2 and NS1K405S overexpression vectors have been cloned into the pcDNA3.1 vector backbone and have been previously described (22). All plasmids were transfected into A9 cells (0.5 micrograms) during isoleucine deprivation at 12 hours post-synchronization. CDC7 inhibitor PHA 767491 Hydrochloride was purchased from Millipore Sigma and used according to manufacturer’s instructions.

DNA Fiber Assay

Murine A9 cells were synchronized in G0/G1 in isoleucine deficient media, then released into complete media and transfected or infected according to experimental requirements [as described above and previously (53)]. Single molecule DNA Fiber Analysis experiments were
performed according to established protocols (54). At the end of the infection period, cells were pulse in 20mM IdU for 20 minutes, immediately followed by pulsing with 50mM CldU for 20 minutes. Cells were then pelleted for 5 minutes at 5000xg and resuspended in 200 \( \mu \)L of complete media and stored on ice. 2 \( \mu \)L of cell solution was pipetted onto positively charged slides, then mixed with 6 \( \mu \)L of DNA Lysis Buffer and allowed to lyse for 5 minutes. Slides were then tilted so the fibers spread down the slide and allowed to air dry for 15 minutes. The DNA was then fixed to the slides with a 3:1 methanol:acetic acid solution. The DNA on the slides was denatured in 2.5 M HCl for 1 hour at room temperature before being blocked in 3% BSA in PBS for 30 minutes. After blocking, the cells were stained with Abcam anti-BrdU (1:1000) and BD Biosciences mouse anti-BrdU (1:500) at room temperature for 30 minutes, then washed with 0.1% Tween 20 in PBS 3 times and stained with anti-rat Alexa Fluor 488 and anti-mouse IgG1 Alexa Fluor 568 (1:1000) at room temperature for 30 minutes under covered conditions. Samples were washed with 0.1% Tween 20 in PBS 3 times and cover slips were affixed to slides using ProLong Gold Antifade Mountant (Thermo Scientific). Fibers were then imaged with a Leica Stellaris confocal microscope using a 63X oil immersion objective lens. Fiber lengths were measured using Digimizer software (MedCalc Software Ltd).

**Antibodies**

Antibodies used for DNA fiber analysis were: anti-BrdU (BD Biosciences, Clone B44, 347580), anti-BrdU (Abcam, ab6326), Alexa-Fluor 568 conjugated anti-mouse secondary (Thermo Scientific, A11004), Alexa-Fluor 488 conjugated anti-rat secondary (Thermo Scientific, A11006).

Antibodies used for western blot analysis were: Tubulin (Millipore Sigma, clone DM1A, 05-829), NS1 (2C9b monoclonal antibody), phosphor-RPA32 (Cell Signaling, 83745), γH2AX (Abcam, ab11174), HRP-conjugated anti-mouse secondary (Cell Signaling, 7076S), HRP conjugated anti-rabbit secondary (Cell Signaling, 7074S).
Antibodies used for immunofluorescence analysis were: NS1 (2C9b monoclonal antibody), RPA32 (Cell Signaling, 2208S), phosphor-RPA32 (Cell Signaling, 83745S), γH2AX (Abcam, ab11174), Alexa-Fluor 568 conjugated anti-mouse secondary (Thermo Scientific, A11004), Alexa Fluor 488 conjugated anti-rat secondary (Thermo Scientific, A11006), Alexa-Fluor 647 conjugated anti-rabbit secondary (Thermo Scientific, A21245).

Antibodies used for ChIP-qPCR analysis were: RPA (Cell Signaling, 2208S).

**Western blot analysis**

Cell pellets were lysed on ice for 10 minutes in Radio-Immuno-Precipitation Assay (RIPA) buffer. The solid lysate was pelleted by centrifugation for 10 minutes at 5000xg at 4 degrees Celsius. Protein sample concentration was calculated using BCA assay (Bio-Rad) and equal amounts of lysate were loaded per well.

**Immunofluorescence analysis**

MVMp-infected A9 cells were harvested at the indicated timepoints by pre-extracting with CSK Buffer (10 mM PIPES pH 6.8, 100 mM Sodium Chloride, 300 mM Sucrose, 1 mM EGTA, 1mM Magnesium Chloride) for 3 minutes followed by CSK Buffer with 0.5% Triton for 3 minutes. Cells were crosslinked with 4% paraformaldehyde for 10 minutes at room temperature, washed with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. The samples were blocked with 3% BSA in PBS for 30 minutes, incubated with the indicated primary antibodies for 30 minutes and incubated with the indicated secondary antibodies for 30 minutes. Samples were washed with PBS between treatments and finally mounted with Fluoromount solution containing DAPI (SouthernBiotech).

