Expression of the NS5 (VPg) Protein of Murine Norovirus Induces a G1/S Phase Arrest

Colin Davies, Vernon K. Ward *

Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, P. O. Box 56, Dunedin, 9054, New Zealand

* vernon.ward@otago.ac.nz

Abstract

Murine norovirus-1 (MNV-1) is known to subvert host cell division inducing an accumulation of cells in the G0/G1 phase, creating conditions where viral replication is favored. This study identified that NS5 (VPg), is capable of inducing cell cycle arrest in the absence of viral replication or other viral proteins in an analogous manner to MNV-1 infection. NS5 expression induced an accumulation of cells in the G0/G1 phase in an asynchronous population by inhibiting progression at the G1/S restriction point. Furthermore, NS5 expression resulted in a down-regulation of cyclin A expression in asynchronous cells and inhibited cyclin A expression in cells progressing from G1 to S phase. The activity of NS5 on the host cell cycle occurs through an uncharacterized function. Amino acid substitutions of NS5(Y26A) and NS5(F123A) that inhibit the ability for NS5 to attach to RNA and recruit host eukaryotic translation initiation factors, respectively, retained the ability to induce an accumulation of cells in the G0/G1 phase as identified for wild-type NS5. To the best of our knowledge, this is the first report of a VPg protein manipulating the host cell cycle.

Introduction

Noroviruses are non-enveloped viruses from the Caliciviridae family that cause gastroenteritis in a variety of mammals including humans [1–5]. Human norovirus (HuNoV) infections account for significant mortality in the developing world, and in the developed world norovirus outbreaks come with a substantial financial burden [6]. HuNoV research has been hampered by the lack of a reproducible animal or cell culture system that supports viral replication. Using MNV-1 as a model allows norovirus replication and host cell interactions to be studied in cell culture and in small animals [7]. MNV-1 is a positive-sense RNA virus of approximately 7.4 kb, containing four open reading frames (ORF). ORF1 encodes for 6 non-structural proteins (NS1-2, NS3, NS4, NS5, NS6, and NS7) while ORF2 and ORF3 encode the major and minor structural proteins respectively [8]. ORF4 encodes for virulence factor 1, a non-essential protein involved in interactions with host apoptotic pathways [9].

The MNV NS5 (VPg; virus protein, genome linked), is a ~16 kDa protein that is covalently linked to the 5’ end of the genomic and subgenomic RNA [8]. Linkage to the genome is
thought to prevent detection by host pathogen recognition receptors such as RIG-1 and protein kinase R that detect uncapped 5' triphosphorylated RNA, leading to an antiviral response. NS5 additionally has a role in genome replication, acting in place of an RNA 5' cap to provide a free hydroxyl that can be extended by the virally encoded RNA-dependent RNA polymerase (NS7) [10]. The NS5 protein also acts to aid viral translation, recruiting host eukaryotic translation initiation factors to initiate translation of viral proteins [11]. The NS5 protein also contains regions of predicted disorder that are often associated with multiple functions [12, 13].

As more viruses are characterized, it is becoming increasingly common to observe interactions between viral replication and the host cell cycle. Each phase of the cell cycle presents distinctive biological conditions that have a significant impact on viral replication. Many viruses can subvert the host cell division in order to create an environment where viral propagation is preferred. Several RNA viruses, including murine norovirus 1 (MNV-1) have been characterized to manipulate cell cycle progression at the G1/S restriction point, often creating favorable conditions for viral replication [14–21].

Cell cycle progression is a complex process that is tightly controlled by multiple pathways. The G1/S checkpoint controls progression from the first gap phase (G1), a period of substantial cell growth, into the synthesis phase (S) where the host DNA is replicated. Progression through G1/S is predominantly controlled by the phosphorylation status of the retinoblastoma protein (pRb), which is in turn controlled by the activities of cyclins and cyclin-dependent kinases (CDK) (reviewed in [22]). Cyclins are expressed at various stages of cell division and bind to their corresponding CDK and phosphorylate numerous targets including pRb. In early G1 phase, cyclin D family members bind to CDK4/6 and phosphorylate pRb, driving G1 phase progression and expression of E and A cyclins. Cyclin E forms a complex with CDK2, which further phosphorylates pRb to release an E2F transcription factor, driving S phase entry [23]. Cyclin A levels continue to increase during S phase and help drive cell cycle progression through the later stages of the cell cycle, through to the initiation of prophase during mitosis [24, 25].

