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VIRUS-CELL INTERACTIONS

Contribution of DNA Replication to the FAM111A-Mediated Simian Virus 40 Host Range Phenotype

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ABSTRACT Host range (HR) mutants of simian virus 40 (SV40) containing mutations in the C terminus of large T antigen fail to replicate efficiently or form plaques in restrictive cell types. HR mutant viruses exhibit impairments at several stages of the viral life cycle, including early and late gene and protein expression, DNA replication, and virion assembly, although the underlying mechanism for these defects is unknown. Host protein FAM111A, whose depletion rescues early and late gene expression and plaque formation for SV40 HR viruses, has been shown to play a role in cellular DNA replication. SV40 viral DNA replication occurs in the nucleus of infected cells in viral replication centers where viral proteins and cellular replication factors localize. Here, we examined the role of viral replication center formation and DNA replication in the FAM111A-mediated HR phenotype. We found that SV40 HR virus rarely formed viral replication centers in restrictive cells, a phenotype that could be rescued by FAM111A depletion. Furthermore, while FAM111A localized to nucleoli in uninfected cells in a cell cycle-dependent manner, FAM111A relocalized to viral replication centers after infection with SV40 wild-type or HR viruses. We also found that inhibition of viral DNA replication through aphidicolin treatment or through the use of replication-defective SV40 mutants diminished the effects of FAM111A depletion on viral gene expression. These results indicate that FAM111A restricts SV40 HR viral replication center formation and that viral DNA replication contributes to the FAM111A-mediated effect on early gene expression.

IMPORTANCE SV40 has served as a powerful tool for understanding fundamental viral and cellular processes; however, despite extensive study, the SV40 HR mutant phenotype remains poorly understood. Mutations in the C terminus of large T antigen that disrupt binding to the host protein FAM111A render SV40 HR viruses unable to replicate in restrictive cell types. Our work reveals a defect of HR mutant viruses in the formation of viral replication centers that can be rescued by depletion of FAM111A. Furthermore, inhibition of viral DNA replication reduces the effects of FAM111A restriction on viral gene expression. Additionally, FAM111A is a poorly characterized cellular protein whose mutation leads to two severe human syndromes, Kenny-Caffey syndrome and osteocraniostenosis. Our findings regarding the role of FAM111A in restricting viral replication and its localization to nucleoli and viral replication centers provide further insight into FAM111A function that could help reveal the underlying disease-associated mechanisms.

KEYWORDS DNA replication, gene expression, nucleolus, simian virus 40, viral replication, virus-host interactions

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Simian virus 40 (SV40) is the canonical member of *Polyomaviridae*, a family of small, nonenveloped, double-stranded DNA viruses that includes the known human pathogens BK polyomavirus (BKPyV), JC polyomavirus (JCPyV), and Merkel cell polyomavirus (MCPyV) (1, 2). Due to its small genome (~5.2 kb) and simple genetic organization, SV40 has served as a powerful tool for the study of fundamental eukaryotic processes, including DNA replication (3, 4), nuclear localization of proteins (5), function of gene enhancer elements (6, 7), and cellular transformation (reviewed in references 8–10). In addition, the identity and function of numerous cellular proteins have been described as a result of their specific interaction with SV40 viral proteins (11, 12).

The large T antigen (LT) of SV40 is a 708-residue viral protein that plays essential roles in promoting productive viral infection and cellular transformation (1, 2, 8, 10). It contains several discrete functional domains that contribute to the viral life cycle and to the transforming abilities of the virus, in many cases by interacting with cellular proteins and modulating their activity (11–17). The C terminus of SV40 LT (residues 627 to 708) represents a unique domain that encompasses the host range and adenovirus helper functions (18–20). Host range (HR) mutants of SV40 with specific deletions in the C terminus of LT fail to replicate efficiently and do not form plaques in restrictive cell lines, such as African green monkey kidney CV-1P cells or the human osteosarcoma cell line U-2 OS (U2OS here) (18, 21, 22). Under restrictive conditions, host range mutant viruses exhibit impairment at multiple stages of the viral life cycle, including decreased early (LT) and late (VP1 and VP3) gene and protein expression (22–24), impaired viral DNA replication (18), and defective virion assembly (25). Additionally, host range SV40 mutants are defective for the adenovirus helper function and, unlike wild-type SV40, cannot support human adenovirus replication in monkey cells upon coinfection (20, 26). Interestingly, expression in *trans* of the LT C-terminal residues 627 to 708 is able to rescue the host range and adenovirus helper defects exhibited by these viruses (23). However, the underlying cause of the viral host range phenotype is not well understood. Additionally, it is unclear whether the multiple defects observed in host range mutants are independent of or dependent on each other, with defects in one process affecting others. For instance, in the absence of LT early protein, late gene expression is substantially reduced (27), while a reduction in DNA replication efficiency or in late protein levels could lead to a corresponding decrease in viral progeny.

We have reported that the cellular protein FAM111A is a restriction factor for the host range phenotype of SV40 (22). FAM111A binds directly to the LT C terminus, as shown by a yeast-2-hybrid assay. Furthermore, reduced expression of FAM111A by RNA interference (RNAi) leads to rescue of viral gene and protein expression and plaque formation of host range SV40 viruses with various deletions in the LT C terminus. Additionally, certain point mutations in the *FAM111A* gene have been shown to give rise to two human syndromes, Kenny-Caffey and osteocraniostenosis, characterized by hypoparathyroidism and impaired skeletal development (28–30), although the underlying mechanism remains unknown. Recently, it was found that FAM111A associates with newly replicated chromatin during cellular replication and that depletion of FAM111A by RNAi delays DNA replication and S phase entry, supporting a role for FAM111A in cellular DNA synthesis (31).