**Chromatin Immunoprecipitation combined with Quantitative PCR (ChIP-qPCR)**

MVMp-infected A9 cells were crosslinked in 1% Formaldehyde for 10 minutes at room temperature. The crosslinking reactions were quenched in 0.125 M glycine. Cells were lysed in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, protease inhibitor) for 20 minutes on ice and the cell lysates were sonicated using a Diagenode Bioruptor Pico for 60
cycles (30 s on and 30 s off per cycle), before being incubated overnight at 4 degrees C with the antibodies bound to Protein A Dynabeads (Invitrogen). Samples were washed for 3 minutes each at 4 degrees C with low salt wash (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8, 150 mM NaCl), high salt wash (0.01% SDS, 1% Triton X-100, 2 mM EDTA 20 mM Tris-HCl pH8, 500 mM NaCl), lithium chloride wash (0.25M LiCl, 1% NP40, 1% DOC, 1 mM EDTA, 10 mM Tris HCl pH8) and twice with TE buffer. DNA was eluted with SDS elution buffer (1% SDS, 0.1M sodium bicarbonate), crosslinks were reversed using 0.2M NaCl, Proteinase K (NEB) and incubated at 56 degrees C overnight. DNA was purified using PCR Purification Kit (Qiagen) and eluted in 100 µl of Buffer EB (Qiagen). ChIP DNA was quantified by qPCR analysis (Biorad) under the following conditions: 95°C for 5 mins, 95°C for 10 secs and 60°C for 30 secs for 40 cycles. Interaction of RPA molecules with UV-MVM genome was assessed by qPCR assays using primers complementary to the MVMP4 promoter. The primer sequences in 5’ to 3’ direction are: Forward primer- TGATAAGCGGTTCAGGGAGT, Reverse primer- CCAGCCATGGTTAGTTGGTT.

**Immunofluorescence Assays**

Parasynchronized MVMP-infected A9 cells were grown on cover slips in round dishes. Cells were treated with CSK buffer for 3 minutes at room temperature, CSK buffer with Triton X-100 for 3 minutes at room temperature and washed with PBS prior to fixation. Cells were crosslinked in 4% Paraformaldehyde in PBS (EMS; Gibco respectively) at room temperature for 10 minutes, washed in PBS and permeabilized in 1% Triton X-100 (in PBS) for 10 minutes. The samples were blocked in 5% BSA in PBS for 30 minutes, treated with the target antibodies (diluted in the blocking solution) for 30 minutes and then washed in PBS. Samples were treated with the target secondary antibody (diluted in blocking solution) for 30 minutes at room temperature. Samples were washed in PBS before being mounted on glass slides using DAPI Fluoromount (Southern Biotech). Samples were imaged on a Leica Stellaris5 Confocal Microscope using a 63X oil objective.
Statistical analysis

Statistical analysis was performed using Graphpad Prism software.
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FIGURE LEGENDS

Figure 1: MVM infection induces cellular replication stress that precede DDR signals.

(A) Schematic of the timeline of Single-Molecule DNA Fiber Assays during MVM infection. Mouse A9 fibroblasts are synchronized in Isoleucine deficient media for 42 hours before being released into complete DMEM and concurrently infected with MVMp at an MOI of 25 or treated with hydroxyurea (HU) at a concentration of 2µM. At the indicated timepoints post-infection (12, 18, and 24 hours post infection), cells were sequentially pulsed with IdU and CldU and processed for DFA. (B, C) Each datapoint represents the length of a single IdU and/or CldU labelled DNA fiber. The median length of many measurements of IdU and CldU labelled fibers are represented by red and green horizontal bars respectively. The experiment was performed as described by the schematic depicted in (A). At least 150 individual fibers were measured for each condition. Similar results were obtained for three independent biological replicates of MVM infection. Statistical significance was determined by Mann Whitney Wilcoxon test, **** represents P ≤ 0.0001. (D) Representative DFA images of single fibers in Mock infected A9 cells (top panel) compared with MVM infected A9 cells at 24 hpi (bottom panel) with the respective measurements of the IdU and CldU lengths indicated in white text. (E) Western blot showing the levels of NS1 and γH2AX for viral replication and DNA damage, respectively, over the time-course of MVM infection. The top row of γH2AX is taken with a 10 minute exposure (short) and the bottom with a 20 minute exposure (long). Cells pulsed with Hydroxyurea at 2µM concentration is a positive control for DNA damage, as has been previously described (22). Tubulin serves as the loading control for the Western blots. (F) Categorization of DNA fiber types as percentages out of a total of 100% as determined by presence of IdU or CldU, divided into percentages that are progressing replication forks (black), stalled replication forks (red) and new origins (green). (G,H) Non-synchronous U2OS cells were infected with wild-type MVMp or the NS1-deficient (MVM-NS1) mutant of MVM at an MOI of 25 for 24 hours before being pulsed.
with IdU/CldU as described in the schematic in (A), and processed for imaging. At least 150
individual fibers were measured for each condition. Similar results were obtained for three
independent biological replicates of MVM infection. Statistical significance was determined by
Mann Whitney Wilcoxon test, **** represents P ≤ 0.0001.