Recently, we have shown that MNV-1 is able to manipulate the host cell division in murine macrophages, inducing an accumulation of cells in the G0/G1 phase due to an arrest at the G1/S restriction point [20]. Additionally, this G1/S arrest created conditions where MNV-1 replication was favored compared to other stages of the cell cycle. In this study, we show that expression of viral NS5 protein in cell culture induces an accumulation of cells in the G0/G1 phase through a G1/S arrest in an analogous manner to MNV-1 infection. Furthermore, the effects of NS5 on the host cell cycle are independent of the known replication and translation activities attributed to NS5 (VPg).

Materials and Methods

Cells

RAW-Blue cells, a mouse leukemic monocyte macrophage cell line (obtained from InvivoGen, San Diego, CA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific), penicillin (100 U/ml), streptomycin (0.1 mg/ml), normocin (100 μg/ml) and zeocin (200 μg/ml) (Life Technologies). Cells were maintained at 37°C in a 5% CO2 humidified atmosphere and passaged every 48 h.

Cloning and expression of NS1-2 and NS5

MNV-1 NS1-2 and NS5 sequences were amplified from the MNV-1 infectious clone pMNV/C3, [10], derived from MNV strain CW1 (GenBank Accession DQ285629) as described in Ward et al.
[26], and the PCR products ligated into pUC8 and the sequence verified. Primers used for NS1-2 amplification were 5’ GAAATTAAATAGACTCACTATAGTGAAATGAGGATGGC AAGCCCATC 3’ (forward) and 5’ AGCAAGGTCGAAAGGTATTCGAGC 3’ (reverse) and for NS5 5’ GAAATTAAATAGACTCACTATAGGAGATGGGAAAGAAGGGACAAGA 3’ (reverse). Each forward primer added a GAAAT motif and a T7 promoter (underlined) to the 5’ end of the insert for T7 RNA polymerase binding as recommended for mMessage Machine in vitro RNA transcript production (Ambion). Bold sequences indicate start (ATG) or stop (TTA) codons. The expression constructs matched the MNV-1 NS1-2 and NS5 sequences except for the addition of a methionine codon at the 5’ end of the NS5 coding sequence to facilitate translation and stop codons in the NS1-2 and NS5 constructs to terminate translation. The resulting plasmids were transformed into XL1-Blue MRF’ Escherichia coli cells and used as templates for in vitro RNA synthesis.

Cloning of NS5(Y26A) and NS5(F123A). Constructs for the expression of NS5 variants NS5(Y26A) and NS5(F123A) were designed with the requirements for RNA synthesis and translation and were synthesized by GenScript and cloned into a pUC57-Simple vector (GenScript, Piscataway, NJ, USA) [27]. The NS5 sequences (GenBank Accession DQ285629) were flanked at the 5’ end by a BamHI restriction site, a T7 promoter sequence (underlined), a Kozak sequence for optimal RNA translation and a methionine codon (bold) (GGATCCGAA ATTAATAGACTCACTATAGGAGATGGGAAAGAAGGGACAAGA). Flanking the 3’ end of the NS5 sequence was a stop codon (bold) and a HindIII restriction site (TGAAAGCTT). The NS5 variants sequences had alanine substitutions inserted at the tyrosine 26 and phenylalanine 123, named NS5(Y26A) and NS5(Y26A) respectively. The resulting plasmids were transformed into XL1-Blue MRF’ E. coli cells and used as templates for in vitro RNA synthesis.

In vitro RNA synthesis. Plasmids were linearized at the 3’ end of viral genes with EcoRI (for NS1-2), Aval (for NS5) or HindIII (for NS5(Y26A) and NS5(F123A)). Subsequently, messenger RNA (mRNA) transcripts were synthesized from the linearized plasmids using the mMessage mMachine transcription kit (Ambion) and purified using MEGAclear transcription clean-up kit (Ambion).

Electroporation. Transfection of RNA transcripts was performed using a Neon Transfection system (Life Technologies), following manufacturer’s instructions. Briefly, RAW-Blue cells were suspended in resuspension buffer and approximately 1 × 10^6 cells transfected with 4–6 μg of RNA using 1 pulse at 1730 V and 20 mA. Transfected cells were added to 2 ml of pre-warmed medium in a 6-well plate and incubated for the times indicated.