Like cellular DNA replication, SV40 viral DNA replication occurs in the nucleus of infected cells and is dependent on specific interactions with host cell proteins. Viral genomes are replicated in distinct subnuclear foci, called viral replication centers, that readily incorporate the deoxynucleoside EdU and stain positive for LT as well as various host proteins required for replication (32–36). Viral DNA replication starts soon after the initial expression of LT early in infection and is initiated by the formation of a double hexamer of LT binding to the SV40 origin of replication through its DNA-binding domain (residues 131 to 260) (35, 37). The subsequent unwinding and elongation of the DNA template is coordinated by the LT helicase domain (residues 251 to 627) (16) and the tightly orchestrated interaction of LT with various cellular replication proteins, including DNA polymerase-α-primase complex (38, 39), replication protein A complex (40, 41), and DNA topoisomerase I (42). Here, we examined viral replication center

formation and the role of viral DNA replication in the FAM111A-mediated SV40 host range phenotype.

RESULTS

SV40 host range viruses exhibit defects in viral replication center formation. SV40 host range viruses exhibit numerous defects at several stages of the viral life cycle. To determine if host range viruses are also defective for viral replication center formation in restrictive cell lines, we infected U2OS cells with SV40 wild-type strain 776 (WT) or HR684, a previously characterized HR mutant that lacks the last 24 residues of the LT C terminus (23). U2OS cells stably expressing either a short hairpin RNA (shRNA) against FAM111A (U2OS-KD) or scrambled shRNA (U2OS-ctrl) were pretreated with the monosyaloganglioside GM1 to increase infectivity of SV40 in human cells (43, 44) and infected with SV40 WT or HR684 at a multiplicity of infection (MOI) of 15. Cells were pulsed with EdU for 5 min prior to fixation at 24, 48, and 72 h postinfection (hpi), stained with an antibody against LT, and then imaged using spinning-disk confocal microscopy.

We observed that mock-infected U2OS-ctrl cells were either negative for EdU signal or displayed small pan-nuclear EdU puncta that likely represent foci of cellular replication (Fig. 1A). SV40 WT-infected cells exhibited LT signal that was present throughout the nucleoplasm but was excluded from areas of low 4′,6-diamidino-2-phenylindole (DAPI) intensity that represent nucleoli (45) (Fig. 1B). By 24 hpi, WT-infected U2OS cells showed discrete nuclear EdU foci that costained with LT, indicative of viral replication centers. The viral replication centers grew in size as infection progressed to 48 and 72 hpi, when they occupied most of the nucleus. A small fraction of U2OS-ctrl cells infected with HR684 virus also exhibited a pan-nuclear LT signal that could be detected as early as 24 hpi (Fig. 1C). However, there were no detectable EdU-positive viral replication centers at 24 hpi, even in cells with robust LT signal. Viral replication centers appeared in a few HR684 LT-positive cells at 48 and 72 hpi but were much smaller in size than WT replication centers at similar times postinfection. Even in cells where levels of HR684 LT were high, viral replication centers were absent or, if present, were smaller in size.

RNAi-mediated depletion of FAM111A rescues early and late viral gene and protein expression and plaque formation of SV40 HR viruses (22). To determine if reduced levels of FAM111A could also rescue the development of viral replication centers induced by the host range mutant, we infected U2OS-KD cells stably expressing an shRNA against FAM111A with WT and HR684 viruses. We did not observe an increase in the size of viral replication centers induced by WT SV40 at 48 hpi in U2OS-KD cells compared to U2OS-ctrl cells with wild-type levels of FAM111A (Fig. 1D). In contrast, we saw a dramatic increase in the size of viral replication centers in U2OS-KD cells infected with HR684 virus, comparable to the viral replication centers induced by WT virus at 48 hpi. Thus, depletion of FAM111A supported development of HR684 viral replication centers and increased their size.

FAM111A restricts the formation and frequency of SV40 host range viral replication centers. Compared to WT virus, we found few HR684-infected U2OS-ctrl cells with detectable LT signal and presence of viral replication centers throughout the course of infection. To quantify this effect, we infected U2OS-ctrl and U2OS-KD cells with SV40 WT and HR684 as described above and imaged samples by epifluorescence microscopy. For each condition, we selected random fields and counted the total number of cells based on DAPI staining. We also counted the number of cells expressing detectable LT signal and the number of LT-positive cells with detectable viral replication centers. The percentage of LT-positive cells out of all cells and the percentage of cells with viral replication centers out of all LT-positive cells from an average of two representative experiments are shown in the graphs in Fig. 2A and B, respectively. Under restrictive conditions in U2OS-ctrl, we saw large differences between the percentage of WT- and HR684-infected cells that were LT positive and replication center positive at all times postinfection. While approximately 70% of WT-infected U2OS-ctrl