**Figure 2: MVM-induced replication stress is reversible by Cdc7 inhibition.**
(A) Schematic of the Single-Molecule DNA Fiber Assays using the CDC7 inhibitor PHA 767491
(Millipore Sigma). Mouse A9 fibroblasts were synchronized in Isoleucine deficient media for 42
hours before being released into complete DMEM media and concurrently infected with MVMp
at an MOI of 25. At the indicated 20 hpi timepoint, 5μM of iCDC7 was added to the cells for 4
hours. At 24 hpi, MVM infected iCdc7 pulsed cells were sequentially pulsed with IdU and CldU
for 20 minutes each before being processed for DFA analysis. (B, C) Each datapoint represents
the length of a single IdU and/or CldU labelled DNA fiber. The median length of many
measurements of IdU and CldU labelled fibers are represented by red and green horizontal bars
respectively. The experiment was performed as described by the schematic depicted in (A). At
least 150 individual fibers were measured for each condition. Similar results were obtained for
three independent biological replicates of MVM infection. Statistical significance was determined
by Mann Whitney Wilcoxon test, **** represents P ≤ 0.0001. (D) Categorization of DNA fiber
types as percentages out of a total of 100% as determined by presence of IdU or CldU, divided
into percentages that are progressing replication forks (black), stalled replication forks (red) and
new origins (green). (E) Western blot showing the levels of NS1 and γH2AX for viral replication
and cellular DNA damage, respectively, upon pulsing with iCDC7 as shown in the schematic in
(A) prior to MVM infection. Tubulin serves as loading control for the western blots. (F) Samples
from lanes 3 and 4 in panel (E) were processed for formation of viral replication centers by NS1
staining. Cells infected with MVM at 24 hpi under mock-treated conditional (top panel) were
compared to those pulsed with iCdc7 (bottom panel) by NS1 staining (red). White broken line
demarcates the nuclear border, which was identified by DAPI staining (blue). The white scalebar represents 10 microns.

**Figure 3: Ectopically expressed MVM Non-structural proteins NS1 and NS2 cause replication fork shortening.**

(A) Schematic of the timeline of the Single-Molecule DNA Fiber Assays during ectopic expression of MVM non-structural proteins. Mouse A9 fibroblasts were transfected with 0.5 μg of pUC18 and NS1, NS2, or NS1K405 expression vectors using Lipo293D transfection reagent or infected with MVM at an MOI of 25 for 24 hours. At 24 hours post-transfection, transfected cells were sequentially pulsed with IdU and CldU for 20 minutes each before being processed for DFA analysis. (B, C) Each datapoint represents the length of a single IdU and/or CldU labelled DNA fiber. The median length of at least 150 measurements of IdU and CldU labelled fibers from each treatment are represented by red and green horizontal bars respectively. The experiment was performed as described by the schematic depicted in (A). At least 150 individual fibers were measured for each condition. Similar results were obtained for three independent biological replicates of MVM infection. Statistical significance of the data was determined by Mann Whitney Wilcoxon test, **** represents P ≤ 0.0001, ns represents not statistically significant. (D) Categorization of DNA fiber types as percentages out of a total of 100% as determined by presence of IdU or CldU, divided into percentages that are progressing replication forks (black), stalled replication forks (red) and new origins (green).