Western blot analysis. Transfected cells were collected posttransfection and washed twice in Dulbecco’s phosphate buffered saline (dPBS). Cells were lysed directly in 25 μl dPBS and 25 μl sample buffer (120 mM Tris-HCl [pH 6.8], 5% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue), boiled for 10 minutes and separated by SDS-PAGE electrophoresis. Proteins were transferred to nitrocellulose membranes (Amersham Hybond-C Extra; GE Healthcare) and detected with the corresponding primary and secondary antibodies. The following primary antibodies were used; cyclin A (H-432) (sc-751; Santa Cruz), actin (I-19) (sc-1616; Santa Cruz), GFP (ab6556; Abcam), MNV-1 anti-NS1-2 [13] and MNV-1 anti-NS5 [13]. Secondary antibodies used were 680RD donkey anti-goat IgG (926–68074; LI-COR) and 800CW donkey anti-rabbit IgG (926–32213; LI-COR).

Cell Cycle Analysis

Synchronization of cells. To synchronize cells to the G1 phase, approximately 2 × 10^6 cells were seeded in 25-cm² flasks and treated with 3 mM sodium butyrate (N-butyrate) (B5887; Sigma) for 20 h [28, 29].
Cell cycle analysis by flow cytometry. Nuclear DNA content was measured by propidium iodide staining and fluorescence-activated cell sorting (FACS) as previously described [20]. Briefly, cells were scraped, washed in dPBS and fixed in 3 ml of cold 70% absolute ethanol overnight. Fixed cells were washed in dPBS and stained in 50 μg/ml propidium iodide (P4170; Sigma) and 0.1 mg/ml RNase A (R4875; Sigma) for 45 min at 37°C in 5% CO₂. Stained cells were washed and analyzed using fluorescence-activated cell sorting (FACS); data was analyzed with MODfit LT 3.0 software (Verity Software House).

Statistical and densitometric analyses. Data is presented as means and standard deviations (SD). Results were analyzed with either a Student’s t-test or a one-way ANOVA with the appropriate post-test as stated. P values of ≤0.05 were considered statistically significant. Western blots are shown for one of three independent experiments. Band analysis for each protein was quantified using Image Studio Lite software. Each protein quantification was first normalized against actin loading before comparisons for changes (recorded as a percentage of mock-transfected) were made.

Results

Expression of NS5 induces a G₀/G₁ phase arrest

MNV-1 infection of RAW-Blue cells induces an arrest at the G₁/S restriction point, increasing the G₀/G₁ population in order to change the internal cellular environment to favor viral replication [20]. We sought to determine if a viral encoded protein was responsible by analysis of the non-structural proteins of MNV-1 for effects to the host cell cycle. The NS5 protein from caliciviruses is essential to viral RNA transcription and translation and is essential for calicivirus replication. The NS5 protein has been shown to bind host eukaryotic translation initiation factors, recruiting these proteins for preferential viral translation and potentially inhibiting host protein expression [11, 30]. We hypothesized that an inhibition of host protein expression may contribute to a cell cycle arrest. The effect of NS5 on the host cell cycle was therefore determined by transfection of RAW-Blue cells with RNA transcripts, encoding individual viral genes, NS1-2 from MNV-1 was included as a negative control (Fig 1A). NS1-2 and NS5 were detected by their corresponding antibodies 18 h posttransfection (Fig 1C). Expression of NS5 increased the population of cells in the G₀/G₁ phase by 28% and decreased the S phase by 27% when compared to the mock-transfected population (Fig 1B). Furthermore, NS5 expression decreased cyclin A expression by 68% when compared to the mock-transfected control (Fig 1D). The cyclin A protein governs S phase entry and progression, so a decrease in expression would imply a decrease in S phase entry, further indicating NS5 as the protein responsible for the MNV-1 induced cell cycle manipulation. NS1-2 had no significant effects on the host cell cycle or cyclin A expression.

NS5 inhibits cyclin A expression and induces an arrest at the G₁/S restriction point