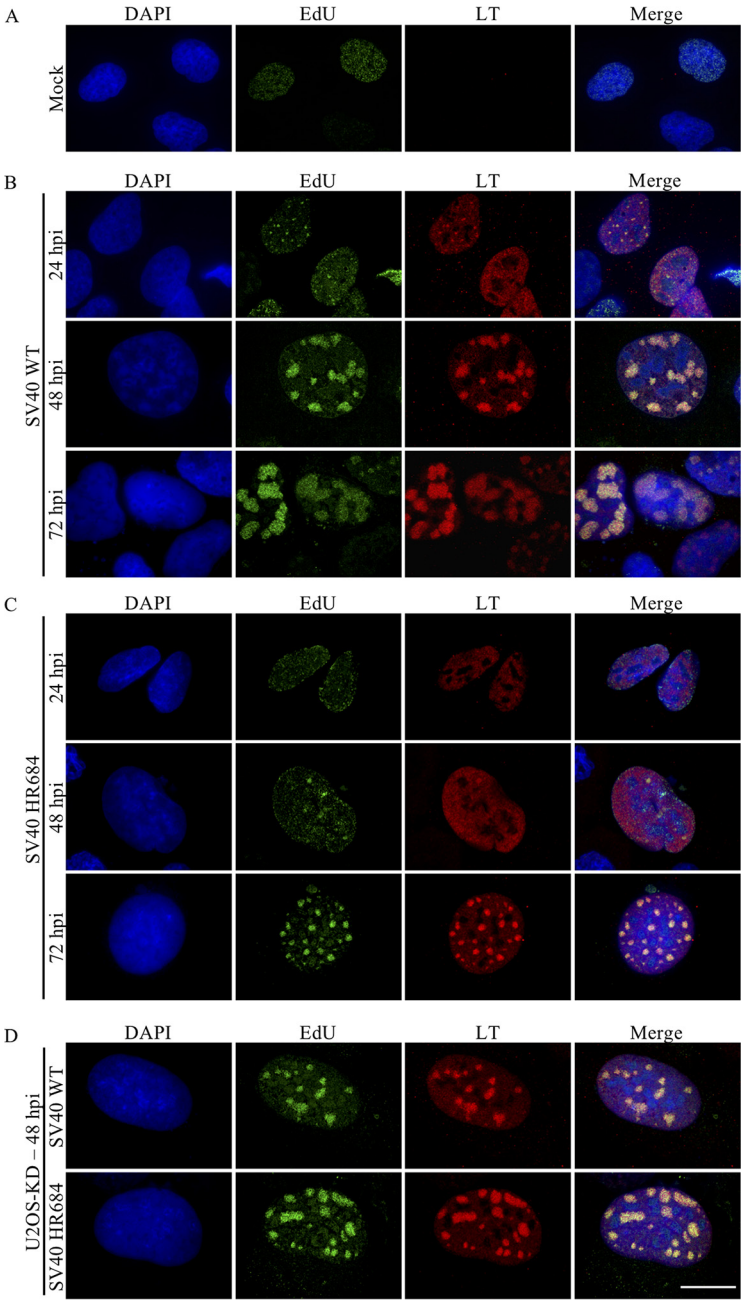

FIG 1 SV40 host range mutants are defective for viral replication center formation. (A to D) Immunofluorescence staining of mock-infected U2OS-ctrl (A), SV40 WT-infected U2OS-ctrl (B), SV40 HR684-infected U2OS-ctrl (C), and SV40 WT-infected or SV40 HR684-infected U2OS-KD (D). Cells were infected at an MOI of 15, pulsed with 20 mM EdU for 5 min prior to fixation at the indicated times postinfection, and then stained with DAPI and an antibody against LT. Single optical slices of all samples were imaged by spinning-disk confocal microscopy at ×100 magnification. Scale bar, 20 μm.

cells showed LT expression at various times postinfection, only 10 to 25% of host range virus-infected U2OS-ctrl cells showed detectable LT (Fig. 2A). At 24 hpi, viral replication centers began to appear in approximately 4% of WT-infected U2OS-ctrl cells but were completely absent from HR684-infected U2OS-ctrl cells (Fig. 2B). Furthermore, between 48 and 96 hpi, HR684 viral replication centers appeared in less than 5% of LT-expressing U2OS-ctrl cells but in 35 to 55% of those for WT. While FAM111A depletion did not have an effect on HR684 LT expression between 24 and 72 hpi, it resulted in an increase in the percentage of HR684 LT-positive cells to 59% at late stages of infection (96 hpi).