**Figure 4: The non-replicating MVM genome binds cellular RPA and induces replication stress.**

(A) Schematic of a Single-Molecule DNA Fiber Assay timeline. Mouse A9 fibroblasts were synchronized in Isoleucine deficient media for 42 hours, then released into complete media and infected with MVM or UV-MVM at an MOI of 25 for 18 hours. At 24 hours post-transfection, transfected cells were sequentially pulsed with IdU and CldU for 20 minutes each before being processed for DFA analysis. (B, C) Each datapoint represents the length of a single IdU and/or
CldU labelled DNA fiber. The median length of at least 150 measurements of IdU and CldU tracks are represented by red and green horizontal bars respectively. The experiment was performed as described by the schematic depicted in (A). At least 150 individual fibers were measured for each condition. Similar results were obtained for three independent biological replicates of MVM infection. Statistical significance of the data was determined by Mann Whitney Wilcoxon test, **** represents P \leq 0.0001. (D) Categorization of DNA fiber types as percentages out of a total of 100\% as determined by presence of IdU or CldU, divided into percentages that are progressing replication forks (black), stalled replication forks (red) and new origins (green). (E) ChIP-qPCR of RPA binding to the UV-MVM genome at 24 hpi assessed at the MVMP4 promoter compared with IgG as the background control. Measurements are represented as percent pulldown of input chromatin. (F) Immunofluorescence analysis of MVM infected A9 cells at 16 hpi stained with antibodies detecting NS1 (red), total RPA (green) and DNA damage markers phosphorylated RPA (top; cyan) or $\gamma$H2AX (bottom; cyan). The nuclear boundary is demarcated by white dotted line and the nucleus is stained with DAPI (blue).

**Figure 5: RPA overexpression rescues MVM-induced replication stress.**

(A) Schematic of the Single-Molecule DNA Fiber Assay where RPA is overexpressed in A9 cells prior to infection. Mouse A9 fibroblasts were synchronized in Isoleucine deficient media during which they were transfected with an RPA expression vector at 12 hours post-initiation of synchronization. Cells were released into complete DMEM media at 42 hours when they were concurrently infected with UV-MVM at an MOI of 25. Cells were then infected with UV-MVM at an MOI of 25 for 24 hours. Cells were pulsed with IdU and CldU before completing the rest of the DFA protocol. (B, C) Each datapoint represents the length of a single IdU and/or CldU labelled DNA fiber. The median length of at least 150 measurements of IdU and CldU tracks are represented by red and green horizontal bars respectively. The experiment was performed as described by the schematic depicted in (A). At least 150 individual fibers were measured for each condition. Similar results were obtained for three independent biological replicates of MVM.
infection. Statistical significance of the dataset was determined by Mann Whitney Wilcoxon test, **** represents $P \leq 0.0001$. (D) Categorization of DNA fiber types as percentages out of a total of 100% as determined by presence of IdU or CldU, divided into percentages that are progressing replication forks (black), stalled replication forks (red) and new origins (green). (E) Western blot analysis of MVM infection during ectopic expression of RPA32 showing cellular levels of NS1 as a marker for virus replication, phospho-RPA32 levels due to expression and induction of cellular DNA damage and total Tubulin levels in the cell as loading control for the immunoblot.
REFERENCES


Figure 2

A

Parasynchronize cells - 42 hrs | MVM Infection | iCDC7

IdU | ClDU | 20 mins | 20 mins

B

15

(*)

10

5

0

IdU length (µm)

Mock | MVM | iCDC7 | MVM + iCDC7

C

15

(*)

10

5

0

ClDU length (µm)

Mock | MVM | iCDC7 | iMVD | MVM

D

Percent of Fiber Type (%)

100

75

50

25

0

Progressing | Stalled | New Origin

Mock | MVM | iCDC7 | iCDC7 | MVM

E

MVM

Mock | iCDC7 | Mock | iCDC7

NS1

γH2AX

TUBULIN

F

DAPI | NS1 | Merge

Mock

iCDC7

1 2 3 4
Figure 3

(A) Transfect Expression Vectors or MVM Infection

(B) IdU length (µm)

(C) CldU length (µm)

(D) Percent of Fiber Type (%)

- Black: Progressing
- Red: Stalled
- Green: New Origin
Figure 4

A

Parasynchronization of cells – 42 hrs

MVM or UV-MVM Infection

IdU

CldU

20 mins

20 mins

B

C

D

E

F

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