If the NS5 protein is responsible for the cell cycle arrest, then it should induce its cell cycle arrest at the G₁/S restriction point, as observed during MNV-1 infections [20]. The transition through G₁/S is a highly regulated checkpoint during cell division and is often targeted by viruses to induce changes to the host cell cycle [14, 18, 31, 32]. The transition of cells through the G₁/S restriction point was examined in cells expressing NS5. Cells were synchronized to the G₁ phase through N-butyrate treatment, released from the arrest and transfected with NS5, NS1-2 and GFP coding RNA. NS1-2 was used as a viral control protein that did not affect the cell cycle and GFP as a non-viral RNA negative control. N-butyrate was also added to released populations and used as a positive control. Cells were then analyzed for their transition from
Fig 1. Expression of MNV-1 NS5 induces a G0/G1 phase arrest. Approximately 1 × 10^6 RAW-Blue cells were transfected with 4–6 μg of NS1-2 or NS5 RNA transcripts. Mock-transfected (MT) cells were seeded at the time of transfection (negative control). (A) Cells were collected 18 hours posttransfection for FACS analysis of the cell cycle. The histograms presented are from one of three experiments. (B) The histograms from (A) were analysed with MODfit LT 3.0, and the percentage of cells in each phase of the cell cycle shown. Statistical significance was determined for comparisons between transfected populations and the corresponding mock-transfected population using a one-way ANOVA with Dunnett's post-test. **, P ≤ 0.01. (C)Expression of NS5, NS1-2 and cyclin A expression was determined by Western blot analysis. Actin was used as a loading control. (D) Cyclin A levels from three experiments were quantified with Image Studio Lite (LI-COR) and normalised against the results for actin and results presented as means and SD from three independent experiments. Statistical significance was determined for comparisons between transfected populations and the mock-transfected population using a one-sample t-test. *, P ≤ 0.05.

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G1 into S phase by FACS analysis of the host cell cycle. GFP, NS1-2 and NS5 RNA transcripts were translated into protein and detected from 15 to 24 hours post-G1 release (Fig 2A). The N-butyrate treated cells remained predominantly in G1 phase post-release while the mock-transfected cells came out of the G1 arrest, indicated by a decrease in the G0/G1 population and an increase in the S phase cells post-release (Fig 2C). Transfection of GFP and NS1-2 RNA had no effect on the transition of cells from G1 into S phase compared to the mock-transfected population, with both GFP and NS1-2 transfected populations progressing into S phase, with a substantial decrease in the G0/G1 population observed at 21 hours post-release, similar to the mock-transfected population (Fig 2C). In contrast, transfection of NS5 RNA induced a G1/S phase arrest, as indicated by cells remaining in the G1 phase post-release, with no increase in the S phase population. At 24 hours post-release in NS5 transfected populations, 73% of cells remained in the G0/G1 phase compared to 46% in the mock-transfected population (Fig 2C).

Because cyclin A expression is inhibited by MNV-1 infection in cells transitioning through the G1/S restriction point [20], cells were analyzed post-G1 release for cyclin A expression by Western blot analysis. Expression of NS5 inhibited the accumulation of cyclin A in populations progressing from G1 to S phase. In a G1 arrested population expression of cyclin A is low, indicated by undetectable levels of cyclin A in N-butyrate treated cells (Fig 2D). In mock-transfected, GFP and NS1-2 transfected populations, cyclin A expression increased from 18 to 24 hours post-release as cells entered S phase. In NS5 transfected cells, cyclin A levels remained below detectable limits post-release, as transfected cells remained in G0/G1 phase and did not progress into S phase. These results indicate that NS5 is responsible the cell cycle arrest induced by MNV-1. Not only does NS5 expression increase the G0/G1 population, but it also inhibits progression at the G1/S restriction point and inhibits cyclin A expression.

NS5 association with host eukaryotic initiation factors does not influence NS5 induced cell cycle arrest

A well-characterized function of NS5 in viral replication is to recruit host eukaryotic initiation factors for preferential translation of viral proteins [30, 33]. It was hypothesized that the manipulation of the host cell cycle by NS5 could be driven by its association with host eukaryotic initiation factor eIF4G, leading to inhibition of host translation, as seen with plant VPg proteins [34], and thus potentially inducing a cell cycle arrest. The ability of MNV NS5 to bind to eIF4G can be abolished through the introduction of a phenylalanine to alanine substitution at position 123 (NS5(F123A)) [30]. Not only does the NS5(F123A) substitution inhibit binding to scaffold protein eIF4G, it abolishes viral replication. We predicted that the introduction of the NS5(F123A) substitution could inhibit its cell cycle control. RNA transcripts encoding WT NS5, NS5(F123A) and NS1-2 were generated, transfected into an asynchronous cell population and their cell cycle effects analyzed by flow cytometry. Both NS5 and NS5(F123A) could be detected by the α-NS5 antibody (Fig 3C). Expression of viral NS1-2 had no effect on the host cell cycle while expression of both NS5 and the NS5(F123A) variant increased the G0/G1 population by ~22% and decreased the S phase population proportionally when compared to the mock-transfected population (Fig 3B). Furthermore, the NS5(F123A) variant decreased cyclin A protein expression by 67% when compared to the mock-transfected population in a synonymous manner to NS5, strongly implying that the host eukaryotic initiation factor binding domain of NS5 does not play a role in its cell cycle manipulation (Fig 3D).