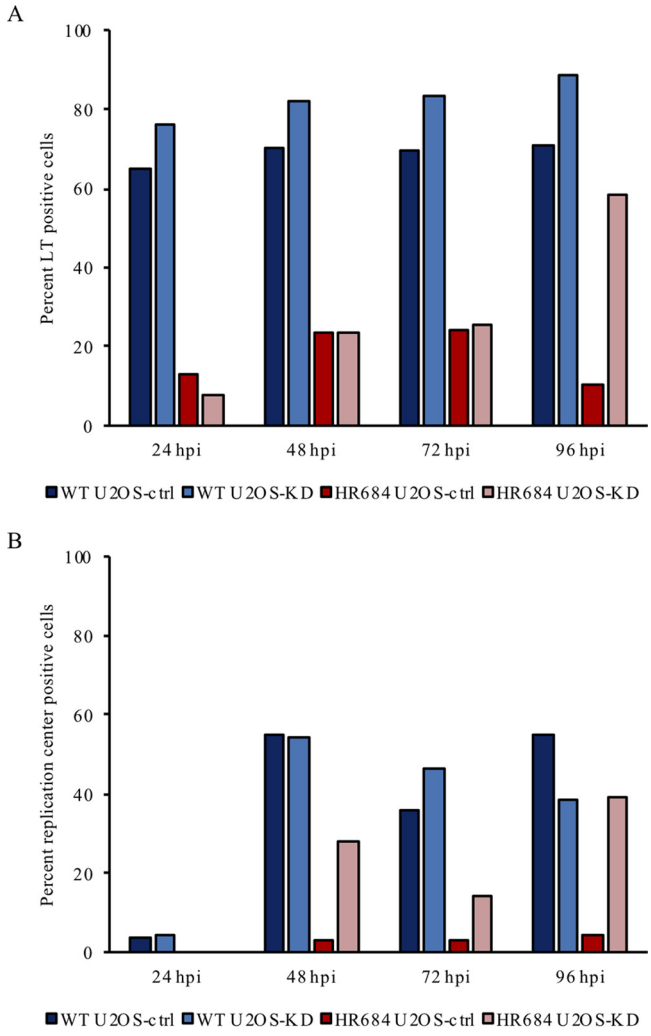

FIG 2 FAM111A restricts the formation and frequency of SV40 host range viral replication centers. (A and B) Graphs of percent LT-positive U2OS-ctrl and U2OS-KD cells infected with SV40 WT or HR684 (A) and percent viral replication center-positive U2OS-ctrl and U2OS-KD cells infected with SV40 WT or HR684 (B). Cells were infected at an MOI of 15, fixed at the indicated times postinfection, and stained with DAPI and an antibody for LT. All samples were imaged by epifluorescence microscopy at ×20 magnification and quantified using ImageJ software. Quantifications are averages from two independent experiments.

FAM111A depletion also had an effect on the percentage of host range viral replication center-positive cells, which increased to 13 to 40% of LT-expressing U2OS-KD cells between 48 and 96 hpi. In contrast, for WT virus, depletion of FAM111A did not appear to have a strong effect on either the frequency of LT-positive cells or the frequency of infected cells containing viral replication centers. Thus, FAM111A depletion resulted in increased HR684 LT expression and viral replication center frequency.

FAM111A localizes to nucleoli in uninfected cells. A previous study reported an association between FAM111A and replicating cellular DNA and found that overexpressed, green fluorescent protein (GFP)-tagged FAM111A was visible by immunofluorescence (IF) and appeared in pan-nuclear puncta (31). To determine the localization of the endogenous FAM111A protein in both uninfected and SV40-infected cells, we used specific antibodies to FAM111A. In uninfected cells, FAM111A appeared as discrete clusters of small puncta in the nucleus of U2OS-ctrl cells (Fig. 3A, top). These puncta were not pan-nuclear but instead appeared in areas of low DAPI intensity and colocalized with nucleolin, a marker of the nucleolar compartment, indicating that FAM111A could localize to nucleoli in uninfected cells. Depletion of FAM111A in

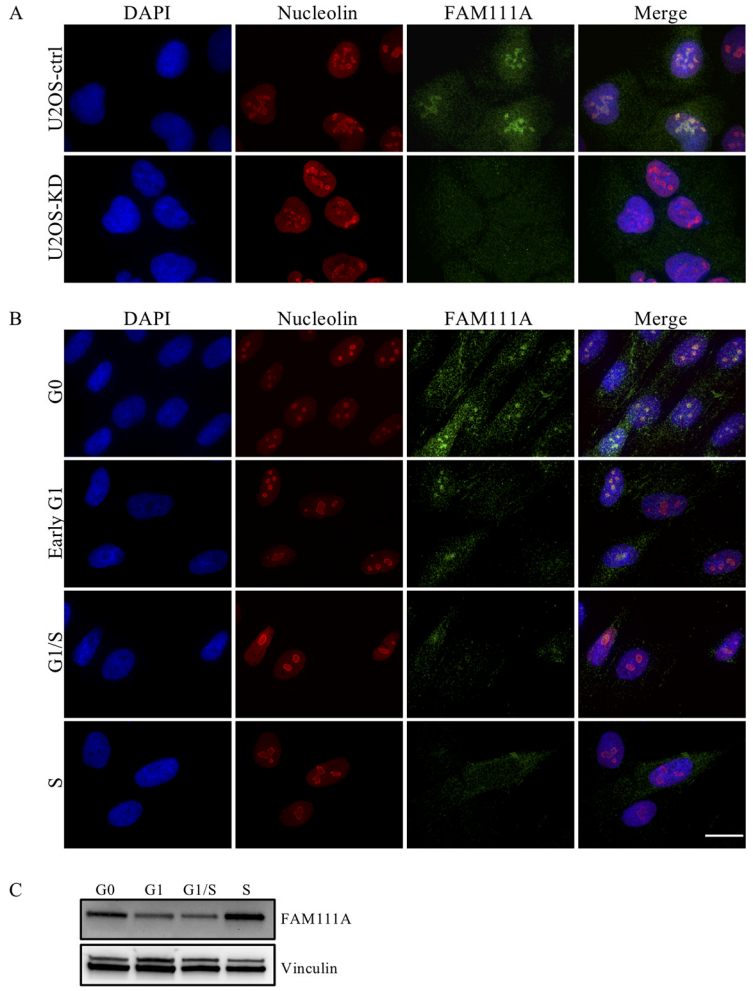

FIG 3 FAM111A localizes to nucleoli in a cell cycle-dependent manner. (A and B) Immunofluorescence staining of U2OS-ctrl (A, top) or U2OS-KD (A, bottom) and synchronized HFF cells (B) in G_0, early G_1, G_1/S, and S phase. Cells were stained with DAPI and antibodies for nucleolin and FAM111A. Single optical slices of all samples were imaged by spinning-disk confocal microscopy at ×100 magnification. Scale bar, 20 μm. (C) Western blot of synchronized HFF cells enriched for G_0, early G_1, G_1/S, and S phase blotted with antibodies for FAM111A and vinculin loading control.

U2OS-KD cells led to an absence of signal in nucleoli and throughout the nucleus, indicating that the FAM111A antibody is specific (Fig. 3A, bottom).

We observed that the FAM111A nucleolar signal was present in some, but not all, U2OS-ctrl cells. Since FAM111A levels are cell cycle regulated (22), we suspected that the cellular localization of FAM111A varies during the cell cycle. To assess the localization of FAM111A during the cell cycle, we used primary human foreskin fibroblasts (HFFs) that could be arrested in the G_0 quiescent phase by serum starvation and contact inhibition. In serum-starved, quiescent HFFs, the FAM111A signal was detectable in the nucleoli of most cells, as determined by colocalization with nucleolin (Fig. 3B). At 6 h postplating into fresh media, when the cells had entered into the early G_1 phase of the cell cycle, a nucleolar signal for FAM111A was still present but in a smaller proportion of cells. By 12 h after cell cycle entry, when cells had entered the G_1/S phase, the FAM111A signal was barely detectable and largely absent from most cells. At 24 h after plating, when cells had advanced to S phase, the FAM111A signal was undetectable above background in either nucleoli or other parts of the nucleoplasm. To determine whether the absence of FAM111A IF signal during G_1/S and S phase was a result of decreased overall protein levels, an immunoblot for FAM111A was performed with lysates from the synchronized HFFs. As shown in Fig. 3C, protein levels were

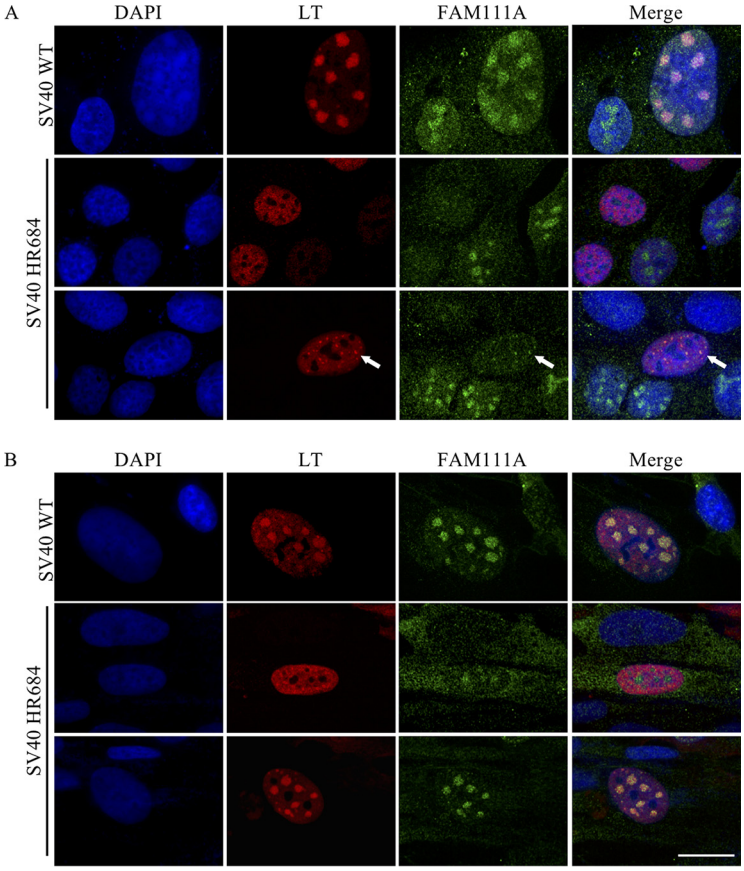

FIG 4 FAM111A colocalizes with LT in WT and HR684 viral replication centers. Immunofluorescence staining of U2OS-ctrl cells infected with WT SV40 (top) or HR684 (middle and bottom) at 72 hpi (A) and HFF cells infected with SV40 WT (top) or SV40 HR684 (middle and bottom) at 72 hpi (B). Cells were infected at an MOI of 15 and stained with DAPI and antibodies for LT and FAM111A. Single optical slices of all samples were imaged by confocal microscopy at ×100 magnification. White arrows indicate viral replication centers. Scale bar, 20 μm.

detectable at all phases of the cell cycle, with a notable increase in protein levels in S phase cells, consistent with previous reports that FAM111A levels increased as cells entered the cell cycle (22). Thus, the absence of FAM111A from nucleoli in G_1/S and S phase was not due to decreased overall levels of FAM111A.

FAM111A localizes to viral replication centers in SV40-infected cells. Since FAM111A can bind to LT and to newly replicated chromatin (22, 31), we considered whether SV40 infection, which induces viral replication centers with active viral DNA replication, alters the cellular localization of FAM111A. U2OS-ctrl cells were infected with WT and HR684 viruses and imaged at 72 hpi using a confocal microscope. In cells with no detectable LT signal, FAM111A was preponderantly present in nucleoli (Fig. 4A, top). In cells expressing LT but without any viral replication centers, the FAM111A signal was either detectable in nucleoli or was absent (Fig. 4A, middle). This pattern of either nucleolar or absent FAM111A signal was seen in both WT- and HR684-infected cells with pan-nuclear LT signal but without detectable viral replication centers. In contrast, in cells containing viral replication centers, FAM111A was consistently present at both WT and HR684 viral replication centers, where it colocalized with LT (Fig. 4A, top and bottom).