NS5 nucleotidylation to RNA via Y26 plays no role in inducing host cell cycle effects

The NS5 protein from MNV is covalently attached at the 5′ terminus of viral RNA, acting as a cap to prime RNA synthesis [8]. Attachment of NS5 to viral RNA occurs via the tyrosine
residue at position 26 (Y26) in MNV, lying within a highly conserved EYDE motif in calici-
viruses [10, 35, 36]. Substitution of the Y26 residue with an alanine residue NS5(Y26A) pre-
vents the formation of NS5-viral RNA [10, 37]. The nucleotidylation of NS5 at Y26 is likely

Fig 2. The MNV-1 protein NS5 induces a G1/S arrest and inhibits cyclin A expression. Approximately 7.5 × 10^6 RAW-Blue cells were
seeded in 75 cm² flasks and treated with 3 mM N-butyrate for 20 h to synchronize cells into the G1 phase. Following synchronization, cells were
washed 3 times with 5 ml of complete medium and incubated for 5 h. After incubation, cells were recovered, counted and approximately 1 × 10^6
cells transfected with 4–6 μg of GFP, NS1-2 or NS5 RNA transcripts. A population of cells had 3 mM of N-butyrate added at the time of
transfection (positive control) and mock-transfected (MT) cells were seeded at the time of transfection (negative control). (A) At the indicated h
post-release (h.p.r.) cells were harvested for Western blot analysis of viral protein expression. The blots presented are representative blots from
three experiments. (B) Cells were collected post-release at the indicated times for FACS analysis of the cell cycle. The histograms presented are
from one of three experiments. (C) The histograms from (B) were analyzed with MODfit LT 3.0, and the percentage of cells in each phase of the
cell cycle shown. The results are means and SD from three independent experiments. Statistical significance was determined for comparisons
between transfected populations and the corresponding mock-transfected time point using a one-way ANOVA with Dunnett’s post-test. **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001. (D) Cells were harvested at the indicated h.p.r. for Western blot analysis of cyclin A and actin
expression. The blot presented is a representative blot from three experiments.

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Fig 3. The eukaryotic initiation factor binding domain is not responsible for the NS5 induced cell cycle effects. Approximately 1 × 10⁶ RAW-Blue cells were transfected with 4–6 μg of NS1-2, WT NS5 and NS5(F123A) RNA transcripts. Mock-transfected (MT) cells were seeded at the time of transfection (negative control). (A) Cells were collected 18 hours posttransfection for FACS analysis of the cell cycle. The histograms presented are from one of three experiments. (B) The histograms from (A) were analysed with MODfit LT 3.0, and the percentage of cells in each phase of the cell cycle shown. Statistical significance was determined for comparisons between transfected populations and the corresponding mock-transfected population using a one-way ANOVA with
contingent upon viral RNA polymerase (NS7), which is not present in the expression system used in this study. To further confirm that Y26 residue is not involved in the cell cycle arrest through an unidentified mechanism, an alanine substitution at Y26 was introduced (NS5(Y26)). Although the viral genome and NS7 protein is absent in the transfection, we could not exclude that NS5 could be attaching via the Y26 residue to host RNA. RNA encoding WT NS5, NS5(Y26A) and NS1-2 were synthesized, transfected into an asynchronous cell population and their cell cycle effects analyzed by flow cytometry. Both NS5 and NS5(Y26A) could be detected by the $\alpha$-NS5 antibody (Fig 4C). Expression of viral NS1-2 had no effect on the host cell cycle while both NS5 and NS5(Y26A) expression increased the G0/G1 population by ~20% and decreased the S phase population proportionally when compared to the mock-transfected population (Fig 4B). Furthermore, WT NS5 and NS5(Y26A) decreased cyclin A protein expression by 69% and 73% respectively when compared to the mock-transfected population (Fig 4D). These results, combined with the absence of viral RNA polymerase, imply that nucleotidylation to RNA via the Y26 residue has no role in NS5 ability to manipulate the host cell cycle.