To determine if SV40 had a similar effect on FAM111A localization in normal cells, we infected HFFs with WT and HR684 viruses at an MOI of 15. Although the infection rate was lower than in U2OS cells, with fewer cells expressing detectable LT signal, WT virus was able to induce high levels of LT and formation of large viral replication centers in

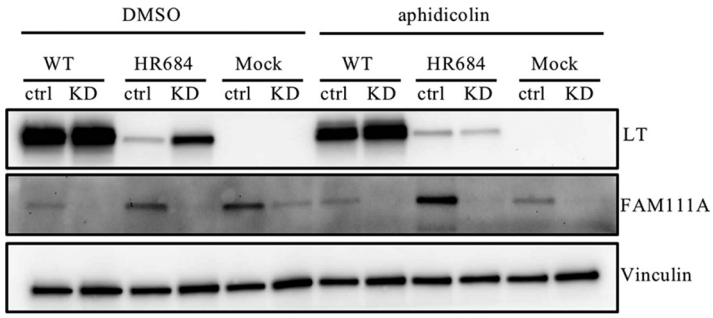

FIG 5 Inhibition of cellular and viral DNA replication affects the FAM111A-mediated early gene expression phenotype of host range mutants. Shown is a Western blot of lysates of U2OS-ctrl and U2OS-KD cells treated with 10 μM aphidicolin or DMSO and infected with SV40 WT or SV40 HR684 at an MOI of 15 or mock infected. Cells were harvested at 48 hpi, lysed, and immunoblotted with antibodies for LT, FAM111A, and vinculin loading control.

individual cells (Fig. 4B, top). As in U2OS-ctrl cells, FAM111A colocalized with WT LT in viral replication centers in HFFs. Infection of HFFs with HR684 led to expression of LT in fewer cells than the WT, with viral replication centers rarely detectable at 72 hpi. Again, FAM111A was either present in nucleoli or localized to viral replication centers when they formed (Fig. 4B, middle and bottom). Thus, FAM111A localized to viral replication centers of both SV40 WT and HR684 in two different restrictive cell types during infection.

Inhibition of cellular and viral DNA replication affects the FAM111A-mediated early gene expression phenotype of host range mutants. Since viral replication center formation was impaired for HR684 in the presence of FAM111A, and given that viral DNA replication, which occurs in viral replication centers (32, 36), is known to be decreased for host range viruses (18), we considered whether defects in host range viral DNA replication affected other FAM111A-mediated viral defects, such as early gene expression. To address this, we treated U2OS-ctrl and U2OS-KD cells infected with either SV40 WT or HR684 with aphidicolin, a selective inhibitor of DNA polymerase-α that blocks both cellular and SV40 viral DNA replication (46, 47), and assessed LT protein expression by immunoblotting at 48 hpi.

Consistent with prior reports, WT virus expressed higher levels of LT protein than HR684 virus in both U2OS-ctrl and U2OS-KD cells treated with a dimethyl sulfoxide (DMSO) control (Fig. 5). In addition, depletion of FAM111A in DMSO-treated U2OS-KD cells led to increased LT levels for the host range virus, similar to results previously reported (22). We did not observe a strong effect of aphidicolin treatment on the levels of either WT or HR684 LT expression in U2OS-ctrl cells compared to those in DMSO-treated samples. However, we found that aphidicolin treatment reduced the rescue of HR684 LT levels upon FAM111A depletion in U2OS-KD cells compared to those of U2OS-ctrl cells. This result indicates that aphidicolin-mediated inhibition of DNA replication reduced the impact of FAM111A depletion on LT expression.

SV40 LT replication-defective mutations affect the FAM111A-mediated early gene expression phenotype of host range mutants. Since aphidicolin inhibits both cellular and viral DNA replication and causes cell cycle arrest during S phase, we considered that it may have multiple effects on host range viruses. To explore the specific contribution of viral DNA replication to FAM111A-mediated host range LT expression defects without affecting cellular replication, we used replication-defective SV40 mutants. Point substitution mutations in LT were generated that specifically reduce LT DNA helicase activity (D474N) (48) or LT binding to the host polymerase/primase complex (K550A) (49) or to the viral origin of replication (A149V) (50). These 3 mutations were separately introduced into the SV40 WT and HR684 genomes, rendering the encoded LT proteins unable to support viral DNA replication.

U2OS-ctrl and U2OS-KD cells were transfected with the parental and replication-defective genomes and harvested at 48 h posttransfection (hpt). As expected, the

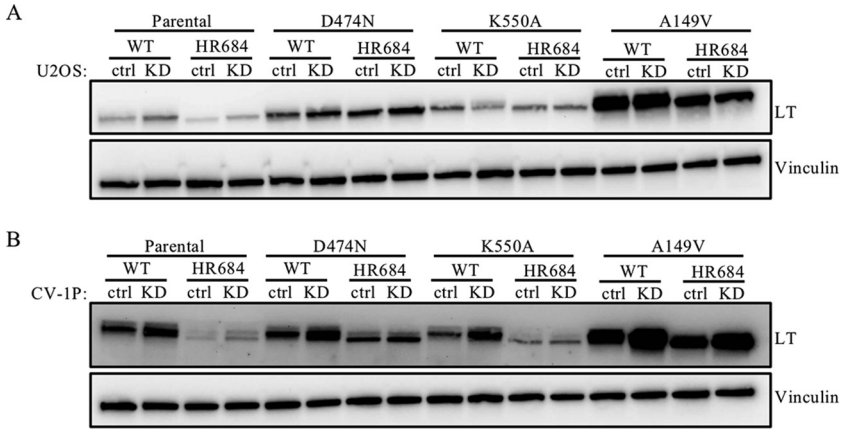

FIG 6 SV40 LT replication-defective mutations affect the FAM111A-mediated early gene expression phenotype of host range mutants. (A and B) Western blot of lysates from U2OS-ctrl or U2OS-KD (A) and CV-1P-ctrl or CV-1P-KD (B) cells transfected with SV40 WT or HR684 replication-competent parental genomes or genomes containing the replication-defective LT point substitution mutations D474N, K550A, and A149V. Cells were harvested at 48 hpt, lysed, and immunoblotted with antibodies for LT and vinculin loading control.

replication-competent, parental HR684 had lower levels of LT expression than WT virus in U2OS-ctrl cells and slightly increased levels of LT in U2OS-KD cells (Fig. 6A). The replication-defective D474N and K550A mutants showed little difference in the levels of WT and HR684 LT levels in both U2OS-ctrl and U2OS-KD cells, while the third mutant, A149V, retained differential expression levels between the WT and HR684 LT. Of note, the levels of the DNA-binding-defective mutant A149V LT were much higher than those of the parental or other mutant forms, reflective of the inability of this mutant LT to repress its own transcription (51). FAM111A knockdown had less of an effect on the levels of early gene expression for the three replication-defective host range mutants, indicating that inhibition of viral DNA replication reduced the impact of FAM111A depletion on LT expression in U2OS cells.

To assess the impact of the replication-defective LT in restrictive monkey cells, a similar experiment was performed in CV-1P cells. CV-1P cells with knockdown of FAM111A (CV-1P-KD) or scrambled control short hairpin RNA (CV-1P-ctrl) (22) were transfected with parental or replication-defective viral genomes. As shown in Fig. 6B, levels of WT LT were higher than those of HR684 LT in the parental background as well as in each of the replication-defective mutant backgrounds in both control cells and FAM111A knockdown cells. Furthermore, the levels of WT LT were higher in CV-1P-KD than CV-1P-ctrl cells for the parental viral DNA as well as for each of the replication-defective mutants. We observed a rescue of parental HR684 LT levels in CV-1P-KD compared to those in CV-1P-ctrl. We also saw a rescue in LT levels for HR684 D474N and A149V, but not for K550A, in FAM111A knockdown cells compared to control CV-1P cells.

DISCUSSION

SV40 host range virus defects have been reported at several distinct stages of the viral life cycle in restrictive cells, including early and late gene expression (22–24), DNA replication (18), and virion assembly and release (25). Here, we have shown that formation of viral replication centers is also severely impaired for SV40 host range mutants. A majority of restrictive cells infected with host range virus showed no detectable viral replication centers, even at late times postinfection and even when LT signal was clearly present in the nucleus. In the few HR684-infected cells where viral replication centers formed, their onset was delayed and their size was smaller than those of WT-infected cells at similar times postinfection. This indicates that while host range restriction reduces the number of cells expressing LT, it can also block the

formation of viral replication centers and can continue to impair their growth as infection proceeds.

We have previously reported that the cellular protein FAM111A functions as an SV40 host range restriction factor that, when depleted by RNAi, can increase levels of SV40 host range viral early and late gene and protein expression as well as restore the ability of host range viruses to undergo lytic replication in restrictive cells (22). Here, we extend these results and demonstrate that FAM111A also restricts the formation of viral replication centers. Knockdown of FAM111A led to an increase in the frequency of HR684-infected cells with detectable LT and viral replication centers. FAM111A depletion also led to an increase in the size of HR684 viral replication centers in individual cells. These results further demonstrate that impaired viral replication center formation is a feature of SV40 host range mutants and that it is mediated by FAM111A restriction.

In addition to the FAM111A impact on virus replication centers, we observed that the FAM111A localization pattern changes in uninfected and infected cells. In the absence of infection, FAM111A localizes to nucleoli in a cell cycle-dependent manner. Nucleolar FAM111A signal is present during G_0 and G_1 but becomes undetectable in G_1/S and S phase, likely as a result of protein dispersal, rather than due to a decrease in overall protein levels due to degradation or turnover. We were unable to detect pan-nuclear FAM111A-positive foci, as described by Alabert et al. (31). This may be due to the lower signal level of the endogenous protein. Although it is unclear why FAM111A localizes to nucleoli during G_0 and early G_1, several other proteins have been reported to be sequestered in nucleoli and relocalized to other cellular sites when their activity is required (52, 53). If FAM111A has a specific role in DNA replication, then sequestration of FAM111A to the nucleolus during G_0 and G_1 may reflect an inactive role during the nonreplicative phases of the cell cycle.

We detected FAM111A in the viral replication centers of both WT and HR684 virus. The presence of FAM111A at viral replication centers could reflect its role in DNA replication and be a result of its binding to newly replicated viral chromatin. Alternatively, since the C-terminal domain of WT LT binds directly to FAM111A, it could recruit FAM111A to LT-positive replication centers; however, this would not explain the presence of FAM111A at HR684 replication centers, since the C-terminally truncated LT is unable to bind FAM111A. Interestingly, FAM111A appears to have an inhibitory effect on viral replication for SV40 host range mutants. This is consistent with FAM111A being an SV40 host range restriction factor but contrasts with its role in uninfected cells, where it has been reported to promote cellular DNA replication (31). The reason for the differential effect of FAM111A on cellular and viral replication is unclear, but it could reflect FAM111A interfering with LT binding to cellular factors required for SV40 DNA replication.

We observed that inhibition of viral DNA replication had an effect on early gene expression. When viral DNA replication was inhibited either by aphidicolin or replication-defective LT mutations, differences in LT expression between WT and HR mutant were attenuated. The strength of this effect varied across treatments and cell types, likely due to the complex nature of SV40 gene expression regulation, where SV40 LT functions to repress its own transcription (51). We also observed that in the absence of DNA replication, depletion of FAM111A had less of a rescue effect on HR684 LT expression. This effect was more pronounced in U2OS cells than CV-1P cells, where FAM111A knockdown increased LT expression for two out of the three HR684 replication-defective mutants. The differences between the U2OS and CV-1P experiments possibly reflect the more efficient degree of host range restriction in the CV-1P cells. Decreased rescue of host range protein expression by FAM111A knockdown in the absence of viral DNA replication supports a role for FAM111A in restricting viral DNA replication and is consistent with the observation that FAM111A inhibits viral replication center formation.