**Discussion**

We recently identified that MNV-1 was able to manipulate the host cell cycle, causing an arrest and accumulation in an early cell growth phase leading to enhanced viral replication [20]. This study identifies that the NS5 protein from MNV-1 is able to induce a cell cycle arrest analogous to that of MNV-1 infection in a mouse macrophage cell line, in the absence of other viral factors. Changes to the host cell cycle post NS5 expression was nearly identical to that observed under MNV-1 infection with the G0/G1 population increasing by 24% and 28% and the corresponding S phase population decreasing by 26% and 27% respectively. Expression of NS5 alone was sufficient to induce an accumulation of cells in the G0/G1 phase and reduce cyclin A expression in an asynchronous population. Further confirmation of NS5 as the cell cycle regulator comes from analyzing the ability of NS5 to inhibit the G1/S transition and prevent cyclin A accumulating in a population progressing from G1 into S phase. These effects on the host cell cycle are consistent with the observed effects of MNV-1 infection, showing NS5 to be a causative agent of this cell cycle arrest.

It was initially hypothesized that the cell cycle arrest induced by NS5 was due to its known interaction with host eIF4G. The NS5 protein from several caliciviruses has been documented to aid in viral protein translation, through binding to several host eukaryotic initiation factors and recruiting ribosomes to the site of viral replication [11, 30, 38–40]. The concentration of host eukaryotic initiation factors by VPg proteins is predicted to impair host translation [36]. Shut-off of host protein translation has been shown to induce cell cycle arrests in several cell phases including G1 and G2/M [41, 42]. We analyzed the impact on the host cell cycle of NS5 binding to host eukaryotic initiation factors through the introduction of an amino acid substitution that was known to impede binding to eIF4G [30]. The NS5(F123A) substitution had no effect on the ability of NS5 to induce a G1 arrest indicating an alternative mechanism is responsible for the cell cycle manipulation.

Another well characterized function of NS5 is in priming of RNA synthesis via attachment to viral genomic and subgenomic RNA. While nucleotidylation of the NS5 Y26 residue is
Fig 4. Nucleotidylation via Y26 is not responsible for the NS5 induced cell cycle effects. Approximately $1 \times 10^6$ RAW-Blue cells were transfected with 4–6 μg of NS1-2, WT NS5 and NS5(Y26A) RNA transcripts. A mock-transfected (MT) control was seeded at the time of transfection (negative control). (A) Cells were collected 18 hours posttransfection for FACS analysis of the cell cycle. The data are from one of three experiments. (B) The histograms from (A) were analysed with MODfit LT 3.0, and the percentage of cells in each phase of the cell cycle shown. Statistical significance was determined for comparisons between transfected populations and the corresponding mock-transfected population using a one-way ANOVA with Dunnett's post-test. *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$. (C) Expression of NS1-2, WT NS5, NS5(Y26A) and cyclin A was determined by Western blot analysis. Actin was used as a loading control. (D) Cyclin A levels from three experiments were quantified with...
unlikely in the absence of viral RNA polymerase (NS7) we wanted to confirm that Y26 had no role in cell cycle arrest. Attachment to host RNA could result in changes in protein expression, leading to a cell cycle arrest. The NS5(Y26A) protein is unable to undergo the nucleotidylation reaction that attaches NS5 to viral RNA but still induced cell cycle changes in an analogous manner to WT NS5. This indicates that attachment to RNA via the Y26 residue is not the cause of cell cycle affects. MNV NS5 is indicated to be located in the perinuclear region, so an interaction with host DNA is unlikely [43]. Because the NS5(Y26A) and NS5(F123A) substitutions could still induce cell cycle control suggests that the NS5 induced G1/S arrest occurs through an as yet uncharacterized activity.

In this study, our data discovers a viral protein that independent of other viral proteins is able to induce cell cycle manipulation and identifies a new function of the multi-faceted NS5 protein. The detailed mechanism of how NS5 is inhibiting cell cycle progression may lie with the observed decrease in cyclin A expression with further studies needed to examine this interaction. Nevertheless, these results reinforce the importance of the multifunctional NS5 in norovirus replication.

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**Author Contributions**

*Conceptualization:* CD VKW.

*Data curation:* CD.

*Formal analysis:* CD.

*Funding acquisition:* CD VKW.

*Investigation:* CD.

*Methodology:* CD VKW.

*Project administration:* CD VKW.

*Resources:* VKW.

*Supervision:* VKW.

*Visualization:* CD.

*Writing – original draft:* CD.

*Writing – review & editing:* CD VKW.

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