In conclusion, we have shown that viral replication center formation is impaired for SV40 host range mutants. We have also shown that FAM111A restricts viral replication center formation for SV40 host range mutants and that it may play an inhibitory role

in SV40 viral DNA replication, possibly through its localization to viral replication centers, where viral DNA replication occurs. Our findings do not exclude the possibility that FAM111A also restricts viral gene expression or other stages of the host range virus life cycle. Future studies could help shed light on the role of FAM111A in SV40 viral restriction, cellular replication, and human disease.

MATERIALS AND METHODS

Cells. CV-1P and U2OS cells stably expressing either a scrambled shRNA (ctrl) or an shRNA specific to FAM111A (KD) were previously described (22). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning Cellgro) supplemented with penicillin and streptomycin and 10% fetal bovine serum (FBS) (Sigma) for CV-1P and U2OS or 15% FBS for HFFs.

Plasmids and viral DNA. Replication-defective mutations were introduced by PCR in the SV40 genomic DNA (strain 776) cloned into the BamHI site of pBluescript KS (Stratagene). For transfections, 300 ng plasmid DNA was used. Lipofectamine 2000 transfection reagent (Invitrogen) was used according to the manufacturer's protocol.

Viruses and viral infections. SV40 wild-type strain 776 (WT) and SV40 HR684 viruses were propagated and titers determined by plaque assay on permissive BSC40 cells, as previously described (54). All viral infections were performed using virus stocks diluted in DMEM supplemented with 2% FBS and were allowed to proceed for 2 h at 37°C with manual shaking every 15 min. At the end of 2 h, the media containing the virus was aspirated and replaced with fresh DMEM containing 10% FBS. U2OS and HFF cells were incubated with 20 μM monosialoganglioside GM1 (Sigma) overnight prior to infection (43, 44) and then infected with the WT or HR684 at an MOI of 15. To inhibit DNA replication, cells were treated with 10 μM aphidicolin or DMSO control for 18 h prior to infection and maintained in aphidicolin or DMSO postinfection until harvesting.

Antibodies. The following antibodies and antibody concentrations were used: LT mouse monoclonal antibody (PAb416; Abcam), 1:50 for immunofluorescence (IF) and 1:1,000 for Western blotting (WB); FAM111A rabbit monoclonal antibody (EPR14407; Abcam), 1:100 for IF and 1:1,000 for WB; vinculin mouse monoclonal antibody (H-10; Santa Cruz), 1:30,000 for WB; nucleolin mouse monoclonal antibody (4E2; Abcam), 1:100 for IF.

Cell synchronization. Early-passage (<12 passages) primary HFFs were maintained in complete DMEM containing 15% FBS. To generate quiescent cells in G_0, cells were cultured until contact arrested and then serum starved for an additional 48 h in serum-free DMEM. For other time points, cells synchronized by contact inhibition for 48 h were released by splitting at a 1:3 ratio into DMEM containing 15% FBS. The cells were fixed for IF or harvested for WB in G_0 or after splitting at 6 h (early G_1), 12 h (G_1/S), or 24 h (S phase).

Western blotting. Whole-cell lysates were prepared in EBC buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40), supplemented with protease inhibitor cocktail set I (Calbiochem) and phosphatase inhibitor cocktail (Calbiochem), and then run in a gradient gel. Gels were transferred onto nitrocellulose membranes that were blocked in 5% milk–TBS-T (Tris-buffered saline-Tween 20) for 30 min at room temperature (RT) with rocking and then incubated with the appropriate primary antibody diluted in 5% milk–TBS-T overnight at 4°C with rocking. The membranes were incubated for 1 h with appropriate secondary antibodies diluted 1:5,000 in TBS-T. Detection of proteins was performed using the Pierce ECL Western blotting substrate (Thermo), and images were captured on a G-Box (Syngene).

Immunofluorescence assays. U2OS and HFF cells were seeded on glass coverslips in 24-well dishes at a density of 5×10^4 cells/well. For EdU labeling, cells were pulsed with 20 μM EdU for 5 min at 37°C immediately prior to fixation. Cells were fixed at RT in 4% formaldehyde in Dulbecco's phosphate-buffered saline (DPBS) (Boston BioProducts) and then washed 3 times in DPBS. Cells were permeabilized at RT in 0.1% Triton X-100 in DPBS, washed 3 times with DPBS, and blocked overnight in 1% bovine serum albumin (BSA) (Sigma) in DPBS. Primary antibodies were diluted in a mixture of 1% BSA in DPBS and added to coverslips for 1 h at RT with rocking. Primary antibodies were removed and coverslips were washed 3 times with DPBS for 5 min with rocking at RT. The staining procedure was repeated with the appropriate fluorescently labeled Alexa Fluor secondary antibodies (Invitrogen) diluted 1:1,000 in 1% BSA in DPBS (Invitrogen). DAPI (Invitrogen) diluted 1:2,000 in DPBS was applied in the last 10 min of the secondary antibody incubation. After 3 final washes with DPBS, coverslips were mounted on glass slides using ProLong antifade reagent (Invitrogen). Image acquisition was performed using a Nikon Ti spinning-disk confocal laser microscope equipped with an Orca-AG cooled charge-coupled device (CCD) camera (Hamamatsu) or a Nikon Eclipse E800 fluorescence microscope equipped with a CoolSnap HQ2 camera (Photometrics). ImageJ was used for postacquisition analysis.

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